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Fumigant activity of insecticidal principles isolated from cassava (*Manihot esculenta* Crantz) against *Tribolium castaneum* and *Rhyzopertha dominica*

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Abstract
In the midst of logarithmic human population explosion it is very critical for being vigilant in the use of food resource with a motto on sustainability. The Ministry of Food and Civil Supplies, Government of India has projected that a total preventable post-harvest losses of food grains is around 10% of the total production or about 20 MT, which is equivalent to the total food grains produced by Australia annually. Most of the chemical fumigants used for the disinfection of stored-product pests have been phased out due to their adverse effect to man and environment; hence globally there is an intensive search for alternative to synthetic fumigants. Cassava, *Manihot esculenta* Crantz is cultivated for their starchy tubers as food, feed and industrial purposes. Cyanogens are the major category among the many other bitter principles in cassava. Central Tuber Crops Research Institute (CTCRI), Thiruvananthapuram has isolated the cyanogen derivatives and standardised techniques for the generation of Cassava-Bio-Fumigant (CBF) from this plant against the insect pests in many horticultural crops. In the current investigation, CBF was isolated, toxicity estimated and tested against *Tribolium castaneum* and *Rhyzopertha dominica* and the lethal doses were standardised. LD_{50} of CBF against the insect *T. castaneum* was higher over *R. Dominica* in experiments with food, without food and for respective larva. The CBF is more effective on treated insects in without food condition.

Keywords: Cassava bio-fumigant; Bio-pesticide; Pest management; Lethality study

Introduction

The Global demand of food grain is increasing with the increase in population growth, thus creates a never-satisfying need on agricultural produce. The world population by the year 2050 will be 9.1 billion as per WHO and to develop a policy to feed them will be a big challenge on agricultural research (parfitt *et al.,* 2010) [5]. As reported by FAO- world bank-2010 the post-harvest loss (PHL) of food grains is 1.3 billion tons per year. Fumigation is the major practice for pest control programme in flour and mill produce in many countries dueto their good dispersion and penetration properties into treated materials. Owing to the low cost and ease of application, phosphine (PH_3) is the most widely used fumigant. Low dosage of many currently used structural fumigants has reduced their ovicidal activity on grain pests. Wide and repeated usage of commercially used fumigant (PH_3) generates resistance for several populations of storage pests from various parts of the world (Jagadeesana *et al.,* 2012) [3]. The ineffectiveness of chemical fumigants follows a quick rebound of pest population is an alarm. (James *et al.* 2010) [6].

As of every states of pesticide; the fumigants are the most dangerous category since they easily get entrained into human body through inhalation. Several reports including both research as well as medical-case are available stating the hazardous status of very common chemical fumigant pesticides. It's really unfortunate to abandon such synthetics after victimising many human lives. "Methyl bromide (MB) is to be phased out by 2015 from the entire globe" is a statement from the Montreal protocol (2015) but regular bann-extentions are still continuing in India. It was also very critical to counter affect the resistance achieved by pests over many of the single compound synthetic fumigants. Even from the early 1970's FAO report there is an increase of pest population showing resistance to phosphine (single compound synthetic fumigant) worldwide (Yosep *et al.* 2012) [14], still no successive alternative to phosphine get functionally established though some encouraging results are there by Koul (2004),

Khamrunissa *et al.* (2006) [7] and Zhaorigetu *et al*. (2011) [15].
Cassava (*Manihot esculenta* Crantz) is the staple food for more than 500 million people in the tropics. Tuber is the edible part of the plant and the leaf is a cockatiel of many nitrogenous toxic compounds including cyanogens, the precursor of cyanide. The cyanogenic potential of cassava leaves ranges from 2 to 1000ppm. (Ngugi *et al.*, 2015) [9] (Howard Bradbury *et al.*, 2001) [2] (Paula *et al.*, 2004) [10]. Cyanogenic glucosides, Linamerin and Lotaustralin are the two major compounds in the leaves and roots of cassava. The hydrolysis of Linamerin yields glucose and acetone cyanohydrin in the presence of an enzyme Linamerase or beta-D-glucosidase (EC 3.2.1.21), which is produced by the plant. In neutral or alkaline condition, the acetone cyanohydrin decomposes and liberates HCN as cyanide ion.
In European Union countries, HCN is commercially available and registered as biocide for structural fumigation (Aulicky *et al.*, 2014) [1] and employed in tobacco stores and flour mills. The seed fumigation with HCN experimentally proved that it was not diminished the grain germination and may have seen slightly enhanced with HCN exposure (Zouhar1 *et al.*, 2016) [16]. HCN has good penetration and biological efficacy on construction wood and its infesting pests (stejskal *et al.*, 2012) [11, 12, 16] and has a good activity on Nematodes *Ditylenchus dispaci* infesting semi day garlic (Testen *et al.*, 2014) (13). Various laboratory studies reported that HCN has an excellent biocidal activity against various stored grain pests and their developmental stages (Lindgren and Vincent, (1965) (8); But, under field condition, there is only limited information about the biological efficacy of HCN in relation to stored grain pests (Aulicky *et al.*, 2015) [1]
The test insects *Tribolium castaneum* (TC) and *Rhyzopertha dominica* (RD) are cosmopolitan in distribution and found throughout the world. In India these are considered as serious pests that are found infesting all stored products like seeds, grains, flour, dry fruits, nuts, oil cakes, dry museum specimens and stuffed animals. The infected flour turns greyish-yellow colour or develops red taints which subsequently becomes mouldy and emits offensive pungent smell. In the current study, the toxic principle (Hydrogen Cyanide) in cassava leaf was isolated as a bio-fumigant and their lethal doses on TC and RD were standardised using most suitable statistical analysis.

Materials and Methods

Study insects

Biological efficacy of cassava bio-fumigant (CBF) was done in laboratory over two major stored grain pests' *viz*. *Tribolium castaneum* (TC) and *Rhizopertha dominica* (RD). Samples used throughout the study were a cohort of tenth generation individuals from the culture maintained at the laboratory (Temperature: 32±2 ºC and humidity: 65rel) over their respective diet demands. About 100 – 150 adult insects were released in a rearing glass jar (15cm x 10cm) containing 100g of wheat flour or black gram for TC and RD respectively for feeding and oviposition. The adult insects were removed 14 days after emergence and the larva were allowed to grow by keeping the jar undisturbed till new adults start emerging. Cohorts of adult insects (40 nos.) were used for each bioassay analysis throughout the experiment.

Isolation of Cassava Bio-Fumigant

Cassava leaves were freshly collected, weighed and pulverised with water in an airtight mode. Digestion and extraction was done using pilot plant commissioned at ICAR-CTCRI. The slurry is transferred to a digestion chamber and allowed to undergo a series of enzymatic reactions facilitated by optimum physical conditions assured in the chamber through an automatic programmer installed with the technical support of Indian space research organisation (ISRO). The bioactive fumigant liberated is dried by passing it through a series of moisture absorbing chambers and finally get collected in air tight containers of volume demanded ranging from 1L to 20L.

Estimation of bioactive principle in Cassava Bio-Fumigant (CBF):

The principle insecticidal component in cassava leaf is hydrogen cyanide which gets liberated when the cells break. Hydrolysis of Linamerin and Lotaustralin (figure: 1), the two major cyanogenic compounds in the cassava leaf, by the enzyme linamerase generates hydrogen cyanide in compound form or as free ions. HCN has a vaporising point of 20 ºC (630 mm Hg) and boiling point very close to 26 ºC, thus even in room temperature (30 ºC) it exist in gaseous form. In this experiment, the compressed form of cassava bio fumigant was analysed for the presence of its active component (HCN) by Gas Chromatographic (GC) method. Since GC cannot detect compounds with very low molecular weight (<30.00 g/mol), the accuracy in determining the presence of HCN (27.02 g/mol) cannot be done directly. The procedure is based on the conversion of hydrogen cyanide in cassava bio-fumigant to cyanogen chloride (CN Cl) using Chloramine-T solution (Juan *et al.*, 2006) [4]. Chloramine-T (0.77g) was dissolved in 50ml deionized water and kept in refrigerator in an amber coloured container. The cassava bio-fumigant was passed through 20ml of sodium hydroxide solution (0.1MolL^{-1}) to trap the cyanide content of the fumigant as sodium cyanide (Na CN) (figure:2). After 10minutes the Chloramine-T solution (0.30ml) and n-Hexane (3ml) were added to the Na CN solution as an extraction solvent of cyaonagen chloride (CN Cl). The whole mixture of solution were mixed thoroughly and kept for 20 minutes to separate the active component. A 1.0µl of the sample solution were analysed using GC (Figure: 3). A standard stock solution was made with 500µgL^{-1}cyanogen ion (potassium cyanide) and 50ml, 0.1molL^{-1} sodium hydroxide solution.

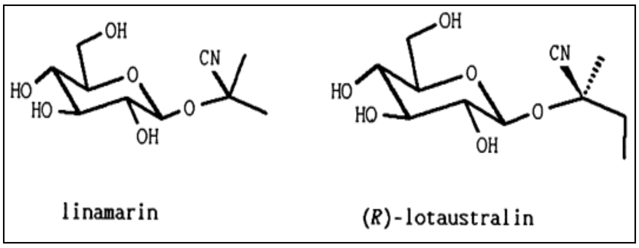

Fig 1

Fig 2

HCN concentrations in each of the collection cylinders are determined prior to filling by alkaline picrate-paper method (Williams and Edward, 1980); in which freshly made (Bradbury *et al*., 1996) [21] alkaline picrate paper (5×1cm) was exposed to the gas evolved from the pilot plant placed midway between the airtight lines for 30minutes. Optical Density (OD) estimation was done using a spectrophotometer (PerkinElmer Lambda 25) at 510 nm absorption. OD to concentration conversion was done by using the equation
Concentration of HCN (ppm) =396 x absorbance x 100/5000gm* (Bradbury *et al*., 1996) [2]
(*weight of cassava leaf)

Lethality estimation of CBF over test insects:
Larvae and adult insects (40 nos each) of same cohort of *Tribolium castaneim* and *Rhizhopertha dominica* were reared in the laboratory and were taken in separate vacuum containers (5L each). Insects were maintained with and without food accordingly and the cassava bio-fumigant of different concentrations was treated against the test insects. Three replications of each concentration were observed, mortality was monitored at different time intervals and the lethal concentrations where standardized.

Results
Estimation of HCN in cassava bio-fumigant:
In this novel method for estimation of Hydrogen cyanide as Cyanogen chloride using gas chromatography the peak for CN Cl perfectly distinguishes it from the base line at retention time of 4.7 minutes (fig. 1). The concentration of Chloramine-T had a significant role in the yield of Cyanogen chloride. The maximum amount of Cyanogen chloride was obtained with 0.3ml Chloramine-T for 40.0 mg ml-1 solution. Considering the concentration of samples, 0.30 ml Chloramine-T was chosen as the preferred concentration which is chosen by Juan (2006) [18] on the work on determination of cyanide content in cigarette smoke.

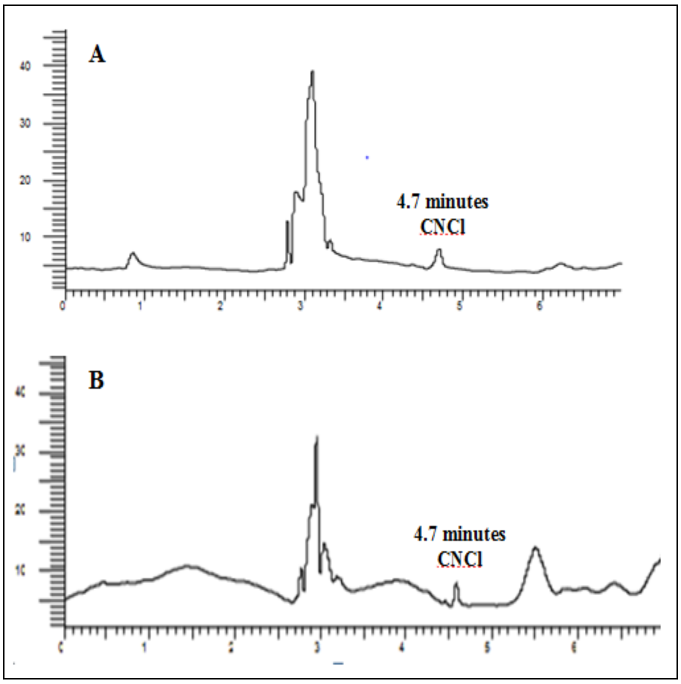

Fig 3: A: Gas chromatogram showing peak of standard cyanide; **B:** cyanide in cassava bio-fumigant.

Quantity estimation of HCN in cassava bio fumigant:
Evolution of HCN fumes from the cassava leaves fascilitated by enzymes at optimum physical conditions in the digestion chamber was detected by the the change in colour of yellow picrate paper placed in the passage tube to brick red colour. The release of active component begins at a temperature of 40 ºC. Spectrophotometric analysis gives the concentration of HCN fumes passed through the path in a unit time. The absorbance value and its corresponding concentration (ppm) of active component indicated that the release of active component increses with the increase in temperature (Table: 1).

Table 1: Concentration of casava bifumigant at different temperature.

Temperature (ºC)	Absorbance (OD)	Concentration (ppm)
40	0.58^{M}	4.60^{M}
45	0.74^{M}	5.87^{M}
50	0.84^{M}	6.66^{M}
55	1.27^{L}	10.06^{L}
60	1.87^{K}	14.78^{K}
65	2.28^{J}	18.07^{J}
70	2.55^{IJ}	20.18^{IJ}
75	2.68^{HI}	21.25^{HI}
80	2.54^{IJ}	20.11^{IJ}
85	3.03^{H}	24.01^{H}
90	3.49^{G}	27.68^{G}
95	4.14^{F}	32.82^{F}
100	5.09^{E}	40.30^{E}
105	6.92^{D}	54.84^{D}
110	8.96^{C}	70.98^{C}
115	10.00^{B}	79.20^{B}
120	11.96^{A}	94.75^{A}
General Mean	4.06	32.13
CV (%)	5.79	5.79

The Duncan's Multiple Range Test (P<0.05) was used and the significant differences in each column are indicated by different letters. There were 3 replicates for each variable (mean).

Lethality study of Cassava bio-fumigant on test insects
The insecticidal property of cassava bio-fumigant at different concentrations (ppm) was studied against the larvae and adults of test insects *Tribolium castaneim* and *Rhizhopertha dominica* and their mortality was estimated (Table: 2).

Table 2: Mortality of test insects by the treatment of cassava bio-fumigant.

Concentration (ppm)	*Tribolium castaneim*			*Rhizhopertha dominica*		
	Adult		Larvae normally fed	Adult		Larvae normally fed
	with food	without food		with food	without food	
0.00	-	-	0.00^{J}	-	-	0.00^{L}
4.60	-	-	4.33^{I}	-	-	2.67^{K}
5.87	-	-	7.67^{H}	-	-	4.33^{K}
6.66	-	-	10.33^{G}	-	-	6.67^{J}
10.06	-	-	14.00^{F}	-	-	9.00^{I}
14.78	-	-	17.00^{E}	-	-	13.33^{H}
18.07	-	-	19.67^{D}	-	-	16.67^{G}
20.18	-	-	23.67^{C}	-	-	18.33^{G}
21.25	-	-	34.33^{B}	0.00^{H}	0.00^{H}	22.33^{F}
20.11	-	-	39.33^{A}	4.00^{G}	1.67^{G}	25.33^{E}
24.01	0.00^{F}	-	40.00^{A}	12.00^{F}	4.33^{F}	28.67^{D}
27.68	4.33^{E}	0.00^{E}	-	12.67^{F}	9.00^{E}	32.67^{C}
32.82	6.00^{E}	19.67^{D}	-	16.67^{E}	14.00^{D}	36.33^{B}
40.3	19.33^{D}	20.33^{C}	-	18.67^{D}	14.67^{D}	40.00^{A}
54.84	20.00^{D}	26.67^{B}	-	29.67^{C}	40.00^{C}	-
70.98	23.33^{C}	40.00^{A}	-	34.67^{B}	-	-
79.2	36.67^{B}	40.00^{A}	-	40.00^{A}	-	-
94.75	40.00^{A}	40.00^{A}	-	-	-	-
Mean	8.80	10.29	28.84	12.25	10.14	24.43
CV (%)	11.15	5.79	3.06	7.22	7.24	3.61

The Duncan's Multiple Range Test (P<0.05) was used and the significant differences in each column are indicated by different letters. There were 3 replicates for each variable (mean). Cells kept blank (-) are insignificant.

In the first set of experiment, the treatment of cassava bio-fumigant on *T. castaneum* without food, 100% of mortality was obtained at 30minute after treatment (MAT) on 70.98ppm concentration. 100% mortality is obtained in TC with food at 934.75ppm concentration on 35MAT. The mortality rate of TC without food is greater than that of TC with food. While treating the cassava bio-fumigant on TC larva, death begins merely after10 minutes of treatment and 100% of mortality was obtained after 35 minutes of treatment at a very low concentration (24.01ppm), indicating that the larvae of TC is more susceptible to cassava bio-fumigant than adults (Fig:6).

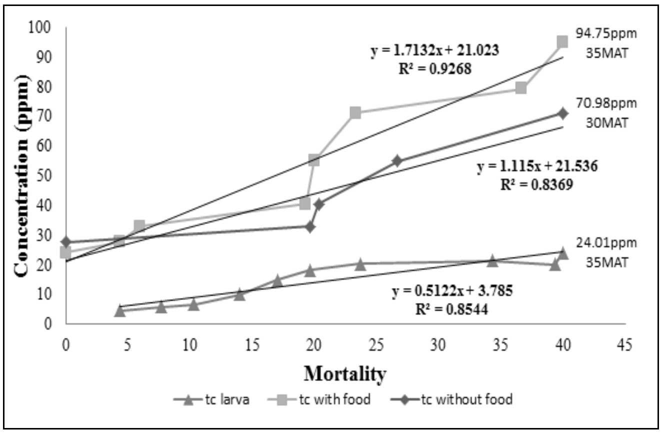

Fig 6: Concentration of cassava bio-fumigant against *Tribolium castaneum*

In the second set of experiment, the cassva bio-fumigant was treated against *R. dominica* without food and 100% mortality was obtained after 15 minutes of treatment with a concentration of 54.84ppm and that of with food was 79.2ppm after 20 minutes of treatment. The larvae of RD show 100% mortality at a concentration of 40.3ppm after 20 minutes of treatment (Fig.7).

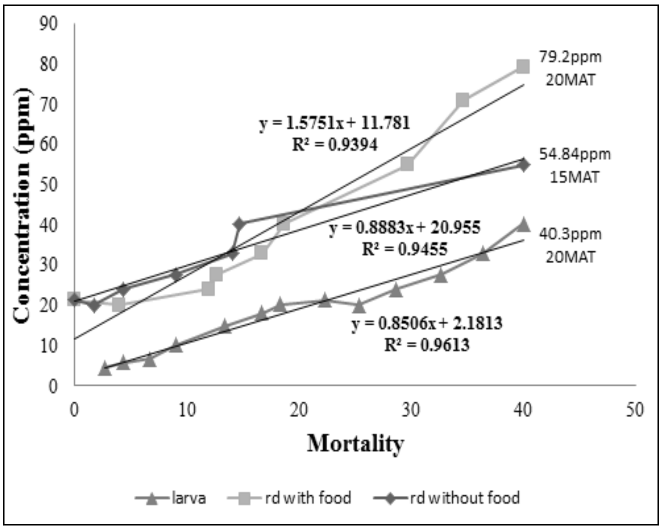

Fig 7: Concentration of cassava bio fumigant against *Rhizhopertha dominica*

When comparing the mortality rates of both the test insects, the adult *Rhizhopertha dominica* is more susceptible to cassava bio-fumigant than *Tribolium castaneim* in both with and without food experimental conditions. From the study it was observed that the mortality rate of test insects decreases in the presence of food indicates the absorbance of active component by the moisture content in the food grains, which can easily get removed during heating or cooking. The larva of both insects has a high mortality rate with cassava bio-fumigant which prevents the emergence of second generation in the treated food grains.

Estimation of lethality limits of cassava bio-fumigant on test insects:

The lethal concentrations of cassava bio-fumigant against *T. castaneim* and *R. dominica* was standardised by conducting concentration *v/s* mortality studies (Table 3).

Table 3: Lethal Concentration of Cassava Bio-fumigant on test insects

		Tribolium castaneim			*Rhizhopertha dominica*		
		LC_{50}	LC_{90}	LC_{99}	LC_{50}	LC_{90}	LC_{99}
Adult	**Without food**	33.7±0.2	48.7±0.3	72.7±0.4	23.1±0.2	27.8±0.2	34.1±0.2
	With food	42.4±0.5	67.1±0.6	110.3±0.5	23.6±0.3	31.6±0.3	43.4±0.4
Larvae		7.0±0.1	13.7±0.2	28.4±0.2	6.2±0.1	12.8±0.1	28.0±0.2

Analysis: SAS 9.3 statistical software. There were 3 replicates for each variable.

The lethal concentrations of cassava bio-fumigant against the two test insects shows, in all the experimental conditions the insect RD are more susceptible to cassava bio-fumigant than TC. The larva of test insects was more susceptible to the bio-fumigant than adults hence prevents the emergence of second generation in the treated food grains.

Discussion

Cyanogens are the main active principles in cassava bio fumigant and this has been an approved chemical for the pest management strategies. In California, cyanohydrins has been using in trees under tents against scale insects (Woglum, 1949). Lehmann (1959) reported that in a controlled laboratory experiment conducted for two years by incorporating its regular feeds with 300 ppm of HCN, no symptoms of poisoning was reported. FAO (1984, 1989) reported cyanogen is an effective fumigant than the other synthetic or complex-organic fumigants. As reported by FAO in 1989 a continuous exposure of Cyanogens for more than two hours in 100ppm only causes anoxia to humans. While using cassava bio fumigant, only 67.1±0.6 ppm and 31.6±0.3ppm is enough for the complete mortality of the test insects *T. castaneim* and *R. dominica* respectively and hence the hazards due to the application of cassava bio fumigant is negligible.

Cyanogens, being an efficient fumigant, are normally not advisable for spray in open fields, but it gives confirmed result under confined fumigation. In the present study we examined the effect of cassava bio fumigant in 5L confined containers and the success of the experiment leads to large scale use of cassava bio fumigant in warehouses as a remedy for the post-harvest lost of food grains by insect pests. Although a number of plant species have been reported to possess insecticidal properties, non-availability of the raw materials in sufficient quantities and cumbersome procedures in the extraction procedures impede their commercial exploitation, but cassava sets against this dictum, as the raw materials availability is abundant and the extractability of active principle is remarkably high. The green pesticide extracted from cassava will be a glaring contribution to the effort for searching novel molecules to protect the mother earth from the clutches of synthetic pesticides

Conclusion

Fumigants dissipate evenly into the whole batch of grain products and kill the pests, including the larval stages harbouring inside the kernels. Due to its ozone depletory nature of methyl bromide and ethylene di-bromide, were banned in industrialized countries that singled out phosphine as a widely acclaimed fumigant. But insects particularly stored grain pests are getting resistance over phosphine. Hence globally there is an intensive search for alternative to synthetic fumigants. The current study scientifically supports the utility of cassava leaves as a source for the isolation of insecticidal principles and to formulate an eco-friendly bio-fumigant for the management of major stored grain pests such as *Tribolium castaneum* and *Rhyzopertha dominica*. Popularity of this bio-fumigant will lead for a clean environment, and also fetch additional income to cassava farmers.

Disclosure Statement

The authors are not aware of any biases that might be perceived as affecting the objectivity of this research article.

Competing Interest

I declare that the authors have no competing interests as defined by this publishing group, or other interests that might be perceived to influence the results and/or discussion reported in this article.

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