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Research Article

Efficacy of cassava leaf bio-fumigant against *Tribolium castaneum*, *Rhyzopertha dominica*, and their symbiotic bacteria in comparison to phosphine fumigation

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ABSTRACT: Symbiotic bacteria in insects aid in detoxifying insecticides, contributing to resistance against synthetic pesticides. This study focuses on *Tribolium castaneum* and *Rhyzopertha dominica*, two major grain pests resistant to phosphine. A biofumigant that was developed from cassava leaves was tested against above two stored grain pests and with its proven pesticidal properties were further evaluated against endosymbiotic bacteria associated with these insects. The response of characterised compound of Cassava Leaf Bio-fumigant (CLBf) against *in vitro* culture of endosymbiotic bacteria was compared to phosphine. Mortality assays revealed that CLBf achieved 100% mortality for *T. castaneum* and *R. dominica* at concentrations of 49.84 ppm and 35.6 ppm, respectively, within a 5-minute exposure. The antibacterial effects of CLBf and phosphine were assessed by measuring the growth inhibition zones on Mueller Hinton agar plates. CLBf exhibited significant antibacterial activity against the endosymbionts of both insects, whereas the bacterial strains showed resistance to phosphine. The study highlights the role of endosymbiotic bacteria in pesticide detoxification.

KEYWORDS: Biofumigant, cassava leaves, endosymbiotic bacteria, insect resistance, phosphine

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INTRODUCTION

Food grain is a fundamental necessity for human survival, and its global demand is rising with the burgeoning population, exerting tremendous pressure on agricultural production. This escalating need impacts the world economy at both the micro and macro levels (Basavaraja *et al.*, 2007; Parfitt *et al.*, 2010). A critical issue in agriculture is postharvest loss, with approximately one-third of the global food produced annually (worth about one trillion US dollars) going unconsumed due to postharvest operations (Aulakh *et al.*, 2013). Insect infestations pose a significant threat to stored grain products, causing about 30% to 40% of grain losses (Abass *et al.*, 2014). This study focuses on two cosmopolitan pests, *Tribolium castaneum* (the red flour beetle) and *Rhyzopertha dominica* (the lesser grain borer), which infest grains and flour, causing discolouration, mold growth, and a pungent smell. Currently, synthetic fumigants like methyl bromide and phosphine are the primary methods for controlling these beetles in the storage grain industry (Lessard, 2016; Rajendran & Sriranjini, 2008). However, due to its ozone-depleting characteristics, in many developed countries, the application of methyl bromide has been banned, leaving phosphine as the most widely used fumigant (Bulathsinghala & Shaw, 2014). Despite its effectiveness, the rapid development of resistance by stored-product insect pests against phosphine presents a significant challenge (Collins *et al.*, 2002). Around 15 insect species have been reported for the resistance development to phosphine, resulting in a complete loss of efficacy (Nayak *et al.*, 2013; Benhalima *et al.*, 2004). *T. castaneum* and *R. dominica* have also developed strong resistance to phosphine due to its prolonged use (Opit *et al.*, 2012; Subramanyam, 1996; Zettler, 1993). According to a 1970s FAO report, pest populations showing resistance to phosphine have increased, and no effective alternative has yet been established (Mau *et al.*, 2012).

Endosymbiotic associations between insects and bacteria are common in nature. Bacteria exhibit primary and secondary symbiotic associations with insects. Primary symbionts are obligate, vertically transmitted through maternal inheritance, have ancient associations with their

hosts, are confined to specialised cells, and are crucial for the reproduction and survival of the host (Fukatsu *et al.*, 2000; Zchori-fein & Brown, 2002). In contrast, secondary symbionts are facultative, can be cultured *in vitro*, and function more effectively with their hosts (Malke, 1967; Douglas, 2007). These symbionts play crucial roles in their host's physiology, ecology, and evolution, facilitating food digestion, nutrient absorption, and protection from natural enemies (Chiel *et al.*, 2007; Zchori-Fein *et al.*, 2014; Douglas, 2015). Endosymbiotic bacteria contribute to insecticide resistance in insects by secreting enzyme complexes that detoxify xenobiotics (Kikuchi *et al.*, 2005; Sreerag *et al.*, 2014). In light of the increasing resurgence of pests against synthetic fumigants like phosphine, there is a global effort to find safe, economical, and viable alternatives, including botanicals, antifeedants, and insect growth regulators. The application of the materials of naturally occurring plants from insect pests for the protection of agricultural products is an age-old practice. Because they are readily available, biodegradable, and least harmful to creatures other than the intended target, botanicals are recommended. Secondary compounds found in some plants function as larvicides, antifeedants, oviposition deterrents, and insect development regulators (Krishnan & Ajesh, 2023). Selected phytochemicals have been confirmed useful against a wide range of insect pests, including thousands of terpenoids, phenolics, and alkaloids. Although these chemicals in plants do not play major roles in growth, photosynthesis, or another basic aspect of physiology, their bioactivity against insects, nematodes, and phytopathogens is comprehensively documented (Krishnan *et al.*, 2017).

Cassava (*Manihot esculenta* Crantz) is a staple food, with the tuber being the edible part of the plant. The leaves contain various nitrogenous toxic compounds, including cyanogens, the precursors of cyanide. Both the leaves and roots are rich sources of cyanoglycosides like linamarin and lotoustralin (Cho *et al.*, 2013). Linamarin breaks down in the presence of the subcellular enzyme linamarase, forming acetone-cyanohydrin, which spontaneously or enzymatically decomposes into acetone and volatile Hydrogen Cyanide (HCN), toxic to many organisms (Yu, 2014). After eliminating the toxic principle by sun drying, some farmers use the leaves as cattle feed. In this investigation, the active principles from cassava leaves were isolated to generate a biofumigant against stored grain pests. The study focused on the molecular characterisation of major stored grain pests, *T. castaneum* and *R. dominica*, the endosymbiotic bacteria they harbour, and their insecticide resistance properties against synthetic phosphine and cassava biofumigant. This approach aims to provide a safer, more sustainable alternative to synthetic fumigants, addressing the issue of resistance development in these pests.

MATERIALS AND METHODS

Test insects

Tribolium castaneum and *R. dominica* were selected for this study (Figure 1). These insects were collected from a stock culture maintained in the laboratory under controlled conditions (temperature: 32±2°C and relative humidity: 70±5%), with an *Ad-libitum* food supply (Ajesh *et al.*, 2020).

Molecular identification of insects

Genomic DNA was isolated using a modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Gawel & Jarret, 1991). Individual insect samples were homogenised in 500 µL lysis buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.1% 2-mercaptoethanol) and suspended in the same buffer. The suspension was incubated at 65°C for 1 hour, then centrifuged at 10,000 rpm for 10 min. An equal volume of chloroform: alcohol (24:1) was added to the supernatant, then centrifuged at 6,000 rpm for 15 min at room temperature. The upper aqueous layer was transferred to a fresh microcentrifuge tube, and DNA was precipitated by adding 40 µL of sodium acetate and 600 µL of 95% ethanol. The tubes were stored at -20°C for 20 min, followed by centrifugation at 8,000 rpm for 10 min. The supernatant was discarded, and the resulting pellet was washed with 70% ethanol and then dissolved in 50 µL of DNase-, RNase-, and protease-free molecular biology water. The intact genomic DNA was quantified using a Nanodrop ND-1000 (Thermo Scientific, Belgium) and diluted with sterile water to obtain a working solution of 50-100 ng/µL.

PCR was performed using a thermal cycler (AB-Applied Biosystems, Veriti 96 wells) with the following cycling conditions: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 s, annealing at 47°C for 45 s, and extension at 72°C for 45 s; and a final extension at 72°C for 10 min, followed by a hold at 4°C. Primers specific to mitochondrial cytochrome oxidase (COX-1) were used:

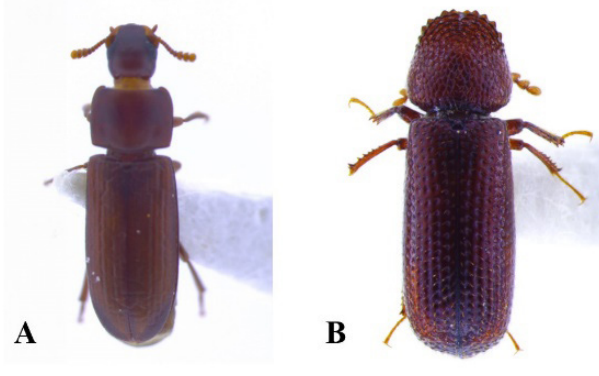


Figure 1. Test insects: **(A)** *T. castaneum* and **(B)** *R. dominica*.

LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer *et al.*, 1994). PCR was conducted in a 25 µL total reaction volume containing 20 pmol of each primer, 1.0 µL of 20 mM dNTP, 2.5 µL of 10X buffer, and 1.0 µL of 1.0 U Taq DNA polymerase (Fermentas Life Sciences, Maryland, USA). The amplified products were resolved on a 1.0% agarose gel, stained with ethidium bromide (10 µg/mL), and visualised using a gel documentation system (UVP).

The mitochondrial COX-1 gene sequences were generated by sequencing the PCR products on an Applied Biosystems 3500 Genetic Analyzer (Big Dye Terminator v 3.1). The nucleotide sequences were compared with those in the NCBI databases using the Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed from the aligned sequences using MEGA 6.06 software (Tamura *et al.*, 2013). The mitochondrial COX-1 gene sequences have been deposited in the NCBI GenBank.

Isolation of cassava bio-fumigant

The active principles were isolated from cassava leaves using a Pilot Plant unit designed by the Crop Protection division of ICAR-Central Tuber Crops Research Institute (Figure 2). Freshly collected cassava leaves were weighed and directly loaded into the mixer-cum-grinder of the extraction unit to pulverise with a predetermined quantity of water. The resulting slurry was transferred to the digestion chamber through a confined outlet located at the lower side wall of the grinder near its bottom. In this chamber, the slurry was agitated with water and allowed to undergo a series of enzymatic reactions facilitated by optimum physical conditions, ensured by an automatic programmer with technical support from the Indian Space Research Organisation (ISRO) (Krishnan *et al.,* 2015, 2016, 2017). The gaseous active principle liberated passed through a series of moisture-absorbing chambers containing desiccant silica gel. Finally, the anhydrous fumigant was compressed into a storage tank by a pressure pump and could be later filled into small portable cylinders (Ajesh *et al.,* 2018).

Estimation of bioactive principle in cassava bio-fumigant (CLBf)

The primary insecticidal component in cassava leaves is Hydrogen Cyanide (HCN), which is liberated when the cells are broken. Hydrolysis of linamarin and lotaustralin, the two major cyanogenic compounds in cassava leaves, by the enzyme linamarase generates hydrogen cyanide in compound form or as free ions. In this experiment, the compressed form of cassava bio-fumigant was analysed for the presence of its active component using a Gas Chromatographic (GC) method. Since GC cannot detect compounds with a very low molecular weight (<30.00 g/mol), accurate determination of HCN (27.02 g/mol) presence cannot be done directly. The procedure was based on the conversion of hydrogen cyanide in cassava bio-fumigant to cyanogen chloride (CNCl) using chloramine-T solution (Juan *et al.*, 2006). Chloramine-T (0.77 g) was dissolved in 50 ml of deionised water and stored in a refrigerator in an amber-coloured container. The cassava bio-fumigant was passed through 20 ml of sodium hydroxide solution (0.1 M) to trap the cyanide content of the fumigant as sodium cyanide (NaCN). After 10 min, chloramine-T solution (0.30 ml) and n-Hexane (3 ml) were added to the NaCN solution as an extraction solvent for cyanogen chloride. The whole mixture was mixed thoroughly and allowed to separate for 20 min. A 1.0 µl sample of the solution was analysed using GC. A standard stock solution was made with 500 µg/L cyanogen ion (potassium cyanide) and 50 ml, 0.1 M sodium hydroxide solution.

Lethality estimation of chemical insecticides and cassava bio-fumigant

The synthetic fumigant phosphine and CLBf were tested on cohorts of *T. castaneum* and *R. dominica* reared in the

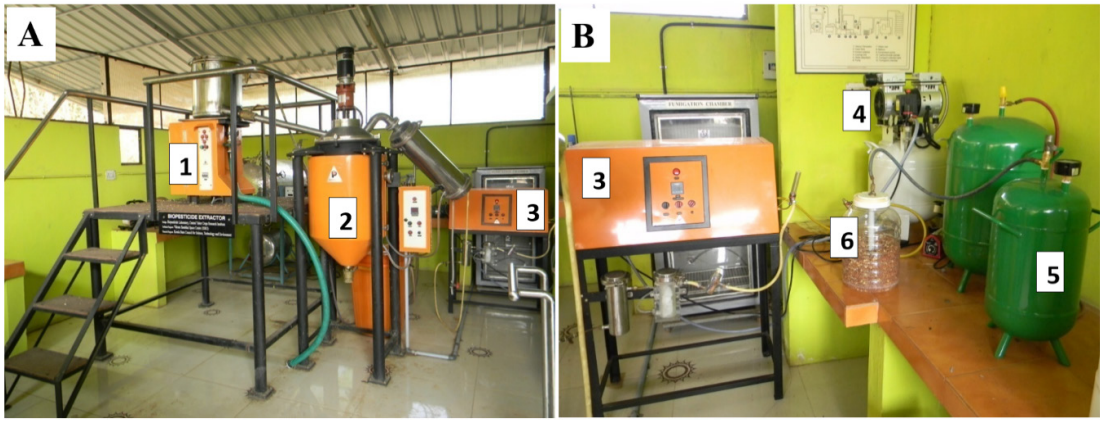


Figure 2. Cassava bio-fumigant extraction pilot plant installed at ICAR-CTCRI. **(A)** Digester unit with **(1)** Grinder-cum-feeder, **(2)** Thermo-regulated-boiler, **(3)** Electronically controlled de-humidifier, **(B)** Fume collection unit with **(4)** Regulated compressor, **(5)** Storage Cylinders and **(6)** Treatment unit/s.

laboratory. CLBf extraction was done in an aqueous medium as cassava leaf distillate, which is volatile in nature, and used to estimate the mortality of the insects. Fifty individuals of each insect species were placed in a 100 ml plastic container. The containers were kept undisturbed for one hour to ensure the free movement and uniform distribution of the adult beetles. A cotton plug soaked with cassava distillate was placed in a perforated fumigation tube and then inserted into the container. The concentration (ppm) of HCN liberated from the cotton plug in each container was estimated using a modified picrate-paper method (Mitchell, 1974; Williams & Edwards, 1980). The freshly made alkaline picrate paper (5×1 cm) was exposed to the gas liberated from the cotton plug in each container. The liberated HCN from the cassava leaf distillate converted the yellow sodium picrate on the paper to sodium isopurpurate, which is brick-red in colour. Colorimetric (OD) estimation was done using a spectrophotometer (PerkinElmer Lambda 25) at 510 nm absorption (Haque & Bradbury, 2002). OD to concentration conversion was done using the equation:

Concentration of HCN (ppm) = (396 x absorbance x 100) ÷ 5000 *g**

(*weight of cassava leaf used) (Bradbury *et al.*, 1999).

An aluminium phosphide tablet (3 g) 'Celphos' (Excel Crop Care Ltd., Mumbai, India), which can release 1 g of active phosphine in acidified water, was used as the source of the synthetic fumigant. Fifty individuals of each weevil species placed in a 100 ml plastic container without treatment served as the control.

Isolation of endosymbiotic bacteria

Adults of *T. castaneum* and *R. dominica* collected from the stock culture were surface sterilised with 1% streptomycin followed by chlorine bleach and then homogenised in sterile 0.9% saline. The homogenate was serially diluted tenfold, and an inoculum of 0.1 ml of this dilution was transferred into the respective nutrient broth and kept in a shaker overnight. Flourished colonies were spread onto Petri dishes containing nutrient agar medium, and the plates were incubated at 30 ± 2ºC for 24-48 h. Developed colonies were transferred to nutrient agar slants and preserved as stock cultures.

Phenotypic characterisation of endosymbiotic bacteria

Phenotypic characteristics of each colony were studied from the standard array of morphological characters, including form, margin, colour, elevation, pigmentation, and opacity of the colony, using a stereomicroscope (Carl Zeiss, Stemi 2000C, USA) under 40× magnification. Gram staining identification of unknown bacterial strains was performed using 24-hour bacterial cultures on nutrient broth. Biochemical identification of the bacterial isolates was accomplished using the VITEK system (Pincus, 2014). All chemicals and media were purchased from Himedia Laboratories Limited, Mumbai, India.

Amplification of 16S rDNA

The total genomic DNA of bacteria was extracted according to the protocol described by Reinhardt *et al.* (2008). Bacterial 16S rDNA was amplified using universal primers: forward primer fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′, corresponding to 8-27 of *E. coli*) and reverse primer RP2 (5′-CGGCTACCTTGTTACGACTT-3′, corresponding to 1492-1510 of *E. coli*) (Weisburg *et al.*, 1991). A final volume of 25 µl reaction containing 2.5 µl of 10× Taq buffer A (containing 15 mM MgCl2), 0.5 µl of 10 mM dNTPs (2.5 mM each), 1.0 µl of each primer (20 ng), 2 µl of template DNA, and 0.25 µl of (1U) Taq DNA polymerase (Bangalore GeNei, India) was subjected to PCR. The amplification was done at 92°C for 2 min 10 s followed by 30 cycles of 1 min 10 s at 94°C, 30 s at 49°C, and 2 min at 72°C, followed by a final extension of 10 min at 72°C. The amplified products were resolved on 1% agarose gel containing ethidium bromide (0.5 µg/ml). DNA bands were visualised under a UV transilluminator and documented with a Gel Doc system (Alpha Imager, Alpha Innotech, USA). The size of the amplicon was determined using a 500 bp DNA ladder (Bangalore GeNei, India). The 16S rDNA gene sequences were generated by sequencing the PCR product on an Applied Biosystems 3500 Genetic Analyzer (Big Dye Terminator v 3.1). Nucleotide sequences were compared with those in the NCBI databases using the BLAST. Phylogenetic trees were constructed from the aligned sequences using MEGA 6.06 software (Tamura *et al.*, 2013).

Antibacterial activity

In vitro, the antibacterial activity of phosphine and CLBf on endosymbiotic bacteria was screened using the agar-well diffusion method (Perez *et al.*, 1990) with Mueller Hinton Agar (MHA) obtained from Himedia, Mumbai, India. MHA plates were prepared by pouring 15 ml of the molten medium into sterile Petri plates and allowing them to solidify. The bacterial inoculum suspension was swabbed uniformly on the surface and allowed to dry for 5 min. Wells were made on the agar surface using a 6 mm cork borer. Aqueous cassava leaf distillate and acidified phosphine (50 µl) solution at concentrations of 0.1, 0.5, and 1 ppm were poured into the wells and allowed to diffuse for 5 min. Plates were incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone. Distilled water was used as a control.

Table 1. NCBI-Gen Bank Accession number of insect samples

Insect species	Accession number (NCBI-Gen Bank)
T. castaneum	**MF 278341**
R. dominica	**MT 021973**

RESULTS

Molecular characterisation of insects

Genomic DNA was extracted from the insect samples. The PCR-amplified products were sequenced. All the sequences obtained in automated DNA sequencing were aligned and compared by using BioEdit. The BLAST analysis of sequences obtained was generated, and these sequences were deposited to NCBI-Gen Bank (Table 1).

The insect sample with accession number 'MF 278341' showed a similarity of 99% at 100% query coverage to *MK649851.1 T. castaneum isolate TH4 cytochrome oxidase subunit I gene* (Figure 3). Similarly, the insect sample with the accession number 'MT 021973' also showed a similarity of 99% at 100% query coverage to *MN271971.1 R. dominica OKHIND8955 (COI)* (Figure 4).

Estimation of active principle (HCN) in cassava bio-fumigant

The gas chromatographic analysis of both cassava biofumigant and standard KCN solution showed a peak for CNCl at a retention time of 4.7 min (Figure 5), which confirms that the active principle of CLBf is hydrogen cyanide (Figure 5). The concentration of Chloramine-T had a significant role in the yield of Cyanogen chloride. The maximum amount of Cyanogen chloride was obtained with 0.3 ml Chloramine-T for 40 µg mL^{-1} solution. Considering the concentration of samples, 0.30 ml Chloramine-T was chosen as the preferred concentration, which was chosen by Juan (2006) in the work on the determination of cyanide content in cigarette smoke.

Estimation of mortality of test insects by cassava biofumigant and phosphine

The mortality of the test insects was estimated with different concentrations of CLBf ranges from 0.1 to 57 ppm as indicated in Tables 2 and 3. The mortality of *T. castaneum* and *R. dominica* were observed from 3.56 ppm onwards in 50 and 40 Min After Treatment (MAT), respectively and 100% mortality was attained at 90 MAT. The mortality was increased with an increase in concentration and time of exposure with a positive correlation of 0.93 and 0.88 for *T. castaneum* and *R. dominica*, respectively. The maximum concentration of CLBf to obtain 100% mortality with the least time of exposure was 49.84 ppm for *T. castaneum* and 35.6 ppm for *R. dominica* which concludes the latter is more susceptible to CLBf than the former weevil.

The mortality of tested insects on treatment with phosphine reveals that *R. dominica* is more susceptible to phosphine than *T. castaneum,* as indicated in Tables 4 and 5. Phosphine concentrations ranging from 0.1 to 1.75 ppm were treated, and the mortality begins at a concentration of 0.25 ppm onwards in 70 and 50 MAT for *T. castaneum* and *R. dominica,* respectively. Both the insects exhibit 100% mortality at 90 MAT. The maximum concentration of phosphine to obtain 100% mortality at the least exposure time was 1.5 and 1 ppm for *T. castaneum* and *R. dominica,*

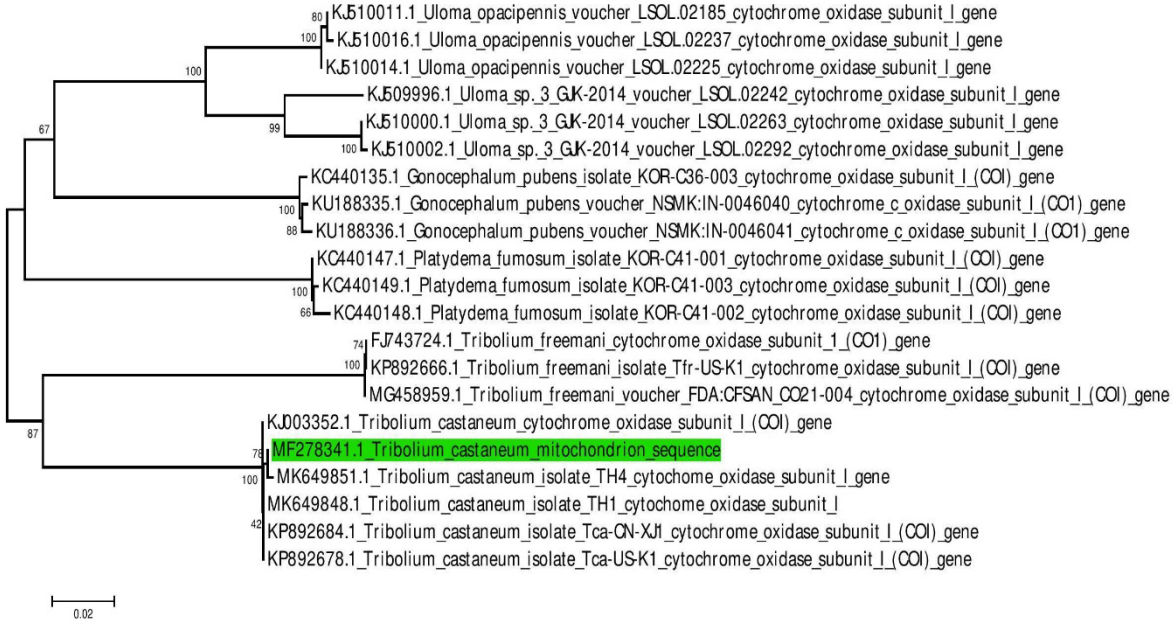


Figure 3. Clustering pattern of *T. castaneum.*

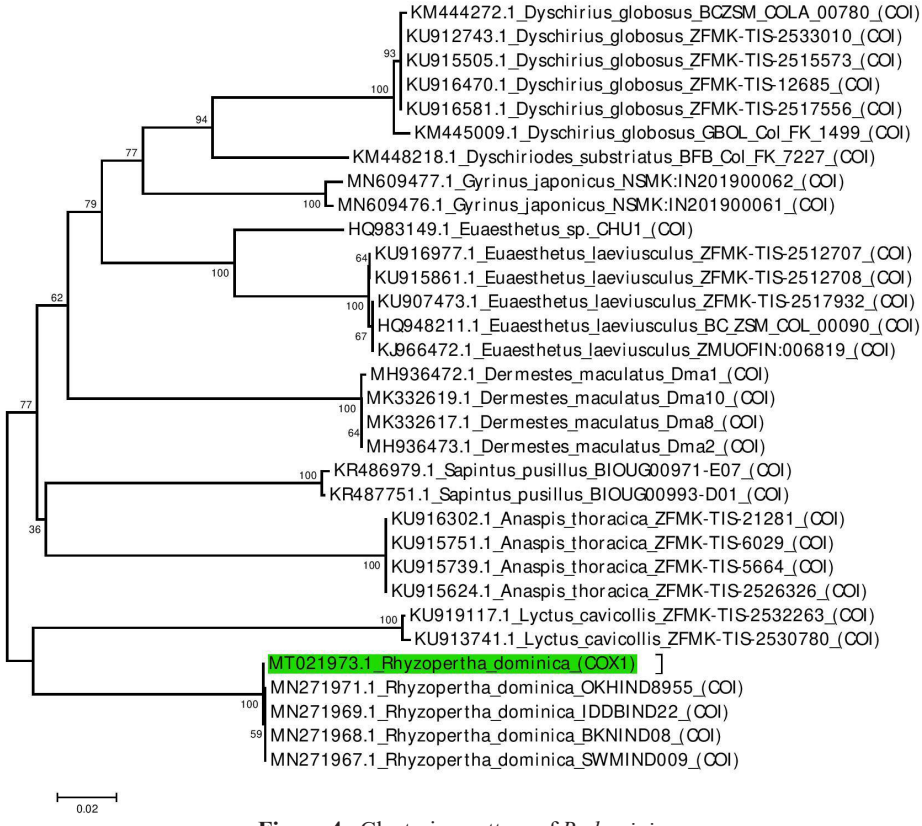


Figure 4. Clustering pattern of *R. dominica.*

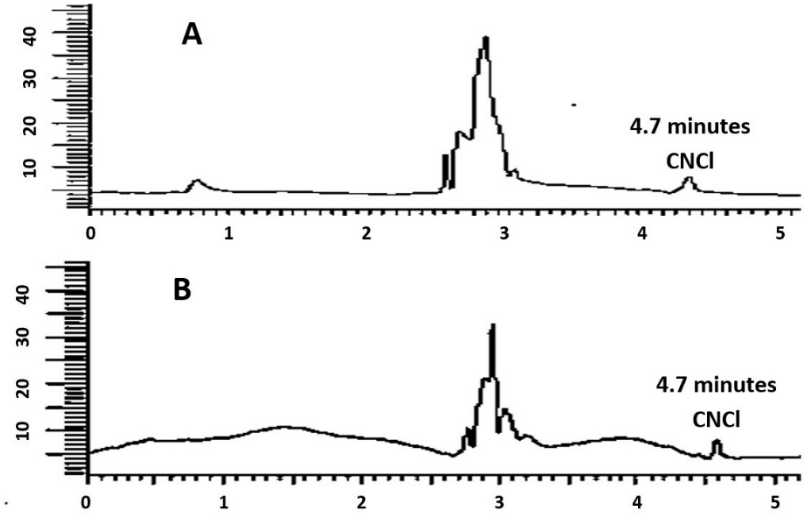


Figure 5. A. Gas chromatogram of potassium cyanide represented as cyanogen chloride and B. Gas chromatogram of cassava bio fumigant represented as cyanogen chloride.

respectively. The mortality of both beetle and weevil increased with respect to the increase in concentration and exposure time with a positive correlation value of 0.93. No mortality was observed in the beetles taken as control.

Isolation of bacteria

Three different bacterial colonies were isolated from *T. castaneum* and one from *R. dominica* (Figure 6), and their phenotypic and molecular characterisation was done.

Phenotypic characterisation

Morphological characters of colonies were observed on Nutrient Agar (NA) plates for each bacterial strain. No pigmentation was observed on any of the bacterial colonies. Colonies formed on NA plates were irregular, regular and circular with convex elevation. The isolates TC3 and TCB were yellowish with transparent opacity, whereas TC2 and RD3 were white in appearance but not transparent (Table 6). All the strains were gram-positive, and except TC3, others were rod-shaped.

Table 2. Effect of CLBf on *T. castaneum*

Cassava biofumigant	Mortality of *T. castaneum*									
	MAT (Minute After Treatment)									
Concentration (ppm)	**5**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
0.178	0.00^{E}	0.00^{E}	0.00^{C}	0.00^{D}	0.00^{D}	0.00^{E}	0.00^{C}	0.00^{D}	0.00^{C}	0.00^{J}
3.56	0.00^{E}	0.00^{E}	0.00^{C}	0.00^{D}	0.00^{D}	6.00^{D}	19.33^{B}	36.00^{C}	45.00^{B}	50.00^{I}
7.12	0.00^{E}	0.00^{E}	0.00^{C}	1.33^{D}	3.00^{D}	12.00^{C}	22.67^{B}	41.00^{B}	47.00^{B}	50.00^{H}
14.24	0.00^{E}	0.00^{E}	0.00^{C}	2.67^{D}	15.33^{C}	32.33^{B}	48.67^{A}	50.00^{A}	50.00^{A}	50.00^{G}
21.36	0.00^{E}	0.00^{E}	0.33^{C}	13.00^{C}	43.00^{B}	49.33^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{F}
28.48	5.00^{D}	9.67^{D}	14.00^{B}	41.67^{B}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{E}
35.6	36.67^{C}	41.33^{C}	47.33^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{D}
42.72	43.33^{B}	47.00^{B}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{C}
49.84	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{B}
56.96	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}
General Mean	18.5	19.8	21.17	25.87	31.13	34.97	39.07	42.7	44.2	45
p-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CV (%)	15.7	7.73	11.32	8.73	6.4	7.11	5.07	3.95	3.48	0
SE(d)	2.372	1.249	1.956	1.843	1.626	2.03	1.616	1.377	1.256	0
LSD at 5%	4.9837	2.6245	4.1087	3.8725	3.4165	4.2649	3.3957	2.8931	2.639	0

Duncan's Multiple Range Test ($p<0.05$) was used, and the significant differences in each row are indicated by different letters. There were 3 replicates for each variable.

Table 3. Effect of CLBf on *Rhyzopertha dominica*

Cassava biofumigant	Mortality of *R. dominica*									
	MAT (Minute After Treatment)									
Concentration (ppm)	**5**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
0.178	0.00^{D}	0.00^{E}	0.00^{E}	0.00^{D}	0.00^{E}	0.00^{D}	0.00^{D}	0.00^{C}	0.00^{C}	0.00^{H}
3.56	0.00^{D}	0.00^{E}	0.00^{E}	0.00^{D}	5.33^{D}	23.67^{C}	36.33^{C}	41.67^{B}	45.67^{B}	50.00^{G}
7.12	0.00^{D}	0.00^{E}	8.33^{D}	16.00^{C}	29.00^{C}	39.00^{B}	45.33^{B}	48.00^{A}	50.00^{A}	50.00^{F}
14.24	0.33^{D}	3.67^{D}	22.33^{C}	37.67^{B}	45.33^{B}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{E}
21.36	18.33^{C}	33.33^{C}	40.67^{B}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{D}
28.48	42.00^{B}	44.67^{B}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{C}
35.6	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{B}
42.72	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}
General Mean	20.08	22.71	27.67	31.71	34.96	39.08	41.46	42.46	43.21	43.75
p-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CV (%)	10.67	7.48	10.67	5.34	6.52	10.12	6.08	4.52	3.31	0
SE(d)	1.749	1.387	2.409	1.382	1.861	3.23	2.059	1.567	1.167	0
LSD at 5%	3.7516	2.9755	5.1678	2.9632	3.992	6.9275	4.4154	3.3615	2.5023	0

Duncan's Multiple Range Test ($p<0.05$) was used, and the significant differences in each row are indicated by different letters. There were 3 replicates for each variable.

Biochemical characteristics of bacteria associated with insects

Biochemical tests were done for each bacterial isolate by VITEK 2 analyser (Table 7). According to the BLAST search, the isolates TC2, TC3, TCB and RD3 were identified as *Staphylococcus arlettae* (MK078607), *Lysinibacillus fusiformis* (MK078608), *Bacillus subtilis* (MF278531) and *Bacillus cereus* (MF278530), respectively, and were cross verified using Bergey's Manual (Bergey & Holt, 2000).

Amlification of 16S rDNA

Sequences with >98% sequence similarity to their nearest phylogenetic neighbour were identified at the species level. The four different strains were affiliated to three different genera such as *Bacillus*, *Lysinibacillus* and *Staphylococcus* by comparative analysis of 16S rDNA sequences. The isolates

Table 4. Effect of phosphine on *T. castaneum*

Phosphine	Mortality of *T. castaneum*									
	MAT (Minute After Treatment)									
Concentration (ppm)	5	10	20	30	40	50	60	70	80	90
0.1	0.00^{C}	0.00^{D}	0.00^{C}	0.00^{C}	0.00^{C}	0.00^{C}	0.00^{C}	0.00^{D}	0.00^{C}	0.00^{H}
0.25	0.00^{C}	0.00^{D}	0.00^{C}	0.00^{C}	0.00^{C}	0.00^{C}	0.00^{C}	25.67^{C}	43.67^{B}	50.00^{G}
0.5	0.00^{C}	0.00^{D}	0.00^{C}	0.00^{C}	0.00^{C}	2.00^{C}	16.00^{B}	33.33^{B}	48.33^{A}	50.00^{F}
0.75	0.00^{C}	0.00^{D}	0.00^{C}	2.33^{C}	8.33^{B}	30.67^{B}	47.33^{A}	50.00^{A}	50.00^{A}	50.00^{E}
1	1.00^{C}	6.00^{C}	14.67^{B}	29.67^{B}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{D}
1.25	30.33^{B}	37.67^{B}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{C}
1.5	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{B}
1.75	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}
General Mean	16.42	17.96	20.58	22.75	26.04	29.08	32.92	38.63	42.75	43.75
p-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CV(%)	16.49	9.42	4.32	8.88	2.83	6.97	4.77	9.55	5.05	0
SE(d)	2.21	1.382	0.727	1.649	0.601	1.656	1.283	3.011	1.764	0
LSD at 5%	4.7404	2.9632	1.5582	3.5361	1.2889	3.5516	2.7524	6.457	3.783	0

Duncan's Multiple Range Test ($p<0.05$) was used, and the significant differences in each row are indicated by different letters. There were 3 replicates for each variable.

Table 5. Effect of phosphine on *R. dominica*

Phosphine	Mortality of *R. dominica*									
	MAT (Minute After Treatment)									
Concentration (ppm)	5	10	20	30	40	50	60	70	80	90
0.1	0.00^{C}	0.00^{C}	0.00^{C}	0.00^{C}	0.00^{C}	0.00^{D}	0.00^{D}	0.00^{D}	0.00^{C}	0.00^{F}
0.25	0.00^{C}	0.00^{C}	0.00^{C}	0.00^{C}	0.00^{C}	1.67^{D}	11.33^{C}	27.67^{C}	43.67^{B}	50.00^{E}
0.5	0.00^{C}	0.00^{C}	0.00^{C}	0.00^{C}	1.67^{C}	13.67^{C}	29.00^{B}	44.33^{B}	50.00^{A}	50.00^{D}
0.75	1.67^{B}	3.00^{B}	9.00^{B}	22.00^{B}	34.33^{B}	43.67^{B}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{C}
1	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{B}
1.25	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}	50.00^{A}
General Mean	16.94	17.17	18.17	20.33	22.67	26.5	31.72	37	40.61	41.67
p-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CV(%)	2.78	4.76	5.95	11.18	8.05	9.67	7.76	5.6	3.07	0
SE(d)	0.385	0.667	0.882	1.856	1.491	2.092	2.009	1.693	1.018	0
LSD at 5%	0.8576	1.4854	1.965	4.1353	3.3215	4.662	4.4769	3.7725	2.269	0

Duncan's Multiple Range Test ($p<0.05$) was used, and the significant differences in each row are indicated by different letters. There were 3 replicates for each variable.

were identified as TC2 - *Staphylococcus arlettae,* TC3 - *Lysinibacillus fusiformis*, TCB - *Bacillus subtilis* and RD3 - *Bacillus cereus*. The phylogenetic relationship of the bacteria based on 16S rRNA gene sequences is shown in Figures 7-10. Biochemical characterisation and BLAST search for these isolates showed the highest match with the respective species, and all the sequences were deposited with NCBI-Gen Bank.

Antibacterial activity of cassava biofumigant and phosphine against endosymbionts

The antibacterial effect of the fumigants was estimated by measuring the zone of growth inhibition on bacteria in the MHA plates (Figure 11). The insecticide-treated MHA plates exhibited a significant zone of growth inhibition in all the selected bacterial strains (Table 7).

The maximum zone of inhibition (24 mm) was with *Bacillus cereus* (RD3) at 1 ppm, but it was 10.66 and 16.66 mm at 0.1 and 0.5 of CLBf, respectively. Whereas in the case of phosphine, the maximum diameter of inhibition was only 16.33mm at 1ppm, which indicates that the growth inhibition

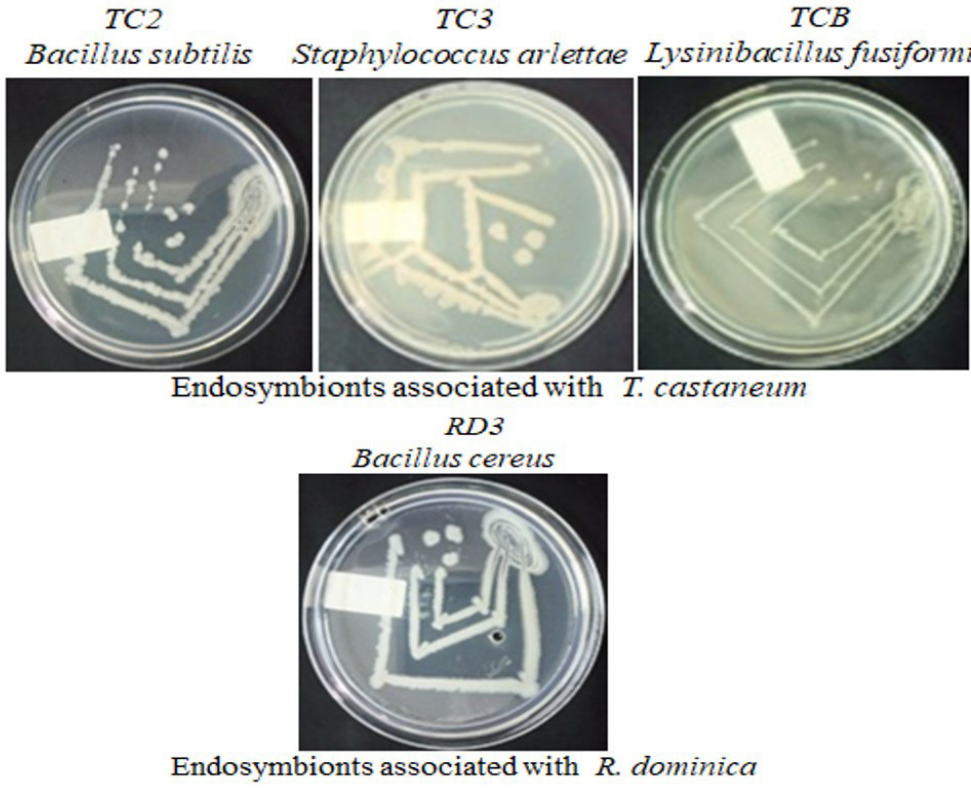


Figure 6. Endosymbiotic bacteria isolated. **(A)** from *T. castaneum* **(1)** *Bacilus subtilis* **(2)** *Staphylococcus arlettae* **(3)** *Lysinibacillus fusiformis*, **(B)** from *R. dominica* **(1)** *Bacillus cereus*.

Table 6. Colony morphology of the bacteria isolated from *T. castaneum* and *R. dominica*

Bacteria	Form	Elevation	Margin	Colour	Pigmentation	Opacity
TC2	Irregular	Convex	Undulate	Whitish	-	Not Transparent
TC3	Circular	Convex	Undulate	Yellowish	-	Transparent
TCB	Irregular	Convex	Entire	Yellowish	-	Transparent
RD3	Regular	Convex	Undulate	Whitish	-	Not Transparent

Table 7. Biochemical characteristics of bacteria isolated from *T. castaneum* and *R. dominica*

S. No.	Biochemical parameters	Bacterial isolates			
		TC2	*TC3*	*TCB*	*RD3*
1	Beta- Xylosidase (BXYL)	+	-	-	-
2	L- Lysine Arylamidase (LysA)	-	-	-	-
3	L- Aspartate Arylamidase (AspA)	-	-	-	-
4	LeucineArylamidase (leuA)	+	-	+	-
5	Phenylalanine Arylamidase (PheA)	+	-	+	-
6	L- ProlineArylamidase (ProA)	-	-	-	-
7	Beta- Galactosidase (BGAL)	-	-	+	-
8	L- PyrrolydonylArylamidase (PyrA)	+	+	+	-
9	Alpha- Galactosidase (AGAL)	+	-	-	-
10	Alanine ARYLAMIDASE (AlaA)	-	-	-	-
11	Tyrosine Arylamidase (TyrA)	+	-	-	-
12	Beta- N- AcetyleGlucosaminidase (BNAG)	-	-	+	-
13	Ala- Phe- Pro Arylamidase (APPA)	+	-	+	-
14	Cyclodextrin (CDEX)	-	-	-	-

Table 7. Continued

15	D- Galactose (DGAL)	-	+	-	-
16	Glycogen (GLYG)	-	-	-	-
17	Myo- Inositol (INO)	-	-	-	-
18	Methyl A- D- Glicopyranoside (MdG)	+	-	-	-
19	Ellman (ELLM)	-	-	+	-
20	Methyl- D- Xyloside (MdX)	-	-	-	-
21	Alpha Mannosidase (AMAN)	-	-	-	-
22	Maltotroice (MTE)	+	-	+	-
23	Glycine Arylamidase (GlyA)	+	-	-	-
24	D- Mannitol (dMAN)	+	+	-	+
25	D- Mannose (dMNE)	+	+	-	-
26	D- Melezitose (dMLZ)	-	-	-	-
27	N- Acetyl D- Glucosamine (NAG)	-	-	+	-
28	Palatinose (PLE)	-	-	-	-
29	L- Rhamnose (lRHA)	-	-	-	-
30	Beta Glucosidase (BGLU)	+	-	-	-
31	Beta Mannosidase (BMAN)	-	-	-	-
32	Phosphoryl Choline (PHC)	-	-	-	-
33	Pyruvate (PVATE)	+	-	+	-
34	Alpha Glucosidase (AGLU)	+	-	+	-
35	D- Tagatose (dTAG)	-	-	-	-
36	D- Trehalose (dTRE)	-	+	+	+
37	Inulin (INU)	-	-	-	-
38	D- Glucose (dGLU)	+	-	+	-
39	D- Ribose (dRIB)	+	+	+	+
40	Putrescine (PSCNa)	-	-	-	-
41	Growth in NaCl 6.5% (NC6.5)	+	-	+	-
42	Kanamycine resistance (KAN)	-	-	+	-
43	Oleandomycine resistance (OLD)	-	-	-	-
44	Esculinhydrolysis (ESC)	+	-	+	-
45	Tetrazolium Red (TTZ)	-	-	-	-
46	Polymixin_B Resistance (POLYB_R)	-	-	+	-
47	Amygdalin (AMY)	-	-	-	-
48	Phosphatidylinositol Phospholipase C (PIPLC)	-	-	-	-
49	D- Xylose (dXLY)	-	-	-	-
50	Arginine Dihydrolase1 (ADH1)	-	+	-	-
51	Beta Galactopyranosidase (BGAR)	-	-	-	-
52	Phosphatase (PHOS)	-	+	-	-
53	Beta Glucuronidase (BGURr)	-	-	-	-
54	Beta Glucuronidase (BGUR)	-	-	-	+
55	D- Sorbitol (dSOR)	-	-	-	-
56	Urease (URE)	-	-	-	-
57	L- Lactate Alkalinization (ILATk)	-	+	-	-
58	Lactose (LAC)	-	+	-	-
59	D- Maltose (dMAL)	-	+	-	+
60	Bacitracin Resistance (BACI)	-	-	-	-
61	Novobiocin Resistance (NOVO)	-	+	-	+
62	Methyl B- D- Glycopyranoside (MBdG)	-	-	-	-
63	Pullulan (PUL)	-	-	-	-
64	D- Raffinose (dRAF)	-	+	-	-
65	O/129 resistance Vibrio. (O129R)	-	+	-	-
66	Salicin (SAL)	-	-	-	-
67	Saccharose (SAC)	-	-	-	+
68	Arginine Dihydrolase-2 (ADH2s)	-	-	-	-
69	Optochinresistance (OPTO)	-	+	-	+

+ Positive to enzyme test; - Negative to enzyme test

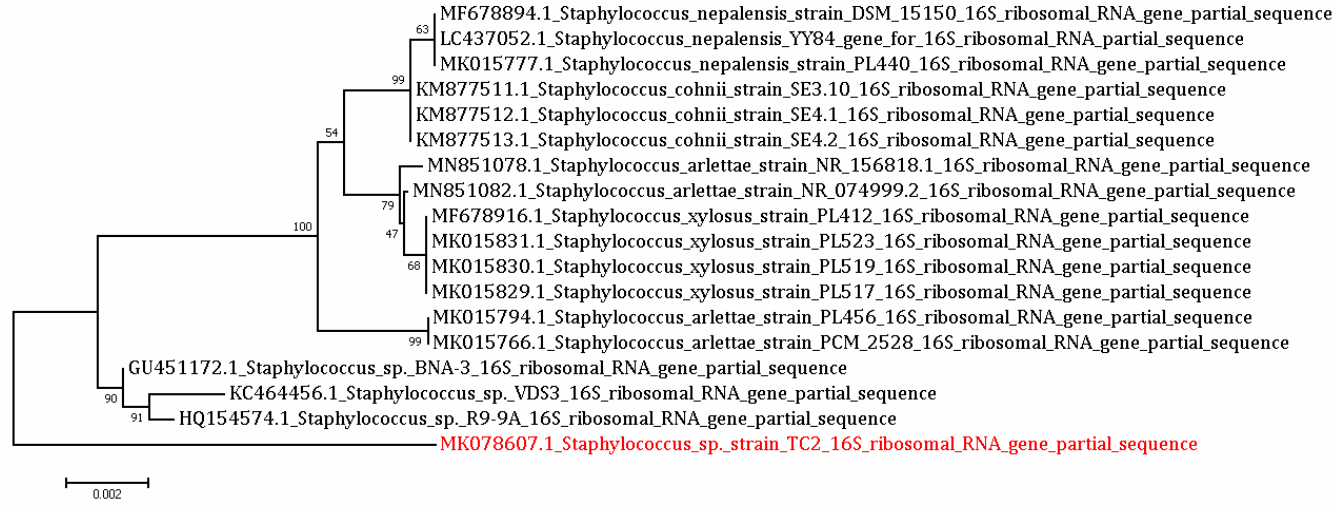


Figure 7. Phylogenetic tree of 16S rRNA gene of TC2- MK.078607 using the neighbour-joining method.

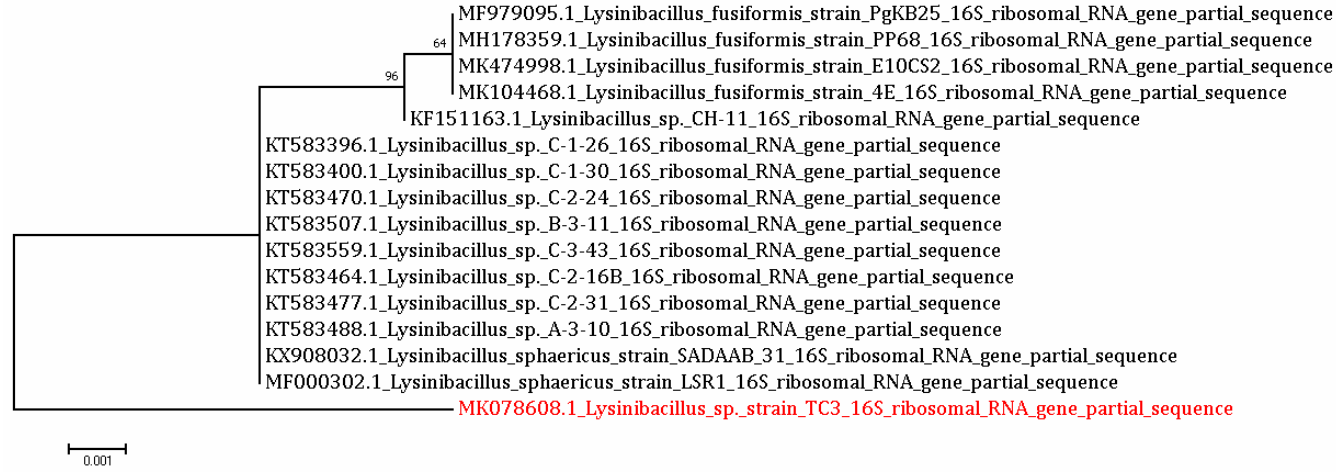


Figure 8. Phylogenetic tree of 16S rRNA gene of TC3- MK.078608 using the neighbour-joining method.

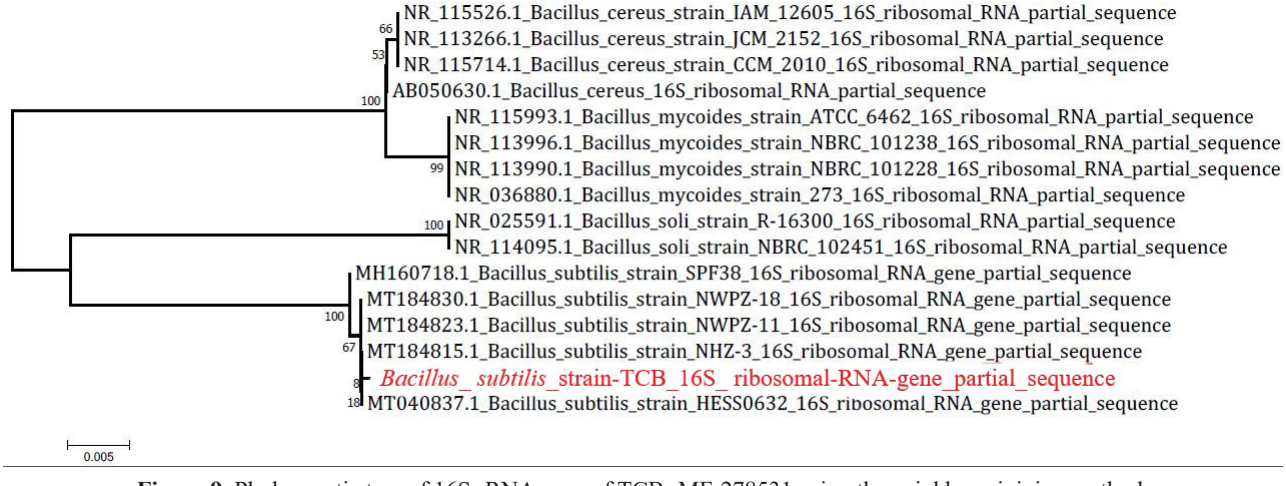


Figure 9. Phylogenetic tree of 16S rRNA gene of TCB- MF-278531 using the neighbour-joining method.

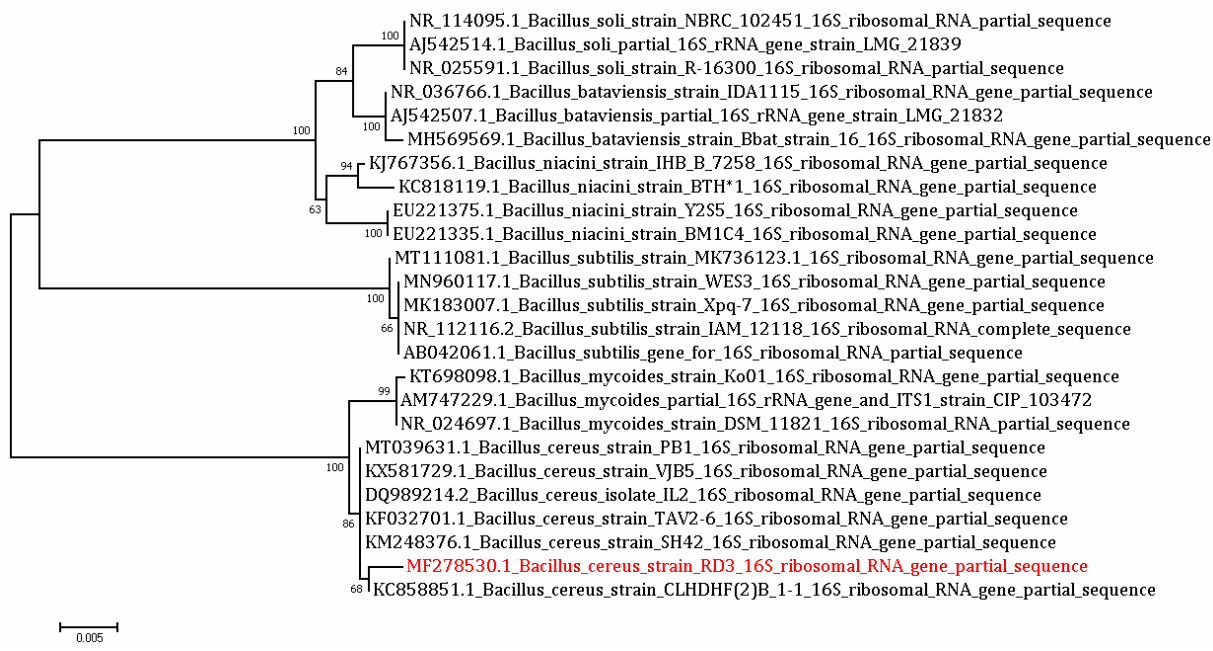


Figure 10. Phylogenetic tree of 16S rRNA gene of RD3- MF.278530 using the neighbour-joining method.

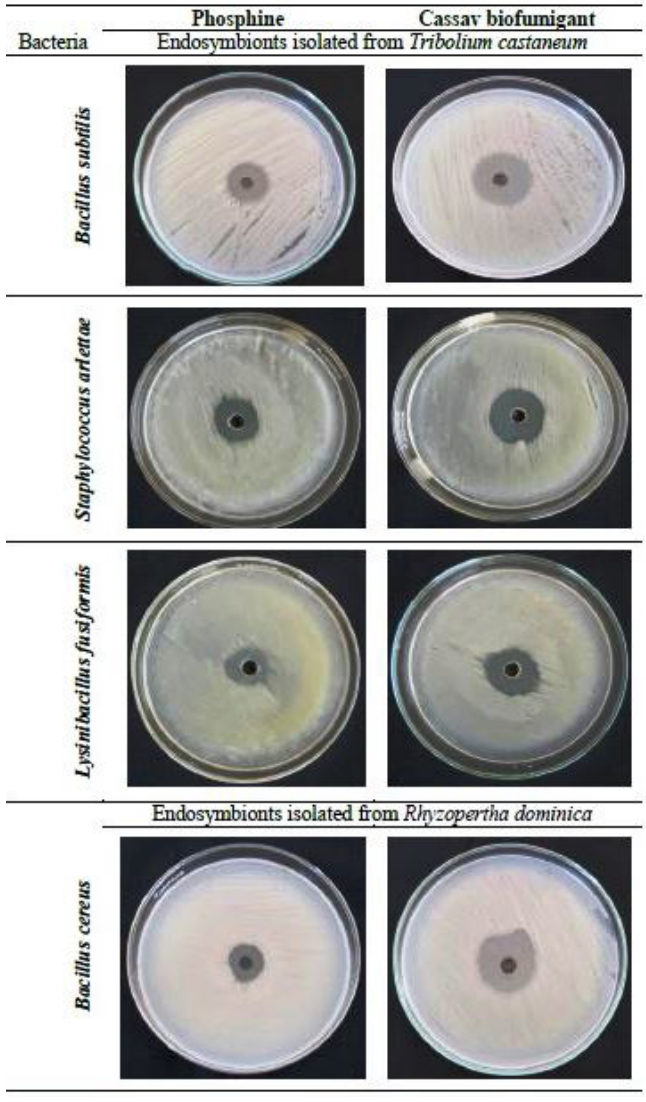


Figure 11. Zone of growth inhibition by fumigants on bacteria in the MHA plates.

Table 7. Antibacterial effect of selected fumigants on bacteria

Endosymbiotic bacteria sp.	Variable concentrations of Cassava bio fumigant			Variable concentrations of Phosphine			Zone of inhibition (mm)
	0.1 ppm	0.5 ppm	1.0 ppm	0.1 ppm	0.5 ppm	1.0 ppm	
Bacillus cereus	10.6±1.2^{b}	16.7±1.2^{a}	24.0±1^{a}	9.6±1.5^{a}	14.6±1.5^{a}	16.3±0.5^{a}	
Staphylococcus arlettae	10.0±1.0^{b}	16.7±1.15^{a}	22.0±1^{b}	7.6±1.6^{b}	11±1^{a}	13.3±1.5^{b}	
Lysinibacillus fusiformis	8.0±1^{b}	12.3±1.2^{b}	16.6±1.5^{c}	5.3±0.8^{c}	12±1.1^{a}	11.3±1.5^{b}	
Bacillus subtilis	15.3±2.1^{a}	18.7±1.5^{a}	20.7±0.5^{b}	9.0±1^{ab}	13.6±2.3^{a}	14.0±2.6^{ab}	
p-Value	0.0036	0.0107	0.0004	0.0024	0.1284	0.0246	
LSD at 5%	2.8058	2.9968	1.854	1.5969	NS	2.766	

Duncan's Multiple Range Test ($p<0.05$) was used, and the significant differences in each row are indicated by different letters. Replication: 3

of CLBf is more effective than phosphine on *Bacillus cereus.*

The growth of three bacterial strains isolated from *T. castaneum* was highly inhibited by CLBf than phosphine. The maximum zone of inhibition was with *Staphylococcus arlettae* (22 mm) at 1 ppm, as against 13.33 mm by phosphine. The least inhibition (16.66 mm) due to CLBf at 1 ppm was noticed on bacterial strains isolated from *T. castaneum.* Among the endosymbionts isolated from *T. castaneum,* high susceptibility was noticed in *Bacillus subtilis* and *Staphylococcus arlettae* with CLBf and phosphine, respectively.

The results conclude that CLBf has more growth inhibitory effect on all the strains than phosphine, and the bacterial strain isolated from *R. dominica* was more susceptible with both the insecticides, which show a higher inhibition zone than strains isolated from *T. castaneum*.

DISCUSSION

The current study describes the comparative antibacterial properties of CLBf and phosphine on endosymbiotic bacteria of *T. castaneum* and *R. dominica;* The same has been evaluated against the aforesaid insects by Ajesh *et al.* in 2024. Endosymbionts are crucial for the normal development and survival of insects (Naik, 2016), and the loss of these microorganisms often results in abnormal development and reduction in the survival of the insect host (Sreerag *et al.*, 2014). The symbiosis between bacteria and insects is frequently found in the orders Coleoptera, Blattaria, Homoptera, and Hymenoptera (Malke, 1967; Douglas, 1998; Zabalou, 2004; Rosenberg *et al.*, 2013). Although reports are available on the isolation of bacteria from *T. castaneum* and *R. dominica*, no work has been reported on the role of these endosymbiotic bacteria on pesticide resistance in insects.

Phenotypic, biochemical, and 16S rDNA sequence analysis of endosymbiotic isolates prove the presence of cultivable bacteria, namely *Staphylococcus arlettae, Lysinibacillus fusiformis* and *Bacillus subtilis* from T. *castaneum* and *Bacillus cereus* from *R. dominica*. Bacteria of the genus *Bacillus* and *Staphylococcus* were previously reported from many insect species, but scanty reports are available on *Lysinibacillus* as an endosymbiont. Gram-positive bacteria, including *B. subtilis,* were previously reported as an endosymbiont of whitefly, *Bemisia tabaci* (Ateyyat *et al.*, 2010) and *Musca domestica* (Banjo *et al.*, 2005). Reports are also available for the presence of *B. subtilis* and *Staphylococcus* in the gut of *Aedes aegypti* and *Bombyx mori,* respectively (Gusmão *et al.*, 2010; Feng *et al.*, 2011). *Bacillus* and *Staphylococcus* strains were well known for their potential to produce sucrose for sticky honeydew secreted by the host insect (Indiragandhi *et al.*, 2010). The dominant endosymbiotic species in *Phlebotomus papatasi* are reported to be *B. cereus* and *B. subtilis* (Mukhopadhyay *et al.*, 2012). *Bacillus* strains are capable of producing amylase enzymes that are involved in the initial breakdown of starch into simple sugars (Amund & Ogunsina, 1987; Oyewole & Odunfa, 1992). *Lysinibacillus* strains promote the growth and larval development of insects (Rajagopal, 2009). Inadequacy of these strains could delay the pupation in *D. melanogaster* larvae (Shin *et al.*, 2011; Wong *et al.*, 2011; Priyanka *et al.*, 2012). The bacterium *Lysinibacillus fusiformis* was reported in the wild predatory ground beetle *Poeciluschalcites* as an endosymbiont (Lehman *et al.*, 2009) and in the larvae of butterflies (Aldahi, 2017). Molecular studies on the gut microbiota of the ladybird beetle, *Coccinella septempunctata,* revealed the association of *L. fusiformis* and *B. cereus* with this insect, and it is said to be crucial for the survival and evolution of insects (Abolee, 2017). The current study also establishes the presence of *L. fusiformis* and *B. cereus* in the gut of *T. castaneum* and *R. dominica,* respectively.

According to Zindel *et al.* (2011), endosymbiotic bacteria are known to play a role in their host insect's defence against infections and predators as well as their involvement in insecticide resistance processes. Exploration of potent endosymbiotic organisms is an important avenue in insect

pest management strategies. During the current investigation, the endosymbiotic bacteria associated with *T. castaneum* and *R. dominica* were isolated and characterised, and their pesticide degradation potential was estimated.

In this study, the zone of growth inhibition in *B. cereus* culture was higher with CLBf than with phosphine. This indicates that the detoxification capacity of *B. cereus* to CLBf was less than that of phosphine. Compared to the treatment with phosphine, a 47.2% higher inhibition zone was noticed in the *B. cereus* culture plate treated with CLBf. This proves that *B. cereus* has a pivotal role in detoxifying phosphine. Reports are available that *B. cereus* colonising in the gut of the moth can break down insecticides (Janmaat & Myers, 2003; Ramya *et al.*, 2016; Van den Bosch & Welte, 2017) As observed in the present investigation. Chen *et al.* (2012) and Selvam *et al.* (2013) also validated the biodegradation effect of *B. cereus* on pyrethroid and fenvalerate. The role of *B. cereus* in detoxifying chlorpyrifos, endosulfan, malathion, dimethoate, and pendimethalin has been reported by Ishag *et al.* (2016). *Bacillus subtilis* was more susceptible to CLBf than phosphine. When compared to the growth inhibition zones of CLBf and phosphine in the culture plate with *B. subtilis, CLBf* shows a 47.1% increase in inhibition when compared to phosphine. Moreover, reports are available that *B. subtilis* can degrade pesticides. (Ishag *et al.*, 2016). Xiao *et al.* (2015) studied the efficiency of *B. subtilis* in detoxifying the beta-cypermethrin, and the Capacity of *B. subtilis* to decompose xenobiotic compounds like 4-chloro-2-nitrophenol, dimethyl formamide, and red M5B dye was reported (Arora, 2012; Varadarajan *et al.*, 2013; Vidhya & Thatheyus, 2013).

The other two strains isolated from *T. castaneum* were *Lysinibacillus fusiformis* and *Staphylococcus arlettae.* The impact of phosphine and CLBf on *Lysinibacillus fusiformis* and *Staphylococcus arlettae* isolated from *T. castaneum* showed that both the bacteria were resistant against phosphine, and the growth inhibition zone was higher in the culture media with CLBf than with phosphine. As in the case of *S. arlettae,* the growth inhibition zone in the plate treated with CLBf was 65.4% higher than that of the phosphine treatment. Whereas in *L. fusiformis,* the inhibition zone was 46.9% higher in CLBf than the treatment with phosphine. The detoxifying capacity of *L. fusiformis* has been widely employed to neutralize toxic compounds such as hydrocarbon (Eric, 2017), sulfonated azo dyes (Saratale *et al.*, 2013), and the herbicide fomesafen in agricultural fields (Liang *et al.*, 2009). Reports are available about the insecticidal degradation property of the *staphylococcus* genus (Hammer & Bowers, 2015; De Almeida *et al.*, 2017), but scanty as in the case of *staphylococcus arlettae*s. Elisangela *et al.* (2009) reported that by applying a sequential microaerophilic/ aerobic process, a facultative *S. arlettae* strain degrades textile azo dyes. Plenty of reports are available on the role of insect endosymbionts in the insecticide detoxification mechanism, but an in-depth study on the degradation of phosphine by endosymbionts is yet to be explored. The current study revealed that the bacterial strains isolated from *T. castaneum* and *R. dominica* have significant tolerance to phosphine. The study also emphasizes the fact that both the test insects can develop resistance to the conventional fumigant phosphine. The CLBf showed a remarkable anti-bacterial effect on the endosymbionts isolated and had a higher mortality of the target pests than the fumigant phosphine. The present investigation concludes that repeated usage of single fumigant phosphine may elicit resistance in *T. castaneum* and *R. dominica,* and hence, the active principle isolated from the cassava leaf can be an effective befitting alternative to synthetic fumigants.

CONCLUSION

The endosymbionts associated with *T. castaneum* are *Staphylococcus arlettae, Lysinibacillus fusiformis* and *Bacillus subtilis* and that from *R. dominica* is *Bacillus cereus*. The endosymbionts harboured in these stored grain pests are capable of imparting resistance to phosphine, which is a globally publicised fumigant. The CLBf showed a significant effect on the suppression of these endosymbionts isolated from the target pests, and hence it can be a promising green alternative strategy for the management of stored-product insect pests, particularly *T. castaneum* and *R. dominica.*

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