Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(2): 394-400

Received: 05-01-2018
Accepted: 08-02-2018

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Chemical constituents and insecticidal activity of the essential oils from *Thevetia neriifolia* Juss. on *Callosobruchus maculatus* (Coleoptera: Chrysomelidae)

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Abstract
Essential oils have been found to possess numerous biological activities which include pesticidal activities against various pests and mosquitoes. Essential oils were obtained from the flower, leaves, stem-bark, epicarp and seed of *Thevetia neriifolia* using hydro-distillation and analysed by Gas Chromatography coupled with Mass Spectroscopy (GC-MS). The insecticidal activity of the oils was evaluated on *Callosobruchus maculatus* (F.) using cultured cowpea seeds under laboratory conditions of 27 ± 2 ^{0}C ambient temperature and 78 ± 5% relative humidity. Parameters including mortality, repellent effects and viability of seeds were assessed and all data were analysed using Analysis of Variance (ANOVA). The percentage yield of essential oils from the flower, stem bark, leaves, epicarp and seed of oils were 0.55%, 0.39%, 0.35%, 0.30% and 0.40% respectively. A total of 11, 15, 17, 7 and 5 compounds were identified in the flower, stem-bark, seed, epicarp and leaf with E-5-Octadecene (25.01%), humulene (62.80%), methyl-11-octadecanoate (24.63%), methyl stearate (36.14%) and hexa-hydrofarnesylacetone (24.88%) being the predominant compounds in the essential oils respectively. The highest percentage mortality (36.11%) of adult *C. maculatus* to the oils was observed with 80% (v/v) concentration at 12, 18 and 24 hour post-treatment from the stem bark which was relatively better than other parts studied. The essential oil from the flower showed the highest pest repellent effect (88.43%) on adult *C. maculatus* at 80% (v/v) treatment when compared to the control. There was no significant difference in the percentage germination of treated and untreated cowpea seeds although the flower and seed oil were most effective at 80% (v/v). Therefore, it can be concluded that the essential oils of *T. neriifolia* were effective as protectant against *C. maculatus* on stored cowpea.

Keywords: Essential oils, *Callosobruchus maculatus, Thevetia neriifolia,* mortality, repellent, viability

1. Introduction
Thevetia neriifolia Juss. (Apocynaceae) is native to West Indies, Mexico, Puerto Rico, and Brazil. It is known as Olomiojo by Yorubas in south west Nigeria. It has been introduced into cultivation in Nigeria for over fifty years basically as an ornamental plant in homes, schools [1]. Some studies have demonstrated the insecticidal property of this plant on some insects and it has been reported that the leaf extract of *T. neriifolia* possess pesticidal activity [2], and larvicidal activity [3, 4]. *Callosobruchus maculatus* is a species of beetle known commonly as the cowpea seed beetle. It is a member of the beetle family, Chrysomelidae, and not a true weevil. The beetle most likely originated in West Africa and the major pests of cowpea in the tropics and sub-tropics due to the favorable climatic conditions [5, 6]. Therefore, it has a cosmopolitan distribution, occurring on every continent except Antarctica and moved around the globe with the trade of legumes and other crops [7]. These pests are responsible for the substantial quantitative and qualitative loses thereby reducing the degree of usefulness and making the seeds unfit either for planting or for human consumption [8]. As soon as they perforate the seeds, subsequent population build-up results into weight loss of stored cowpea seeds within six months especially if no prophylactic measures are put in place [9, 10, 11]. Synthetic chemical control of stored product insect pests including *C. maculatus* has been the most efficient and effective means for the protection of stored produce. But, the persistent use of organic synthetic pesticides has led to wide spread resistance in insect and other arthropod pests, and also caused irreparable damage to the ecosystem [12].

Therefore, pesticides from plant-based extracts have been suggested as an alternative to synthetic insecticides [13] and are thus being encouraged among farmers in the developing nations. Specifically, essential oils have demonstrated toxic effects against store-product insects and agricultural pests. They could be used as insecticides, fumigants, and larvicides and due to their low mammalian toxicity, could also be useful as alternative sources for controlling a number of insect pests including store-product insects [14]. This study was carried out to evaluate the efficacy of the essential oils from *T. neriifolia* against *C. maculatus* in stored cowpea seeds as way to promoting safer and natural pesticides.

2. Materials and Methods

2.1 Plant treatment and essential oil procurement

Fresh *Thevetia neriifolia* leaves, stem-bark, flower, epicarp and seed were harvested at Chemistry department, University of Ibadan, Oyo state, Nigeria in April 2018 when the plant parts were fresh. The plant samples were identified at the herbarium, Botany Department, University of Ibadan and voucher specimens (UIH-22693) of the plant deposited at the herbarium for further reference. The plant samples were air-dried for the period of one week in a shade away from direct sunlight, then pulverized and weighed. The essential oils of *T. neriifolia* were obtained by hydro-distillation using a modified Clevenger's type apparatus. Pulverized samples (200 g each) were used for the extraction. The oils were collected and stored in an amber coloured sample vial while the yield of the oils obtained were calculated and expressed as percentage weight/volume (%w/v) then stored in the refrigerator at 4ºC prior to analysis.

2.2 Gas Chromatography-Mass Spectrometry Analysis (GC-MS)

Gas Chromatography-Mass Spectroscopy analyses of the essential oils were performed with a Varian CP 3800 Gas chromatograph equipped with a HP-5MS capillary column (30m x 0.25mm x 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were met by making the injector and transfer line temperatures at 250^{0}C and 240^{0}C, respectively; oven temperature was programmed from 50^{0}C-240^{0}C and 3ºC/min; carrier gas used was helium at a flow rate of 1mL/min. The volume of the sample injected was 1 µL and the split ratio was 50:1 with split flow at 70.615 ml/min. The total chromatogram was auto-integrated and the constituents were identified by comparison of the GC-MS data with published mass spectral database (NIST 11.L) and literature data [15, 16].

2.3 Source of cowpea seeds and treatment

Untreated cowpea were harvested from a farm in Ogbomosho, Oyo state, Nigeria and identified at the Institute of Agricultural Research and Training (IAR&T), Moor Plantation Ibadan, Oyo State, Nigeria. Prior to experiments, the seeds were disinfested in a deep freezer for one week and later air-dried in the laboratory. They were later cleaned and kept for one week under experimental conditions for acclimation.

2.4 *Callosobruchus maculatus* culture

The research was carried out in Entomology research Laboratory, Department of Crop protection and Environmental Biology, University of Ibadan, Nigeria. The insects were cultured under laboratory conditions of 27 ± 2 ^{0}C ambient temperature and 78 ± 5% relative humidity. Initial culture of adult *C. maculatus* was raised from infested cowpea seeds purchased from Bodija market, Ibadan, Oyo State. Fifty pairs of adult weevil were introduced into 1 L Kilner jars, each containing 250 g of the disinfested seed and kept in the laboratory for one month for the insects to lay eggs and multiply. New generation of the beetles were subsequently reared on cleaned disinfested seeds in the laboratory and culture was maintained throughout the experiment. The jar was covered with wire mesh lid to allow for aeration and replicated three times for ready availability of insects throughout the experiment. Jars were placed on a table whose stands were dipped in oil to prevent contamination by ants.

2.5 Serial dilution of essential oils

Crude essential oil (0.4 mL) was diluted with (1.6 mL) of 95% ethanol to obtain a stock solution. From the stock solution, serial dilution was done to obtain 20.0%, 40.0%, 60.0% and 80.0% with a control containing 0.00% (v/v) ethanol only.

2.6 Mortality effects of the essential oils on *Callosobruchus maculatus*

Twenty grammes (20 g) of cowpea seeds were weighed into forty five plastic jars each. 1.0 mL of concentrations 20.0%, 40.0%, 60.0% and 80.0% (v/v) of the essential oils was applied each to the grains using a 1 mL syringe and homogenized to allow effective saturation of the oil within the grains. Seeds in the control jar were treated with ethanol only. There were five treatments with three replications. The seeds in the jar were infested with twenty adult (1 male: 1 female) *C. maculatus*, and the jars were covered with mesh to prevent escape of cowpea beetles and for aeration. Mortality was recorded at 6, 12, 18 and 24 hours after infestation. The insect were considered dead if they did not move when probed with brush. Dead beetles were removed at each assessment, counted and recorded. Data on percentage mortality were corrected using Abott's formula:

$$PT = \frac{Po - Pc}{100 - Pc} \times 100$$ [38]

Where PT: is the corrected mortality (%).
Pc is the control mortality (%)
Po is the observed mortality (%)

2.7 Viability effects of the essential oils on cowpea seeds

After four days all the grains were removed from the jars in experiment 2.6 above, twenty seeds were randomly selected from each jar and the same concentration were re-applied on them afresh to determine the viability of the seeds with each concentration. The seeds were placed on Petri dishes containing filter paper moistened with water. The Petri-dishes were moistened from time to time to avoid drying and in order to make the conditions for germination available. The number of germinated seeds was recorded and seed viability was calculated. The data recorded were processed in percentages using the formula:

$$\text{Percentage (\%) viability} = \frac{Number\ of\ germinated\ seed}{Number\ of\ seed\ sown} \times 100$$

2.8 Repellent effects of the essential oils on *Callosobruchus maculatus*

Repellence activity of essential oil from *T. neriifolia* against *C. maculatus* was evaluated using the area of preference method described by [17]. The test area consisted of 11.0 cm whatman No. 1 filter paper cut in halves and 200 µL of each of the three concentrations of *T. neriifolia* was applied uniformly to half-filter-paper disc with a micro syringe. The other portions of the paper were treated with ethanol (control). The half discs were air-dried for 10 minutes to allow the solvent to evaporate completely. Full disc were re-made by attaching the treated portions to the untreated halves of the same dimensions with cello tape and each placed in a Petri dish with ten adult beetles released separately at the centre of each filter paper disc in the Petri dish covered. Treatments (concentrations) were arranged in a completely randomized design (CRD) in three replications. The numbers of insects present on the control (untreated) and treated portion discs were recorded after 30 minutes exposure. Percent Repellency (PR) for each replicates was estimated as:

$$PR = \frac{Nc - Nt}{Nc + Nt} \times 100$$

Where (Nt) = number of insects present on the treated half disc and
(Nc) = number of insects present on the untreated (control) half disc,
A negative PR value was taken as zero and data on percent repellence were analyzed

2.9 Data analysis

The results obtained were analysed using Analysis of Variance (ANOVA) and significant mean values were compared at 0.05 significant level using the Least Significant Difference (LSD).

3. Results

The percentage yields of the essential oils of *T. neriifolia* were 0.55%, 0.35%, 0.39%, 0.30% and 0.40% for the flower, leaf, stem-bark, epicarp and seed respectively with different shades of yellow colour for the essential oils from the flower, seed and stem-bark while the oils from other parts are colourless. The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the essential oils from *T. neriifolia* is presented in Table 1. A total of 11 constituents were identified in the flower. The identified compounds made up 90.6% and categorised into ketones, alkenes and sesquiterpenoids. Ketones accounted for 21.89%, alkene (28.76%) while the sesquiterpeniods constituted a larger proportion (49.37%) of the oil with aromandendrene (8.35%), δ-cadinene (3.33%), 9,10-dehydro-isolongifolene (10.28%), γ-gurjunene epoxide-(2) (2.80%), dehydroxy-isocalamendiol (4.34%), 5-methyleneoctahydro-1H-Indene (6.57%) and Isolongifolene-9-one (4.18%). The epicarp essential oil gave 7 constituents representing 84.81% of the identified compounds. The major constituents identified were esters (19.9% methyl palmitate, 8.51% methyl elaidate and 36.14% methyl stearate) while 5 constituents were discerned in the leaf oil which made up 94.63% of the oil. The main constituents were esters and alkene. Esters accounted for 11.63% methyl palmitate and 16.58% methyl stearate while alkene constituted 24.56% octadecene. A total of 15 compounds were identified in the stem bark oil of *T. neriifolia* which made up 98.85% of the oil. The oil constitutes mainly sesquiterpenes, esters and carbonyls. The sesquiterpenes constituted the larger proportion (81.11%) as β-caryophyllene (7.27%), α-humulene (62.8%), β-humulene (0.62%), trans-α-bergamotene (0.78%), α-himachalene (4.26%), β-sesquiphellandrene (1.82%), 9, 10-dehydroisolongifolene (0.95%), caryophyllene oxide (0.48%) and β-elemene (2.13%). Esters accounted for 3.63% methyl palmitate, 5.82% methyl-11-octadecanoate and 1.10% methyl stearate. However, hydrocarbons constitute 3.84% of the oil, 3,4-dimethyl-3-cyclohexenylmethanal (1.1%), E-15-heptadecenal (1.57%) and stigmastan-7-one (0.38%). The seed oil had 17 compounds accounting for 97.2% of the essential oil which are mainly sesquiterpenoids and esters. Sesquiterpenoids accounted for 0.94% α-ionone, 1.53% α-gurjunene, 1.51% sativene, 2.8% trans-β-ionone, 2.13% aromandendrene, 1.93% cubenene while esters constitute 16.82% methyl palmitate, 24.63% methyl-11-octadecanoate, 20.87% methyl stearate and 1.5% methyl eicosanoate. The mortality effect of the essential oils from different parts of *T. neriifolia* on adult *C. maculatus* is reported in Table 2. Mortality was significantly higher ($P < 0.05$) in some of the concentrations compared to the control. The highest mortality (36.11%) was obtained on seed treated with 80% (v/v) concentration of the stem bark essential oils of *T. neriifolia* at 12, 18 and 24 hours of infestation. Although the highest percentage germination (76.67%) was recorded in grains treated with the highest concentration (80% v/v) of the flower and seed oil, this value was not significantly different ($p > 0.05$) from the percentage germination observed in grains treated with other concentrations of the oil as well as the control (Table 3). Apart from at 60% (v/v) of the stem bark of *T. neriifolia*, essential oils from the other parts of the plants at 60% and 80% (v/v) were highly repellent to *C. maculatus.* Furthermore, the highest percentage repellent effect (88.43%) was observed at 80% (v/v) treatment of essential oil from the flower (Table 4).

Table 1: Chemical Constituents of Essential oils of *Thevetia neriifolia.*

		% Compostion				
RT	**Constituents**	**Flower**	**Epicarp**	**Stem Bark**	**Seed**	**Leaf**
7.81	3-Carene	---	---	---	2.15	---
12.16	E-β-Damascene	---	---	--	0.94	---
12.70	β-Caryophyllene	---	---	7.27	---	---
12.76	α-Ionone	---	---	---	4.01	---
13.04	Farnesolformate	---	---	---	2.97	---
13.27	α-Humulene	---	---	62.8	---	---
13.31	α-Gurjunene	---	---	---	1.53	---
13.31	Aromandendrene	8.35	---	---	---	---
13.39	β-Humulene	---	---	0.62	---	---

13.42	Tetra-decamethylcycloheptasiloxane	---	---	---	0.88	---
13.49	Sativene	---	---	---	1.51	---
13.57	Diamino-hydroxyl pyrimidine	3.75	---	---	---	---
13.58	E-β-Ionone	---	---	---	2.80	---
13.61	(E)-α-Bergamotene	---	---	0.78	---	---
13.79	α-Himachalene	---	---	4.26	---	---
13.92	6-Epishybunone	2.83	---	---	---	---
14.00	β-Sesquiphellandrene	---	---	1.82	---	---
14.00	δ-Cadinene	3.33	---	---	---	---
14.80	Hexadecane	---	---	---	0.87	---
14.87	9,10-Dehydroisolongifolene	10.28	---	0.95	---	---
14.95	Aromandendrene	---	---	---	2.13	---
15.22	γ-Gurjunene	2.80	---	---	---	---
15.33	3,4-Dimethyl-3-cyclohexenylmethananl	---	---	1.10	---	---
15.59	Dehydroxy-isocalamendiol	4.34	---	---	---	---
16.05	Cubenene	---	---	---	1.93	---
16.09	5-Methyleneoctahydro-1H-iridene	6.57	---	---	---	---
16.12	Caryophyllene oxide	---	---	0.48	---	---
16.29	Isolongifolene-9-one	4.18	---	---	---	---
16.39	β-Elemene	---	---	2.13	---	---
16.40	E-Damascone	19.06	---	---	---	---
16.48	1-Acronone	---	---	---	---	16.98
16.49	1,4-E-1,7-Cis Ascronone	---	---	---	4.18	---
18.42	Hexahydrofarnesyl acetone	---	---	---	6.59	24.88
19.70	Methyl Palmitate	---	19.90	3.63	16.82	11.63
22.30	1-Octadecene	---	---	---	---	24.56
22.35	(E)-15-Heptadecenal	---	15.37	1.57	---	---
22.38	E-5-Octadecene	25.10	---	---	---	---
22.59	Methyl-11-octadecanoate	---	---	5.82	24.63	---
22.60	Methyl elaidate	---	8.51	---	---	---
22.99	Methyl stearate	---	36.14	1.10	20.87	16.58
29.24	Stigmasta-5,24(28)-dien-3β-ol	---	---	4.52	---	---
33.14	Dimethyl siloxane cyclic trimer	---	2.17	---	0.89	---
33.79	Dimethyl siloxane	---	1.14	---	---	---
33.99	Trimethylsilylbenzene	---	1.58	---	---	---
	Total	90.59	84.81	98.85	95.70	94.63

Table 2: Mortality Effect of the Essential oils *Thevetia neriifolia*

Plant Parts	Hours After Infestation	0.00%	20.00%	40.00%	60.00%	80.00%
Flower	6	0.00 a	5.32 ab	3.33 ab	14.44 b	1.25 a
	12	0.00 a	5.32 a	6.67 a	14.44 a	1.25 a
	18	0.00 a	13.16 a	20.00 a	14.44 a	1.25 a
	24	0.00 a	19.88 a	23.46 a	21.11 a	8.33 a
Epicarp	6	0.00 a	0.00 a	11.96 a	6.67 a	9.76 a
	12	0.00 a	7.31 ab	13.92 ab	15.41 b	14.76 b
	18	0.00 a	7.31 ab	15.88 ab	17.50 b	18.93 b
	24	0.00 a	7.31 ab	15.88 abc	25.00 c	18.93 bc
Stem –bark	6	0.00 a	2.04 ab	8.33 ab	6.67 ab	22.22 b
	12	0.00 a	11.30 ab	29.44 bc	21.11 abc	36.11 c
	18	0.00 a	11.30 ab	29.44 bc	21.11 abc	36.11 c
	24	0.00 a	11.30 ab	29.44 bc	21.11 abc	36.11 c
Seed	6	0.00 a	19.52 c	15.26 bc	5.00 abc	3.89 ab
	12	0.00 a	19.52 a	18.97 a	13.33 a	3.89 a
	18	0.00 a	19.52 a	18.97 a	13.33 a	3.89 a
	24	0.00 a	35.95 b	18.97 ab	13.33 ab	3.89 a
Leaf	6	0.00 a	3.70 a	2.78 a	2.04 a	3.07 a
	12	0.00 a	12.41 a	8.33 a	12.04 a	9.18 a
	18	0.00 a	12.41 a	8.33 a	12.04 a	16.32 a
	24	0.00 a	16.11 a	8.33 a	12.04 a	18.70 a

Means followed by the same letters within a row shows non-significant difference at $P<0.05$ using Least Significant Difference LSD).

Table 3: Viability activity of essential oil from *Thevetia neriifolia* on cowpea seeds

Plant Parts	Viability		
	0%	60%	80%
Flower	63.33 a	66.67 a	76.67 a
Seed	63.33 a	70.00 a	76.67 a
Epicarp	56.67a	65.00 a	66.67 a
Leaves	58.33a	66.67 a	73.33 a
Stem bark	61.00 a	70. 00 a	68.33 a

Means followed by the same letters within a row shows non-significant difference at $P<0.05$ using LSD

Table 4: Repellence activity of essential oil from *Thevetia neriifolia* on cowpea seeds

Plant Parts	Repellence		
	0%	60%	80%
Flower	0.00 a	75.00 b	88.43 c
Seed	0.00 a	57.14 b	69.05 c
Epicarp	0.00 a	69.05 b	75.00 b
Leaves	0.00 a	49.20 b	30.16 ab
Stem bark	0.00 a	22.22 ab	66.67 b

Means followed by the same letters within a row shows non-significant difference at $P<0.05$ using LSD

4. Discussion

The results of the Gas Chromatography-Mass Spectrometry (GC-MS) analysis (table 1) of the essential oils of flower, leaf, stem-bark, epicarp and seed of *T. neriifolia* showed the presence of sesquiterpenoids (81.11%) while the seed, epicarp, leaf and flower oil represented 45-65% esters. The use of essential oils in the preservation of cereals is increasingly recognized [18], in addition to acting as neurotoxins acutely interfering with octopaminergic transmitters in arthropods [19]. A lot of research on the pesticidal activities of essential oils has been conducted and has proven that essential oils could be considered as potent bioactive compounds against various pests and mosquitoes [20-24]. Topical application of the essential oils from *T. neriifolia* used mortality of *C. maculatus* with the stem bark oil possessing the highest mortality effect (36.11%) at 12-18 hour post treatment (table 2), suggesting that the oils can adversely contribute to the survival of the beetles, this agrees with the findings of Ketol *et al*., 2005 [25] who reported 90% mortality of *C. maculatus* at 6.7µL/L due to treatment with *C. schoenanthus* essential oil for 24 hours resulted in 90% of adult mortality [25]. α-Humulene, the major constituents of the stem bark oil, in this study, is a sesquiterpene that is derived from farnesylpyrophosphate (FPP) whose formation is catalysed by sesquiterpene synthesis enzymes [26] and has been studied for potential anti-inflammatory effects [27] as well as anti-cancer effects [28, 29]. Bioassay showed that the leaf oil from *Commiphora leptophloeos* whose major constituents are α-humulene, E-caryophyllene and β-phellandrene exhibited strong oviposition deterrent effects against *A. aegypti* at concentrations between 25 and 100 ppm and possessed good larvicidal activity suggesting that it could be an alternative to synthetic insecticide [30]. Similarly, one of the volatiles reported from the GC-MS analysis of the stem bark, caryophyllene, is a terpenoid released by damaged cotton [31], and the eastern yellow jacket, *Vespulama culifrons*is thought to use plant released substances to locate leaf feeding insects [32]. Caryophyllene from cotton plants similarly attracted adult *Chrysopa* [31]. *Eucelatoria* specie, a tachinid parasitid of *Heliothis* specie, was shown to be attracted to volatile chemicals from cotton, corn and okra [33]. [34] argued that caryophyllene epoxide, (one of the volatiles of the stem-bark of *T. neriifolia*), is a repellent because it is toxic to the alimentary fungus. Similarly, *azadirachtin* lies in effects on deterrent and other chemo-receptors resulting in anti-feedancy and direct effects on most other tissues studied resulting in an overall loss of fitness of the insect [35]. The presence of the mentioned constituents in the essential oils of *T. neriifolia* suggests that they may also possess insecticidal activities. It could also be inferred that the essential oils produced odour which was capable of damaging the respiratory system of the beetles thereby leading to their death. The mortality of *C. maculatus* increased with increasing hours of exposure to the odour. This findings is in agreement with the report of Hummelbrunner and Isman, 2001 [36] that citronella was toxic to *C. maculatus* (LD_{50} = 66.0-111.2µg/insect). From the result of the viability test (table 3), it could be inferred that the essential oils had no negative effect on the germination of cowpea seeds, an indication that the essential oils could be safe to use in protecting cowpea seeds from *C. maculatus.* The significant repellent activity of the essential oils observed from this study agreed with the studies conducted on the effects of volatile oil constituents of *Mentha* species against *C. maculatus* and *Tribolium castanum* reported by [37]. Futhermore, the high repellent effect (table 4) of the essential oil from *T. neriifolia* relative to the control and also suggests the presence of bioactive compounds in the oil that possess some fumigants actions on the beetles, *C. maculatus*.

5. Conclusion

The insecticidal properties of essential oils of *Thevetia neriifolia* manifested by contact with adult *C. maculatus* may be linked to the main volatile compounds (α-humulene, caryophyllene, aromandendrene), of essential oils acting alone or in synergy with other minor constituents (β-sesquiphellandrene, α-gurjunene) present in the plant parts studied. The outcome of this work would be useful in promoting further research in order to develop new agents for pest control based on biologically active chemical compounds from natural sources such as plants, as an alternative to synthetically-made insecticides. This will further reduce the irreparable damages done on the ecosystem and the widespread resistance built by insects over time against synthetic insecticides.

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