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Research

Uptake and Distribution of Carbofuran and Its Metabolites in Watermelon (*Citrullus lanatus*)

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ABSTRACT

Carbofuran is toxic to humans and the environment, and its misuse in agriculture results in the violation of Maximum Residue Limits (MRL) for most crops. The use of carbofuran in Malaysia is to be banned effectively in May 2023. Limited data were published on the uptake and distribution of carbofuran and its metabolites for most crops. Therefore, a study was conducted to assess carbofuran residues in watermelon grown at the Agriculture Research Centre, Semongok. Carbofuran at 17 kg/ha and 34 kg/ha were applied to watermelon planted under the recommended agronomic practices. The plants were sampled at periodic intervals over 63 days after being treated with carbofuran. The carbofuran and their metabolites in the watermelon leaf, stem, and fruits were determined using a liquid chromatography equipped with a triple quadrupole mass spectrometer. The carbofuran residues were observed up to 35 days after application and reached below the quantification level (0.01 mg/kg) at 42 days after application. Residues were concentrated in the roots and stems for both treatments. About 80 to 90% of the carbofuran was metabolized to carbofuran-3-hydroxy in the watermelon leaves, and 50% in the stems. The highest residue level of carbofuran for application at 17 kg/ha was recorded on Day 9 for roots (0.192 mg/kg), Day 21 for leaves (0.057 mg/kg), and Day 5 for stems (0.134 mg/kg). At 34 kg/ha, the highest carbofuran residues concentrations were recorded on Day 5 for root (0.446 mg/kg) and stem (0.151 mg/kg), and Day 3 for leaves at 0.303 mg/kg. Traces of carbofuran residues were detected in the skin and flesh of the fruit. The carbofuran residue levels in watermelon fruit were below the established MRL of 0.01 mg/kg. Our findings suggest that a proper postharvest interval shall be observed.

Key words: Carbofuran, Carbofuran-3-hydroxy, residues, watermelon

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INTRODUCTION

Pesticide has been widely used by farmers to protect their crops from pest and disease attacks. Each year, thousands of tonnes of different types of pesticides are purchased and used by farmers to control pests, diseases, and weeds. The benefits of using pesticides in agriculture included an increased ease of controlling pests and diseases, high crop production, and improved produce quality (Benicha *et al*., 2013). However, the excessive use of pesticides in crop production also become a concern among consumers. The inappropriate level of pesticides applied in agriculture may pose risks such as food contamination, direct human exposure, environmental contamination, and toxic effects on ecosystems (Benicha *et al*., 2013). Over-used or long-term use of any type of pesticide will contribute to the development of pest and disease resistance (Sharma *et al*., 2019; Pathak *et al*., 2022). The adverse effects of pesticides on human and the environment has prompted many countries to impose strict regulation on the use of pesticides and ban certain pesticides such as carbofuran.

Carbofuran is a broad-spectrum carbamate pesticide and was banned in the United States of America, Canada, and European Union countries (Benicha *et al*., 2011; Kitowski *et al*., 2020). In Malaysia, the usage of carbofuran in agriculture will be banned with effect from 1 May 2023. The reason is likely due to the inappropriate use of carbofuran on crops, resulting in a violation of Maximum Residue Limits

(MRL) in vegetables and fruits (The Star, 2022). Carbofuran is used as an insecticide for crops such as corn, brinjal, coconut, cocoa, pepper, paddy, banana, oil palm, and sugarcane. Carbofuran is a stable compound under neutral or acidic conditions but degrades rapidly in alkaline conditions (WHO, 2008). Carbofuran in soil and water is absorbed by plant roots translocated into the leaves and changed to other metabolites (WHO, 2004). Carbofuran 3-hydroxy is the main metabolite of carbofuran (WHO, 2004).

The uptake and dissipation of carbofuran in tomato, groundnut, brinjal, and sugarcane have been published by other researchers. In tomato fruits, residues of carbofuran were found greater than the MRL of 0.1 mg/kg after 5, 8, 14, and 21 days of application (Elsheikh, 2020). No residues were detected after 45 days in the tomato fruits. In groundnut, the combined residues of carbofuran and 3-hydroxycarbofuran reached below the detectable level after 60 days of sowing and remained below the MRL of 0.1 mg/kg in groundnut kernels at harvest (Singh & Karla, 1992). Iqbal *et al*. (2007) reported that a high concentration of carbofuran residue (0.039 mg/kg) was detected in brinjal after Day 3 of suspension application and became negligible after Day 7. In sugarcane, the concentration of carbofuran and carbofuran-3-hydroxy in sugarcane leaf and juice was below the quantification limit at the time of harvest (Saini *et al*., 2020).

Watermelon is one of the most common types of melons and is mainly eaten fresh or made into juices. The watermelon rind is also edible and can be cooked or made into pickled (Johnson *et al*., 2012). The cultivation of watermelon plants requires several types of pesticides including carbofuran to control the pests and diseases infestation. In Thailand, an excessive use of carbofuran was reported in watermelon cultivation (Wanwimolruk *et al*., 2015). However, information about carbofuran and its metabolite residues in watermelon is limited. Therefore, we attempted here to evaluate the carbofuran residual levels in different parts of the watermelon plant and compare them with the MRL established in the Food Legislation. The objective of this study was to assess the uptake, distribution, and accumulation of carbofuran residues in watermelon grown at the Agriculture Research Centre (ARC), Semongok, Kuching, Sarawak, Malaysia.

MATERIALS AND METHODS

Chemicals and reagents

Carbofuran, carbofuran-3-hydroxy, and carbofuran-3-keto standards were purchased from Dr. Ehrenstorfer, Augsburg, Germany. Acetonitrile (LC grade with purity ≥99%), acetone, formic acid, sodium chloride, acetic acid, and florisil (2% deactivated) were procured from Merck KGaA, Germany. Anhydrous magnesium sulfate ($MgSO_4$) (purity ≥98%) was obtained from J.T. Baker, Philipsburg, USA. The carbofuran granules (AGRITOX 3G) were purchased from HEXTAR Chemicals Sdn. Bhd.

Apparatus and instrumentation

A Robot Coupe Blixer®4 food processor was used to homogenize the watermelon samples. A Thermo Jouan Model B4i multifunction centrifuge (Gontier, France) was used to centrifuge the sample extracts. A vortex mixer (IKA VORTEX 3, Germany) was used to ensure the homogeneity of sample extracts.

Liquid chromatography equipped with a triple quadrupole mass spectrometer (LC-MS/MS) was used for the detection and quantification of carbofuran and its metabolite residues in the samples. The analysis was performed using Agilent 1290 LC connected to a triple quadrupole mass spectrometer (MS/MS) Agilent 6495 Series. The LC separations were performed using reversed-phase column ZORBAX Eclipse XDB-C18 (150 mm × 4.6 mm × 5 µm). Gradient elution of acetonitrile and water was programmed at a 1.2 mL/min flow rate, in which one reservoir contained 5mM ammonium formate and 0.01% formic acid in acetonitrile-water (9:1) solution and the other reservoir contained 5mM ammonium formate and 0.01% formic acid in deionized water solution.

Field experiment

The experiment was conducted in an open space field at ARC Semongok, Kuching, Sarawak, Malaysia. The experiment was designed based on the residue trial guidelines published by the Food and Agriculture Organisation, FAO in 2009 (FAO, 2009). The watermelon seeds (Black Lady variety) were sown in germination trays filled with peat moss in a nursery for 2 weeks. The seedlings were treated with granule fertilizer and watered twice a day. A total of 28 raised beds (1.2 m × 6 m) were prepared and dressed with 1.0 to 2.0 kg/m^2 chicken manure, 100 – 200 g/m^2 of dolomite, and 30 g/m^2 of NPK fertilizer (15:15:15). The raised beds were covered with silver-shine plastic mulch. The soil type was clayey red-yellow podzolic with a soil pH of 6.6 (in water) and electrical conductivity (EC) of 123.3 µmhos/cm. The organic matter content was 2.1%.

A total of 196 healthy seedlings were transplanted onto the 28 raised beds with 7 plants/bed in one single row with in-row spacing of 0.8 m and between-row spacing of 2.0 m. The plants were watered using a drip irrigation system. The experiment consisted of two treatments in different plots. In treatment 1 (T1), the plants were treated with carbofuran granules at the recommended dosage of 17 kg/ha; and 34 kg/ha (double the recommended dosage) in treatment 2 (T2). The granules were placed near the roots of 56 selected plants in each treatment when the plants started to bear fruits (3 weeks after transplanting). The remaining 84 plants were kept in control.

Samples collection and preparation

Three watermelon plants were harvested at random from each plot at 0, 1, 3, 5, 7, 9, 14, 21, 28, 35, 42, 49 and 63 days after application. The plants sampled at Day 0 were taken after 2 hr of carbofuran application. The collected plants were separated into fruits, leaves, and stems. For watermelon fruits, the samples were separated into skin and flesh. Carbofuran and its metabolites were extracted from each part according to the modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) method (Chai *et al*., 2011). Ten grams of homogenized samples were weighed into a PTFE centrifuge tube and added with 20 mL of acidified acetonitrile (1%). The mixture was hand-shaken vigorously for 1 min, added with 5.0 g of anhydrous $MgSO_4$ and 1.5 g of NaCl. The mixture was homogenized by vortex mixing for 1 min. The mixture was centrifuged at 1,127 *g* for 1 min. Three grams of anhydrous $MgSO_4$ was added to the supernatant in a new centrifuge tube. For the clean-up process, 2 mL of the supernatant was leached through a Pasteur pipette packed with 0.2 g of deactivated florisil.

Analysis of carbofuran and its metabolite

A standard stock solution consisting of 500 mg/L each of carbofuran, carbofuran-3-hydroxy, and carbofuran-3-keto was prepared by dissolving an appropriate amount of the pesticide standards in residue grade acetonitrile. The stock solution was mixed and diluted to obtain seven multi-working standard solutions of carbofuran and carbofuran-3-hydroxy at concentration levels of 10.0, 1.0, 0.5, 0.1, 0.05, 0.01, and 0.005 mg/L.

An exact 2 µL of the extracted aliquot was injected into the LC-MS/MS. The equipment operation was carried out in positive mode, the N_2 nebulizer curtain, and other gas settings were set according to the recommendation made by the manufacturer, the source temperature was 325°C, and the ion sprays potential at 5500 V. The collision energies were optimized by introducing individual pesticide solutions into the MS instrument. The multiple reaction monitoring (MRM) methods and the optimized MS/MS conditions for the individual pesticides and their retention time (RT) were summarised in Table 1.

Table 1. MRM transitions and other parameters for carbofuran, carbofuran-3-hydroxy, and carbofuran-3-keto

Pesticide	RT[a]	Q1[b]	CE1[c]	Q2[d]	CE2[e]
Carbofuran	4.247	222.10→165.10	20	222.10→123.10	30
Carbofuran-3-hydroxy	3.151	238.11→181.09	10	238.11→163.08	10
Carbofuran-3-keto	3.729	236.10→179.10	10	236.10→161.10	20

[a]RT, retention time.
[b]Q1, quantifier mass transition.
[c]CE1, collision energy corresponding to Q1.
[d]Q2, qualifier mass transition.
[e]CE2, collision energy corresponding to Q2.

Method validation

All validation studies were performed using pesticide-free watermelon fruit. The linearity in the response was studied using matrix-matched calibration standard solutions. Standard calibration curves were constructed for seven working standard solutions with concentrations ranging from 0.005 to 1.0 mg/L. The recoveries of carbofuran and its metabolites were evaluated at three different concentrations. The homogenized pesticide-free watermelon fruit was fortified each with 0.01, 0.05, and 0.10 mg/kg carbofuran, carbofuran-3-hydroxy, and carbofuran-3-keto. Each analyte and fortification level was replicated three times. The matrix-matched calibration standards were used to calculate the recoveries in compensating for any matrix effects arising from matrix interferences or co-extractives. The limit of determination (LOD) was calculated as the analyte concentration that gave a signal-to-noise (S/N) ratio of 3, calculated and verified by analyzing pesticide mixtures at these concentration levels in matrix extracts. The limit of quantification (LOQ) was estimated based on the signal of background noise that was equal to 10 times the noise level.

Statistical analysis

The differences in the carbofuran and its metabolite concentrations in plant parts at selected interval periods were determined by the analysis of variance (ANOVA) using Statistical Analysis System (SAS) version 9.0.

RESULTS AND DISCUSSION

Recoveries

The average recoveries of carbofuran, carbofuran-3-hydroxy, and carbofuran-3-keto residues in watermelon determined using LC-MS/MS at 0.01, 0.05, and 0.1 mg/kg ranged from 70.0 to 90.0% with RSD less than 15.0% (Table 2). These results indicated that the method used in this study is in agreement with the standard pesticide residue analysis guidelines as per SANTE/11312/2021.

Table 2. Recoveries of carbofuran, carbofuran-3-hydroxy, and carbofuran-3-keto at three fortification levels in watermelon fruit

Pesticide	Fortification Level	Recovery, %[a]
Carbofuran	0.01	70.7 ± 2.1
	0.05	80.1 ± 5.5
	0.1	77.0 ± 6.1
Carbofuran-3-hydroxy	0.01	71.7 ± 5.7
	0.05	84.8 ± 12.3
	0.1	86.3 ± 7.1
Carbofuran-3-keto	0.01	82.3 ± 4.7
	0.05	90.2 ± 6.7
	0.1	74.6 ± 3.9

[a]Each value is the mean ± standard deviation of triplicate determinations.

Carbofuran and its metabolite residues in watermelon plants

Only carbofuran and carbofuran-3-hydroxy residues were detected in the watermelon plants (Table 3 & Table 4). The carbofuran residues in all plant parts were detected 35 days after its application (Table 3 & Table 4). Carbofuran-3-keto was not detected even in samples from a plot treated at 34 kg/ha (Table 3 & Table 4). The carbofuran residue concentrations in the leaves, root, and stem increased from Day 1 to Day 9 and gradually decreased to below the quantification level at Day 49 (Table 3 & Table 4). In treatment 1 (17 kg/ha), the highest concentration of the carbofuran residues in the stem was detected on Day 5 at 0.134 mg/kg (Table 3). In root, the highest residue concentrations were detected on Day 9 at 0.192 mg/kg (Table 3); and for leaf on Day 21 at 0.057 mg/kg (Table 3). The application of carbofuran at 34 kg/ha, increased the concentrations of carbofuran residues in root to 0.446 mg/kg on Day 5 (Table 4). The concentrations of carbofuran residues in the stem were the highest at 0.151 mg/kg also on Day 5 (Table 4). The concentrations of carbofuran residues in the leaf were highest on Day 3 at 0.303 mg/kg (Table 4). In both treatments, the carbofuran residues in the skin were the highest on Day 5 and markedly decreased to below the detection limit of Day 7 (Table 3 and Table 4). The highest level of carbofuran residues in the flesh was 0.059 mg/kg, detected on Day 5 after the application of carbofuran at 34 kg/ha (Table 4). The concentration of carbofuran residues in the flesh was below the detection limit at Day 0 after the application rate of 17 kg/ha (Table 3). The rapid degradation of carbofuran and its metabolites after application has been reported for other crops (Singh and Kalra, 1992; Iqbal *et al*., 2007, Saini *et al*., 2020).

At the application rates of 17 kg/ha and 34 kg/ha, the conversion of carbofuran into carbofuran-3-hydroxy in the leaf lasted until Day 35 (Table 3); and for stem lasted until Day 21 (Table 4). The carbofuran-3-hydroxy residues were also detected in roots with the application of carbofuran at 34 kg/ha. Carbofuran-3-hydroxy residues were not detected in roots, skin, and flesh with the application rate of 17 kg/ha (Table 3). Similar results were observed in the watermelon skin and flesh at the application rate of 34 kg/ha (Table 4). In treatment 1, more than 80% of the total residues detected in the leaf on Day 3 belonged to carbofuran-3-hydroxy (Figure 1a). In treatment 2, about 80 to 90% of the carbofuran-3-hydroxy residues were detected in the watermelon leaf from Day 5 to Day 9 (Figure 2a). Similar findings were reported in sugarcane plant on Day 21 after the application of carbofuran (Battu *et al*., 2000), where carbofuran-3-hydroxy residues were found to comprise more than 80% of the total residues present for two types of carbofuran treatment levels in sugarcane plants after 21 days of application. More than 50% of carbofuran was metabolized to carbofuran-3-hydroxy in the stem at Day 14 regardless of the carbofuran application rates (Figure 1b & Figure 2b).

The carbofuran and carbofuran-3-hydroxy residues in all watermelon parts were not detected after 35 days of application. At harvest (42 days after the application of carbofuran), the carbofuran and its metabolites were not detected in the fruit. This finding was also reported for other crops by Singh and Kalra (1992) and Battu *et al*. (2000) where no residues were detected in groundnut and sugarcane juice at the time of harvest, respectively.

Table 3. Carbofuran and carbofuran-3-hydroxy residue concentrations in watermelon plant parts at different interval times after application of 17 kg carbofuran per hectare

Days after application	Leaves	Root	Stem	Skin	Flesh
Carbofuran (mg/kg)					
0	0.019 ± 0.001*	0.06 ± 0.007	0.125 ± 0.001	ND**	ND
1	0.032 ± 0.001	0.123 ± 0.008	0.129 ± 0.002	0.040 ± 0.002	ND
3	0.038 ± 0.002	0.127 ± 0.008	0.133 ± 0.002	0.042 ± 0.002	ND
5	0.039 ± 0.002	0.152 ± 0.006	0.134 ± 0.004	0.056 ± 0.02	0.002 ± 0.001
7	0.040 ± 0.001	0.144 ± 0.005	0.130 ± 0.001	ND	ND
9	0.040 ± 0.001	0.192 ± 0.006	0.129 ± 0.002	ND	ND
14	0.050 ± 0.001	0.111 ± 0.009	0.054 ± 0.001	ND	ND
21	0.057 ± 0.001	0.091 ± 0.007	0.061 ± 0.001	ND	ND
28	0.043 ± 0.001	0.053 ± 0.007	0.044 ± 0.001	ND	ND
35	0.038 ± 0.001	0.039 ± 0.003	0.038 ± 0.002	ND	ND
42	ND	ND	ND	ND	ND
49	ND	ND	ND	ND	ND
63	ND	ND	ND	ND	ND
Carbofuran-3-hydroxy (mg/kg)					
0	ND	ND	ND	ND	ND
1	0.053 ± 0.003	ND	0.050 ± 0.002	ND	ND
3	0.167 ± 0.006	ND	0.062 ± 0.004	ND	ND
5	0.173 ± 0.010	ND	0.068 ± 0.005	ND	ND
7	0.174 ± 0.010	ND	0.106 ± 0.005	ND	ND
9	0.096 ± 0.008	ND	0.062 ± 0.004	ND	ND
14	0.068 ± 0.007	ND	0.057 ± 0.003	ND	ND
21	0.066 ± 0.009	ND	0.051 ± 0.004	ND	ND
28	0.057 ± 0.006	ND	ND	ND	ND
35	0.049 ± 0.003	ND	ND	ND	ND
42	ND	ND	ND	ND	ND
49	ND	ND	ND	ND	ND
63	ND	ND	ND	ND	ND

*Each value is the mean ± standard deviation of triplicate determinations.

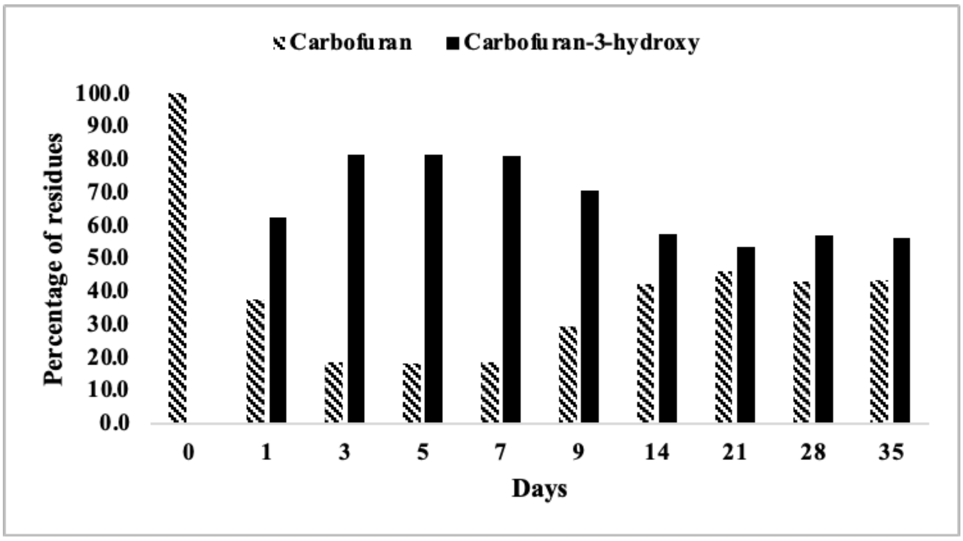
**ND, not detected.

Table 4. Carbofuran and carbofuran-3-hydroxy residue concentrations in watermelon plant parts at different interval times after application of 34 kg carbofuran per hectare

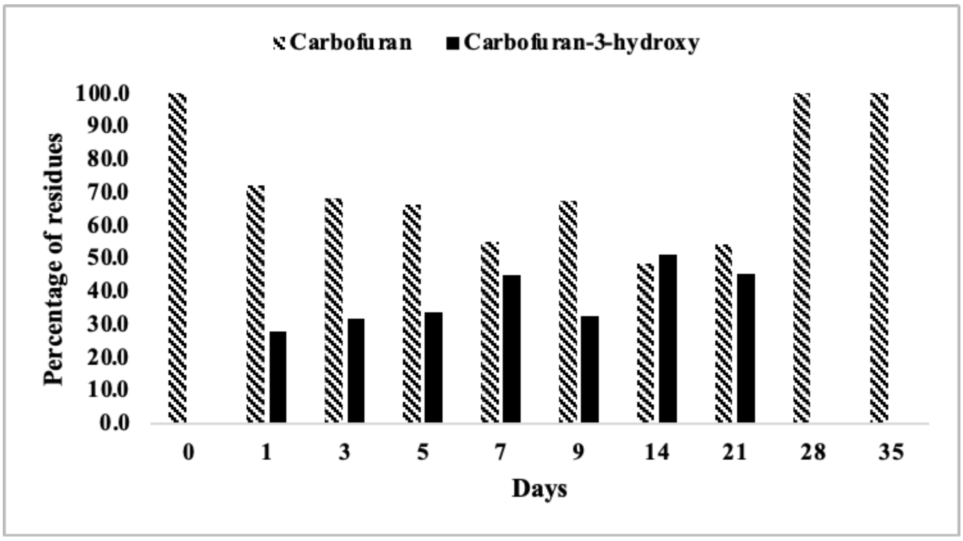
Days after application	Leaves	Root	Stem	Skin	Flesh
Carbofuran (mg/kg)					
0	0.023 ± 0.004[a]	0.06 ± 0.005	0.125 ± 0.001	ND[b]	ND
1	0.055 ± 0.002	0.226 ± 0.006	0.131 ± 0.001	0.040 ± 0.002	ND
3	0.303 ± 0.013	0.220 ± 0.009	0.133 ± 0.001	0.044 ± 0.008	ND
5	0.058 ± 0.004	0.446 ± 0.019	0.151 ± 0.007	0.065 ± 0.008	0.059 ± 0.007
7	0.044 ± 0.003	0.445 ± 0.010	0.145 ± 0.011	ND	ND
9	0.041 ± 0.002	0.212 ± 0.009	0.132 ± 0.002	ND	ND
14	0.052 ± 0.004	0.173 ± 0.009	0.059 ± 0.005	ND	ND
21	0.058 ± 0.001	0.140 ± 0.008	0.060 ± 0.002	ND	ND
28	0.056 ± 0.001	0.093 ± 0.002	0.059 ± 0.001	ND	ND
35	0.043 ± 0.001	0.060 ± 0.009	0.045 ± 0.002	ND	ND
42	ND	ND	ND	ND	ND
49	ND	ND	ND	ND	ND
63	ND	ND	ND	ND	ND
Carbofuran-3-hydroxy (mg/kg)					
0	ND	ND	ND	ND	ND
1	0.054 ± 0.003	0.029 ± 0.006	0.049 ± 0.001	ND	ND
3	0.135 ± 0.002	0.034 ± 0.003	0.055 ± 0.009	ND	ND
5	0.544 ± 0.010	0.026 ± 0.009	0.073 ± 0.006	ND	ND
7	0.642 ± 0.012	0.043 ± 0.012	0.080 ± 0.006	ND	ND
9	0.323 ± 0.006	0.033 ± 0.006	0.074 ± 0.007	ND	ND
14	0.145 ± 0.008	ND	0.055 ± 0.005	ND	ND
21	0.093 ± 0.016	ND	0.044 ± 0.006	ND	ND
28	0.092 ± 0.002	ND	ND	ND	ND
35	0.085 ± 0.005	ND	ND	ND	ND
42	ND	ND	ND	ND	ND
49	ND	ND	ND	ND	ND
63	ND	ND	ND	ND	ND

*Each value is the mean ± standard deviation of 3 replicate determinations.

**ND, not detected.

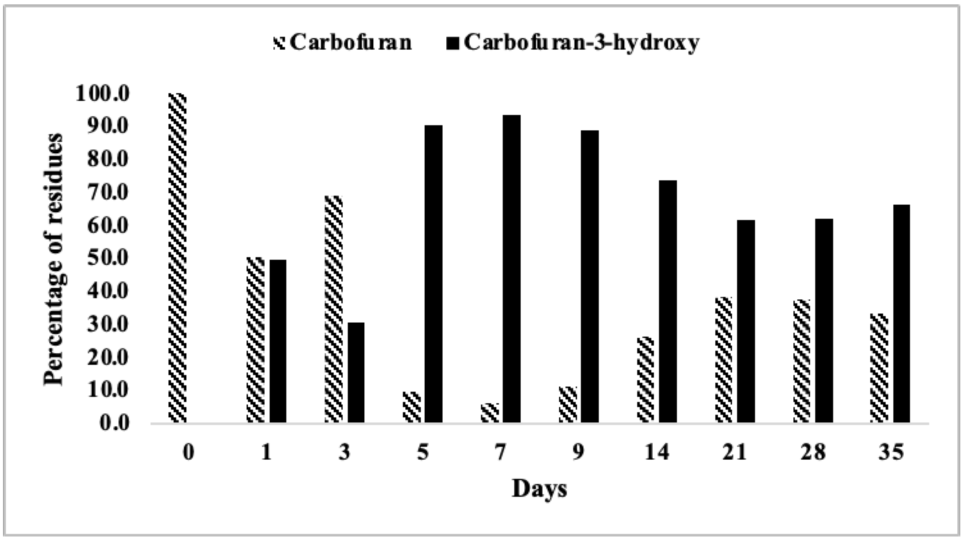


(a)



(b)

Fig. 1. Percentage of carbofuran and carbofuran-3-hydroxy residues in (a) leaves and (b) stems taken at different interval times after the application of carbofuran at 17 kg/ha.



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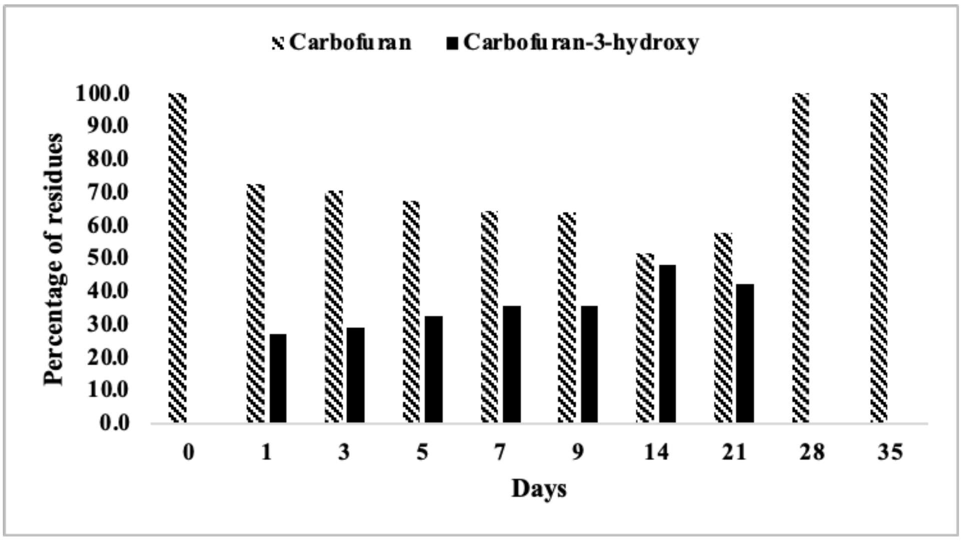

(b)

Fig. 2. Percentage of carbofuran and carbofuran-3-hydroxy residues in (a) leaves and (b) stems taken at different interval times after application of carbofuran at 34 kg/ha.

CONCLUSION

The findings suggested that a single application of carbofuran at either 17 kg/ha or 34 kg/ha during the early fruiting stage was considered acceptable provided the recommended postharvest interval is adhered to. However, due to the ban on carbofuran usage in the agriculture sector in many countries, alternative pesticides should be considered.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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