Final Project Report

Reduction of post-harvest losses in Tomato Fruit by enhancing the shelf-life through the use of fungal probiotics: increasing the economic power of small holder farmers

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EXECUTIVE SUMMARY

Smallholder farmers play a very vital role in the local food production, yet they are not well informed about modern day methods of produce harvesting and handling. Due to this, small holder farmers record low agricultural productivity and high post-harvest losses (PHLs), as a result of significant damage during the harvest and storage of their produce. These challenges faced by smallholder farmers contribute to the drastic food losses and hunger around the world.

One of the most severe challenges that smallholder farmers face is the lack of post-harvest processing and storage equipment. PHLs may be due to high crop perishability, mechanical damage, excessive exposure to high ambient temperature, relative humidity and rain, poor infrastructure, inappropriate post-harvest handling, poor marketing systems, pests (birds, rodents, insects), disease attack (contamination by spoilage fungal and bacteria), insufficient transport facilities, storage and the processing techniques of the product between the farm and distribution. Estimations on PHLs for Africa are often between 20 - 40%. As far back as 2011, PHLs were valued at 1.6 billion US Dollars per year in the Eastern and Southern parts of Africa, destroying about 50% of the perishable crops. Postharvest decay of fresh agricultural produce is a major challenge confronting sustainable food production throughout the world, in the recent times; the use of synthetic fungicides is the chief method of managing postharvest losses

from microbial decay of fresh produce. However, there are concerns and reported proofs of hazardous impacts on the environment and consumers' health, traceable to the use of fungicides as preservatives on perishable agricultural commodities, therefore, healthier and more environmental friendly alternatives are advocated. Some couples of decay prevention and control measures have been resourced by authorities and researchers alike; ultimately, there are more consensus on the utilization of biological control agents (BCAs); obviously because of the perceived advantages over the chemical counterparts. Fungal antagonists (i.e. yeasts and moulds) are favored; as preferred alternative in the management of postharvest decay of farm produce. They have been reported to be isolated from different sources including: fruit surfaces, the roots, the phyllosphere, soil and the sea. Some of these fungal BCAs are commercially available, while some are still at varying stages of developments. Biological control agents use different modes or mechanisms of action whereby competition for nutrients and space, secretion of antifungal compounds and parasitism of the pathogen are the most commonly used mechanisms of antagonistic actions.

The production of tomatoes on the other hand is challenging as the quality and nutritional value of tomato fruit deteriorates during postharvest, resulting in as much as 10-30% reduction in the yield of major tomato crops. Some of the bacterial diseases responsible for PHLs in tomatoes are caused by *Pseudomonas syringae, Xanthomonas campestris, Clavibacter michiganensis etc.* and fungal

diseases caused by *Alternaria solani, Fusarium oxysporum, Rhizopus stolonifer etc.*

Most smallholder farmers in South Africa encounter attack of pathogenic fungi because they have inadequate technical information particularly relating to crop diseases, in order to identify and control those fungi. There are chemical fungicides (Potassium bicarbonate, Calcium chloride, Nitrorous oxide, and Sodium metabisulphite etc.) which are commercially available to control the postharvest losses of tomato fruits. The application of fungicides is progressively becoming restricted: they are expensive, have severe regulation, carcinogenic, high and acute residual toxicity, extended degradation period, environmental pollution and growing public concern about chemical residues in fruit.

Alternatively, there are couple of physical means that have been approved over the years in the control and/or management of postharvest tomato fruits in storage. The Ultra violet (UV) disinfecting and sanitation which upon application decreases contamination but encourage resistance during the packaging of the tomato. There is also the modified/controlled atmosphere that prevent the decay of fruits during storage i.e. the low/high temperature and correct humidity management of the product. Also, the proper postharvest handling to avoid mechanical and physiological damage during storage and transportation. The last but not the least option is the use of recurrent warming to reduce the microbial decay of tomato.

The use of biocontrol agents is gaining grounds as a measure to manage post harvest loss in tomato fruits, the use of fungal antagonists such as *Debaryomyces hanseniiis*; an antagonistic yeast that inhibits the growth of Rhizopus rot in tomato fruit, *Candida guilliermondii* (*Castellani*) *Langeron* antagonistic strain inhibits the growth of gray mold (*Botrytis cinerea*), rhizopus rot (*Rhizopus stolonifer*) and anthracnose rot (*Colletotrichum acutatum*) in tomato fruit, *Cryptococcus laurentii* are biocontrol yeast reported to decrease postharvest fungal diseases of fruits and vegetables such as peach, pear, apple and gray mold in tomato.

These biological control antagonists, are generally recognized as safe (GRAS) is rather promising and gaining recognition among consumers. Fungal antagonistic microorganisms have been successfully used to reduce postharvest diseases of fungi in tomato fruits. These are good substitutes to fungicides, which are expensive and with inherent negative after use on the environment and on consumers.

The reduction of PHLs using fungal antagonists is here reported, it offers double advantages: firstly this fungal antagonist extends the shelf life of tomatoes at storage and secondly, it confers health benefit on consumers. The technology hence reduces the perishability of tomato fruits, from the time of harvest until the time of ingestion for few more days. Therefore, a cheaper, healthier and more affordable biocontrol agent for the control of post-harvest rots in tomato fruits

has been identified, this is expected to give more economic power to the smallholder farmers.

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TIMELINE

Milestone	Activity	Year 1				Year 2				Budget
		Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	
September 2015 to March 2016	Protocol development, optimization, pilot study and sampling									R 250 000.00
April 2016 to September 2016	Experimental runs									R 450 000.00
September 2016 to December 2016	Field trials									R 300 000.00
January 2017 to March 2017	Mass Production of Probiotics									R 250 000.00
April 2017 to July 2017	Creation of roll out plans and pilot plant development at ARC- API									R 200 000.00
July 2017 to March 2018	Compiling results and information dissemination									R 100 000.00

RESEARCH ACTIVITIES

Aims

To isolate, identify, and characterize antagonistic fungal strains that will be available to rural and small-scale farmers for tomato post-harvest management and to further determine the probiotic properties of these antagonistic strains.

Objectives

1. To isolate, identify and characterize fungal strains from tomato fruits using cultural and molecular based approach.
2. To test the antagonistic potentials of the isolated fungal strains against selected tomato fruit spoilage fungi.
3. To conduct spoilage test or pathogenicity test of selected isolates in objective three (2) on tomato fruits.
4. To test the isolates obtained in objective four (3) above for probiotic properties.
5. To conduct field trials on the selected antagonistic fungal probiotic.
6. To produce *en mass* antagonistic-probiotic products (power, pellets, bags etc.)
7. To demonstrate the efficacy and application processes to the rural farmers for marketing.

Methodology

1. Study site and samples

The study was conducted at both GI Microbiological and Biotechnology division, Agricultural Research Council– Animal Production Institute (ARC- API) and the Soil sciences division, Agricultural Research Council– Institution of Soil, Climate and Water (ARC- ISCW). Fresh and rotten fungal infected tomato fruits were harvested from Agricultural Research Council - Vegetable and Ornamental Plants Institute (ARC- VOPI), transported in clean sterile zip-lock plastic bags with ice to ARC-API, for analyses, further analysis were later conducted at ARC-ISCW.

2. Fungal isolation

Fungi were isolated from both fresh and rotten fungal infected tomato fruit. Serial ten-fold dilutions were done from 1g of each tomato blended using a stomacher. The different dilutes were inoculated on potato dextrose agar (PDA), and the plates were incubated at 25°C for 5 days. Distinct fungal growth mass was picked and sub-cultured on sterile PDA plates to obtain pure cultures (El-Komy, *et al.,* 2015). All fungal isolates were stored on PDA at 4°C prior to use (El-katatny & Emam, 2012).

3. Morphological and Molecular identification

Pure fungal isolates were examined, identified and characterized microscopically through slide culture and wet mount techniques (Zivkovic *et al.,* 2010). In slide culture technique, an appressoria were produced, where 10 mm^2 squares of PDA were placed in an empty Petri plate. The edge of the agar was inoculated with spores taken from a sporulating culture, and a sterile cover slip was placed over the inoculated agar (Johnston and Jones, 1997). After 5 days of incubation a 25°C for 5 days, the shape and size of the 100 appressoria formed across the underside of the cover slip were examined microscopically.

4. Molecular-based approach to identify the fungal strains.

(a) Extraction of DNA

Fungal genomic DNA was extracted from mycelium obtained from cultures grown on PDA, incubated at 25°C for 7 days using the ZR Fungal/Bacterial DNA MiniPrep™ extraction kit (Zymo Research, Irvine, California 92614, United States). The Aerial mycelium was removed from each pure culture plate using a sterile needle and placed in a sterile ZR Bashing Bead™ lysis Tube, 750 µl Lysis Solution was added to the tube and vortexed for 15 minutes. The tubes were centrifuged at 10 000 ×g for 1 minute; 400µl of supernatant was transferred to a Zymo-Spin™ IV Spin Filter and centrifuged at 7 000 ×g for 1 minute.

The Zymo-Spin™ IV Spin Filter was transferred to a sterile collection tube and 1 200 µl of Fungal/Bacterial DNA Binding Buffer was added. The supernatant was transferred to a Zymo-Spin™ IIC Column[3] and centrifuged at 10 000 ×g for 1 minute. The column was transferred to a sterile collection tube and 200 µl of DNA Pre-wash Buffer was added to it and centrifuged at 10 000 ×g for 1 minute. To the column 500 µl of Fungal/Bacterial DNA wash Buffer and centrifuged at 10 000 ×g for 1 minute. The Zymo-Spin™ IIC Column[3] was again transferred to a clean 1.5 ml micro-centrifuge tube, 100 µl of DNA Elution Buffer was added to the column and centrifuged at 10 000 ×g for 30 seconds in order to elute the DNA.

Thereafter, DNA was quantified using Qubit™ 2.0 Fluorometer (Invitrogen, Life Technologies, Eugene, OR, USA), where 200 µl of working solution (Qubit™ reagent 1:200 diluted in Qubit™ buffer) was prepared for each standard and sample. To the Qubit™ assay tubes containing 199 µl of working solution, 1 µl of each sample was added and vortexed for 3 seconds. The tubes were incubated at room temperature for 2 minutes; the tubes were therefore inserted in the Qubit™ 2.0 Fluorometer to take readings of the stock concentration of the samples. The integrity and quality of the DNA was further confirmed and ascertained on 1% (w/v) agarose gel.

(b) Amplification of the fungal genomic DNA

The identity of the fungal isolates was confirmed by ITS rDNA sequencing. The 550 bp fragment of ITS rDNA was amplified in a thermo cycler (Bio-Rad Model T100^{TM}, USA) using universal primers of ITS1 (5' TCC GTA GGT GAA CCT GCGG 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al.,*1990). The PCR was carried out in a final volume of 25 µl, containing 10 µM of oligonucleotide primer (1 µl), 12.5 µl of One Taq® 2X Master Mix with Standard buffer (New England Bio-Labs), 1.0 µl template DNA(60 ng/µl) and 9.5 µl of Nuclease-free Water to make the reaction up to 25 µl (Kumar et al., 2008). Amplification was performed in thermocyclers under the condition: initial denaturation at 94°C for 2 minutes, 35 repeat cycles of 94°C for 1 minute, 60°C for 1 minute, 68°C for 3 minutes, final extension at 68°C for 10 minutes and infinite hold at 4°C.

(c) Gel electrophoresis

The 1XTAE buffer (40 mM Tris acetate with 1 mM EDTA, pH 8.0) was prepared. A 1% agarose gel was also prepared where 1 g agarose was added to 100 ml of 1×TAE buffer and dissolved over heat (Microwave for 1 minute, 45 seconds). The 2 µl of ethidium bromide was added to the agarose gel at 6 µl/100 mL for staining. Thereafter, 1 µl of the PCR product was mixed with 1 µl of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol were dissolved in 1XTAE buffer). The solution was then loaded in 1% agarose gel

wells and subjected to electrophoresis at 60 voltages for 1 hour. A ladder (lambda DNA/Eco RI + Hind III) was used as a size standard. DNA was visualized on the agarose gel by trans-illuminator with ultra violet (UV) light and photographed. All fungal pure isolates were maintained on PDA broth with 50% glycerol in 2 ml Eppendorf tubes in the -80°C refrigerator until required.

(d) Sequencing analysis

The amplified products were sequenced using Sanger sequencing (Central Analytical Facility, University of Stellenbosch). The ITS rDNA sequence of the fungal isolates were edited using the program CLUSTALW2 from BioEdit software (Martin & Rygiewicz, 2005). Homologies of ITS rDNA sequence of the isolates were analysed using BLAST (Basic Local Alignment Search Tool) program from GenBank (National Centre for Biotechnology Information, USA) database (www.ncbi.nlm.nih.gov/blast/), and the highest matching sequences were downloaded. The multiple alignments were achieved using the CLSTALW2 program method of the BioEdit software with reference sequences from the GenBank. The MEGA Version 6.0 program (Pennsylvania State University, University Park, PA) was then used to construct a phylogenetic tree using the Neighbor-joining method. The statistical significance was estimated by 1000 bootstrap replications to estimate the stability and support of the branches (Shukla et al., 2010; El-katatny & Emam, 2012). The Phylogenetic trees were used to

demonstrate the evolutionary relationship between genotypes obtained from this study and from the GenBank.

5. *In vitro* antagonistic assay screening of fungal isolates

The test fungal isolates and the known antagonistic fungi inhibition were studied with dual culture plate technique under *in vitro* conditions (Chérif & Benhamou, 1990). The test fungal isolates, the known pathogenic fungi (*Rhizopus stolonifera* ATCC 6227a, *Rhizopus stolonifera* ATCC 6227b, *Geotrichum candidum* ATCC 34614 and *Fusarium solani* ATCC 36031), and known antagonistic fungi (*Cryptococcus laurentii* ATCC 18803, *Aspergillus niger* ATCC 16888, *Candida albicans* ATCC 10231 and *Microsporum canis* ATCC 36299) were grown separately on PDA medium, incubated at 25°C for 5 days before use for dual culture technique.

The 5 mm of agar block was cut from actively growing margin of individual species of test fungal isolates and of known pathogenic fungi, which were inoculated approximately 3 cm away from each other on PDA medium and incubated at 25°C for 7 days. The experiment was replicated in triplicates for each set. Controls were set, where single inoculated cultures of the fungus and the pathogenic fungi were grown against known antagonistic fungi. Calculations

were made when the fungi growth had attained equilibrium (or after 7 days), at which is no further alteration in the growth (Naglot et al., 2015).

The percent inhibition in growth will be calculated according to the following formula

$$PGI = \frac{\text{KR} - \text{R1}}{\text{KR}} \times 100$$

Where PGI represents the percentage (%) growth inhibition, KR represents the distance (measured in mm) from the point of inoculation to the colony margin on the control plate, and R1 is the distance (measured in mm) of fungal growth from the point of inoculation to the colony margin on the treated dishes in the direction of the antagonist (Zivkovic et al., 2010). The fungal isolates that would have inhibited the growth of the pathogenic fungi were subsequently screened for the production of as described below.

6. Screening of fungal isolates for probiotic properties

The antagonistic fungal isolates were further screened for their antifungal resistance, thermo tolerance ability, pH variability and bile salt resistance. All the suspensions used as inoculum were prepares from cultures frown on PDA at 25°C for 5 days.

a. Tolerance to low pH

A suspension was prepared in Potato Dextrose Broth (PDB) and OD was adjusted to 0.6 at 600 nm. The suspension was concentrated by centrifugation at 5000 g for 10 min, washed with buffered phosphate saline (PBS, 10 mM phosphate, pH 7.4), and suspended again in 3 mL of the same buffered solution previously adjusted at pH 2.0, 2.5, 3.0 and 7.4 with NaOH and HCl 2M. Suspensions were incubated for 3 hours, and then aliquots inoculated (1/10, v/v) in PDB and incubated. After 24 hours, the samples were diluted serially, and inoculated onto PDA at 25°C for 3 days to determine the cfu/mL (García-Hernández *et al.,* 2012). The test was performed in triplicate according to completely randomized design and the survival (%) was calculated as:

$$S = \frac{[(\text{cfu/mL})_{\text{PDB+inoculum pH 2.5}} \times 100]}{(\text{cfu/mL})_{\text{PDB+inoculum pH 7.4}}}$$

b. Bile salts tolerance

Suspensions were prepared in buffered phosphate saline and the OD of 0.6 at 600 nm. The suspensions were diluted serially, cultivated in PDA containing 1%, 2% and 3% (w/v) of bile salts (Ox-Gall, Oxoid, UK) and incubated at 25°C for 5 days. The cfu/mL for each treatment were determined (García-Hernández *et al.,* 2012). The assay was performed in triplicate and the survival percent (S) was calculated applying the formula:

$$S = \frac{[(\text{cfu/mL})_{\text{PDA+salts}} \times 100]}{(\text{cfu/mL})_{\text{PDA}}}$$

c. Antifungal Susceptibility Testing

The test to assess the antifungal susceptibility of various fungal isolates to known fungal antibiotics was performed using the disc diffusion method. Fungi were grown in conical flask (150 ml) containing 50 ml of PDB and incubated in a shaking incubator at 25°C for 5 days. Thereafter 100 µl aliquots of prepared fungal cultures were spread on PDA agar plates. The antifungal discs were placed on the surface of agar and incubated at 25°C for 5 days. The antifungal discs used were Amphotericin B (20 µg), Clotrimazole (10 µg), Fluconazole (25 µg), Flucytosine (1 µg), Ketoconazole (10 µg), Mecillinam (10 µg), Nystatin (10 µg) and Penicillin G (10 µg) purchased from Mast discs™ Mast diagnostics. Susceptibility was expressed as minimum fungicidal concentration (MFC).

The results were recorded as average of three readings. The sensitivity of fungal isolates to the fungal antibiotics was determined by measuring the diameter of inhibition zone from the edge to the border of the zone was measured by a ruler. The experiment was repeated three times, and results were recorded as average of the three readings (Makete, et al., 2017). The values >8 mm were considered as not active against the microorganisms (Bhalodia & Shukla, 2011).

7. Storage trials

Tomato fruits were commercially grown and harvested from ARC-VOPI, and were immediately transported to the ARC-API for storage. The fruits were randomly sorted based on size, maturity, free of physical injuries or apparent decay. They were washed in a 2% (v/v) sodium hypochlorite solution for 2 min, rinsed with tap water, and air-dried prior to use (Zhu *et al.,* 2010). The sporekill fungicide was purchased and were used as a control.

a. Fungal isolates

RT10A, RT15, RT24B and RT26 fungi were isolated from natural infected tomato fruits and cultured on PDA AT 25°C for 7 days. Fungal spores were obtained by flooding the surface of the culture with sterile distilled water containing 0.05% (v/v) Tween-80. The suspension was filtered through four layers of sterile cheesecloth and adjusted to a concentration of 5×10^3 spores mL^{-1} using a hemocytometer (Zhu *et al.,* 2010). The suspension was freeze dried for 3 days.

b. Treatment with fungal isolates and controls

The tomatoes were stored at two different storage conditions i.e. the 8°C refrigerator and at room temperature. For quality assessment, 30 tomato fruits were randomly distributed into replicates of 10 fruits for each treatment, the distribution of fruits were done on both the storage systems (Reddy *et al.,* 2000).

The experiment was conducted in triplicates for each treatment at two different storage temperatures (8°C cold room and room temperature). The tomatoes were sprayed with isolated fungal antagonistic bio-preservative and therefore, the spoilage percentage and the weight loss were measured on the 5th, 10th and 15th days of the treatment (Chandra and Chowdhary, 2015).

c. Weight loss

Tomato samples were weighed non-destructively on days 0, 5, 10 and 15 days. The difference between initial and final fruit weight was considered as total weight loss during each storage interval and calculated as percentages on a fresh-weight basis by the standard method according to (Fagundes *et al.,* 2015).

d. Spoilage percentage

The rotten tomatoes in each treatment were counted on days 0, 5, 10 and 15 days. The difference between the treatments after storage interval was calculated as percentages by the standard method according to (Fagundes *et al.,* 2015).

8. Statistical analysis

The data for isolates and pathogens was subjected to an appropriate analysis of variance (ANOVA). The Shapiro-Wilk's test was performed on the standardized residuals to test for deviations from normality (Shapiro and Wilk 1965). In cases where significant deviation from normality was observed and due to skewness,

outliers were removed until it was normal or symmetrically distributed. (Glass et.al. 1972) Student's t-LSDs (Least significant differences) were calculated at a 5% significance level ($P < 0.05$) to compare means of significant source effects (Snedecor & Cochran, 1967). The above analysis was performed using SAS version 9.3 statistical software (SAS, 1999) and Genstat Release 18.

RESULTS AND DISCUSSIONS

Morphological identification

When tomato fruit starts to rot, it begins with a small and dark lesions like a filled with water appearance, which increases in diameter and might have mould over a period of time. Most of the colonies of tomato isolates were aerial, light in color becoming darkened as matured on PDA Agar over 5 days of incubation. Some of the colonies developed dark colors (black, grey, green etc.) around the center of the colony and light colors (white, yellow etc.) on the outer layer of the colonies, including setae (Table: 1).

Table 1: Cultural, Morphological and Microscopic identification of fungal isolates from tomato fruits

Fungal Isolates	Pigmentation		Mycelial Growth			Hyphae	Mold/ Yeast
	Color	Hyaline/ Dematiaceous	Circular/ Irregular	Smooth/ Rough	Aerial/ Vegetative	Aseptate/ Septate	
RT1	White	Hyaline	Circular	Smooth	Aerial	Septate	Mold
RT2	White	Hyaline	Circular	Smooth	Aerial	Septate	Mold
RT4	Purple	Hyaline	Circular	Rough	Aerial	Septate	Mold
RT5	Olive green-white	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT6	Olive green-white	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT7	Green	Dematiaceous	Irregular	Smooth	Aerial	Septate	Mold
RT8	Pink- Cream white	Hyaline	Circular	Rough	Aerial	Septate	Mold
RT9	Grey	Dematiaceous	Irregular	Smooth	Aerial	Septate	Mold
RT10A	Olive green-white	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT10B	White	Hyaline	Circular	Smooth	Aerial	Septate	Mold
RT11	Orange	Dematiaceous	Irregular	Rough	Aerial	Septate	Mold
RT12	Dark brown	Dematiaceous	Irregular	Smooth	Aerial	Aseptate	Mold
RT13	Purple	Hyaline	Circular	Rough	Aerial	Septate	Mold
RT15	Olive green-white	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT16	Purple	Hyaline	Circular	Rough	Aerial	Septate	Mold
RT17	Dark brown	Dematiaceous	Irregular	Smooth	Aerial	Aseptate	Mold
RT18	Pink- Cream white	Hyaline	Circular	Rough	Aerial	Septate	Mold
RT19	Purple	Hyaline	Circular	Rough	Aerial	Septate	Mold
RT20	Black-olive green	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT21	Brown	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT24A	Purple-white	Hyaline	Circular	Rough	Aerial	Septate	Mold
RT24B	White	Hyaline	Circular	Smooth	Aerial	Septate	Mold
RT25	Light grey	Hyaline	Irregular	Rough	Aerial	Aseptate	Mold
RT26	Brown	Dematiaceous	Irregular	Rough	Aerial	Aseptate	Mold
RT27	Light grey	Hyaline	Irregular	Rough	Aerial	Aseptate	Mold
RT29	Black-olive green	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT31	Dark Green	Dematiaceous	Irregular	Smooth	Aerial	Septate	Mold
RT32	Blackish-brown	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT33	Black-olive green	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT34	Light grey	Hyaline	Irregular	Rough	Aerial	Aseptate	Mold
RT35	Black	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT36	Pale brown	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT37	Olive green	Dematiaceous	Irregular	Smooth	Aerial	Septate	Mold
RT38	Olive green	Dematiaceous	Irregular	Smooth	Aerial	Septate	Mold
RT39	Olive green	Dematiaceous	Irregular	Smooth	Aerial	Septate	Mold
RT40	Dark Grey	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT41	Black	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT42	Olive green	Dematiaceous	Irregular	Smooth	Aerial	Septate	Mold
RT43	Blackish-brown	Dematiaceous	Circular	Rough	Aerial	Septate	Mold
RT44	Dark Grey	Dematiaceous	Circular	Rough	Aerial	Septate	Mold

Molecular identification

Forty (40) fungal species were isolated from both fresh and rotten tomatoes, and showed different colonies and mycelial characteristics. The identity of these fungal isolates were further confirmed using the molecular technique. The ITS1

and ITS4 primers amplified a 16S of the genomic DNA of fungal species isolated from tomato fruits. The *Penicillium, Fusarium* were more dominant as compared to *Curvularia, Alternaria, Cladosporium, Lecythophora, Aureobasidium, Byssochlamys, Retroconis* and *Epicoccum* in decreasing order of dominance (Table: 2). These fungal isolates were group to nineteen (19) Operational Taxonomic Units (OTUs) shown in (Table: 3), which were identified and new accession numbers were obtained through Genbank and significantly different from each other.

Table 2: Prevalent of fungal isolates in both fresh and rotten tomato fruits.

Fungal species	**Number of isolates**	**% frequency**
Penicillium	9	22.5
Fusarium	9	22.5
Curvularia	6	15
Alternaria	5	12.5
Cladosporium	4	10
Lecythophora	2	5
Aureobasidium	2	5
Byssochlamys	1	2.5
Retroconis	1	2.5
Epicoccum	1	2.5
Total	**40**	**100**

Table 3: Molecular identification of fungi isolated from tomato fruits

OTU No:	Isolates	Molecular identification	Accession no: Genbank	Similarity %	New Accession no:
1	RT7	*Penicillium citreosulfuratum*	NR_153252	99	MG975610
2	RT9	*Penicillium thomii*	KM396384	98	MG975612
3	RT10A	*Byssochlamys spectabilis*	MG647865	99	MG975613
4	RT10B	*Aureobasidium pullulans*	LC317470	99	MG975614
5	RT11= RT5	*Lecythophora sp.*	JX838853	98	MG975615
6	RT16	*Fusarium oxysporum*	KP780428	87	MG975619
7	RT19 = RT4 = RT13	*Fusarium oxysporum*	MG461555	99	MG975622
8	RT24A = RT18 = RT8 = RT6	*Fusarium verticillioides*	MF682356	99	MG975609
9	RT24B	*Epicoccum thailandicum*	NR_152926	97	MG975626
10	RT26	*Retroconis fusiformis*	EU040239	99	MG975628
11	RT27	*Aureobasidium pullulans*	LC317470	96	MG975629
12	RT32 = RT15 = RT21 = RT17 = RT12	*Curvularia kusanoi*	MF061766	99	MG975620
13	RT34 = RT25 = RT31	*Penicillium crustosum*	JN585931	99	MG975627
14	RT35	*Alternaria brassicicola*	KF542550	86	MG975635
15	RT36 = RT33 = RT29 = RT20	*Alternaria tenuissima*	KX783385	99	MG975636
16	RT41 = RT2 = RT1	*Penicillium sp.*	KM659896	99	MG975641
17	RT42 = RT39 = RT38 = RT37	*Cladosporium sp.*	KX378909	99	MG975639
18	RT43 = RT40	*Curvularia sp.*	MG309756	99	MG975643
19	RT44	*Penicillium griseofulvum*	MF034654	92	MG975644

= ; Identical

Antagonistic assay

The 40 fungal isolates were tested against the 8 fungal pathogens using the dual culture technique. The experiment was conducted in triplicates and repeated 3 times. The 4 fungal pathogens strains (*Fusarium solani* ATCC 36031, *Geotrichum candidum* ATCC 34614, *Rhizopus stolonifer* ATCC 6227a, *Rhizopus*

stolonifer ATCC 6227b) are the American Type Culture Collection (ATCC). The other 4 fungal pathogens strains (*Rhizoctonia solani Fusarium oxysporum, Alternaria solani, Alternaria altenata*) are the ARC-PPRI Collection.

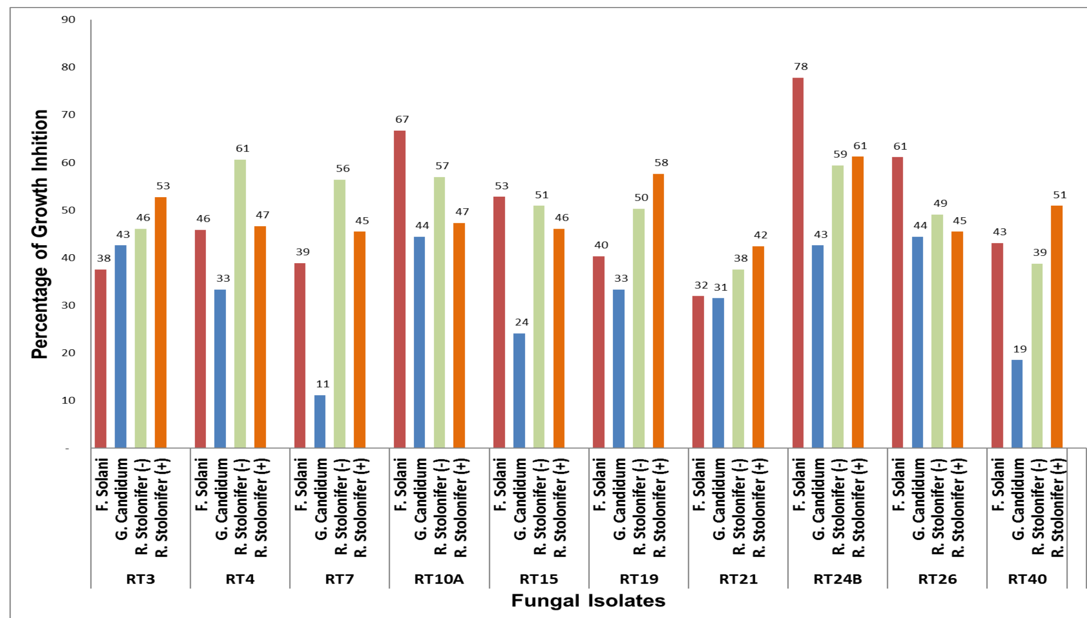

Figure 1: The antagonistic ability of fungal isolates on known ATCC pathogenic fungi of tomato fruits.

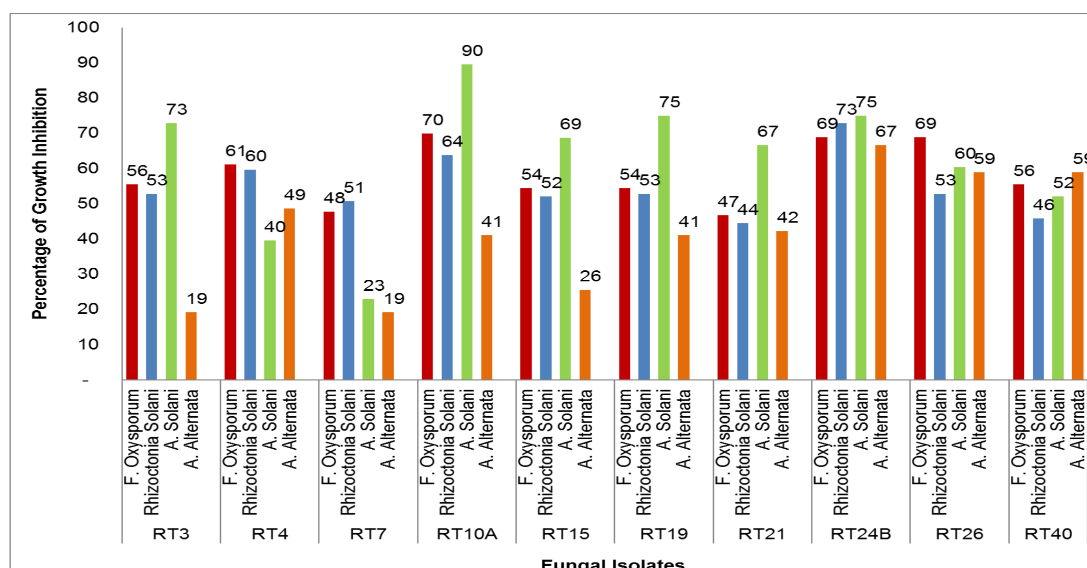

Figure 2: The antagonistic ability of fungal isolates on known S.A. pathogenic fungi of tomato fruits.

Results from the dual culture assay showed that different antagonistic fungi isolated from tomato fruits inhibited most of the fungal pathogens. Among all the 40 fungal isolates only 10 (RT3, RT4, RT7, RT10A, RT15, RT19, RT21, RT 24B, RT26, RT40) fungal strains could antagonise about 2 (25%) to 7(88%) of the 8 pathogenic fungi. Out of the 10 prospective antagonists, only four (4) fungal species (Table 4) were able to antagonize 5 to 7 of the 8 fungal pathogens. The RT10A, RT15 and RT26 inhibited the growth of 5 fungal pathogens. The RT10A inhibited R. Stolonifer ATCC 6227(a) by 57%, *F. Solani* ATCC 36031 by 67%, *R. Solani* by 64%, *F. Oxysporum* by 70% and *A. solani* by 90%. The RT15 inhibited *R. Stolonifer* ATCC 6227(a) by 51%, *R. Solani* by 52%, *F. Solani* ATCC 36031 by 53%, *F. Oxysporum* by 54%, and *A. solani* by 69%. The RT26 inhibited the *R. solani* by 53%, *A. altenata* by 59%, *A. solani* by 60%, *F. solani* by 61%, and *F. oxysporum* by 69%. Whereas, the RT24B could antagonise 7 fungal pathogens, the *R. Stolonifer* ATCC 6227(a) by 59%, *R. Stolonifer* ATCC 6227(b) by 61%, *A. altenata* by 67%, *A. solani* by 69%, *F. Oxysporum* by 73%, *R. Solani* by 75%, *A. solani* by 78%. All these 4 antagonistic fungal isolated were subjected to storage trials.

Table 4: Molecular identification of four antagonistic fungi isolated from tomato fruits

Isolates	Molecular identification	Accession no: Genbank
RT10A	*Byssochlamys spectabilis*	MG975613
RT15	*Curvularia kusanoi*	MG975620
RT24B	*Epicoccum thailandicum*	MG975626
RT26	*Retroconis fusiformis*	MG975628

Probiotic assays

The 4 antagonists were tested for bile tolerance, acid tolerance and antifungal activity. In order to investigate if they are able to confer health benefits to the host when consumed along with the tomato after spraying.

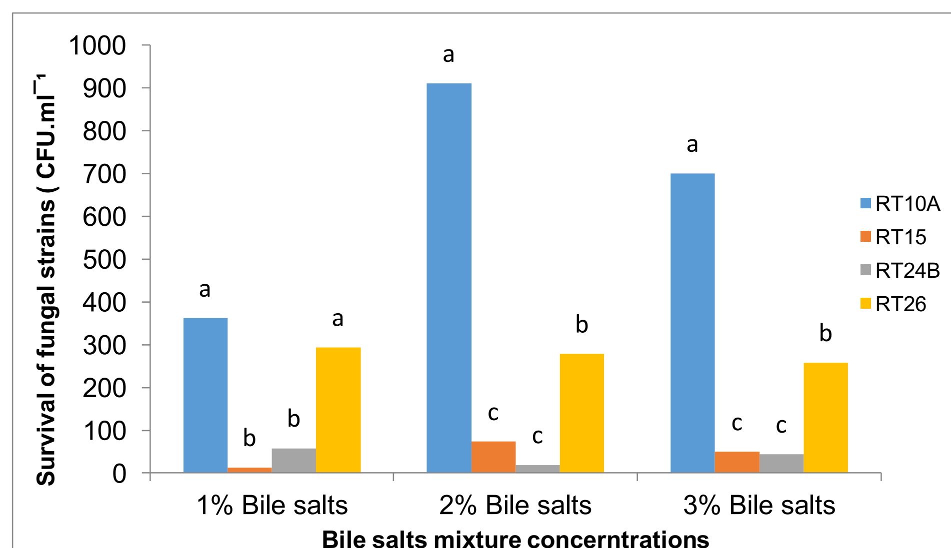

Figure 3: Adaptation of fungal isolates to different bile salts concentrations

The bile salt percentage of intestine of human is 1%. Therefore, the RT10A and RT26 could significantly ($P < 0.05$) survive in all the different bile salts percentages, as compared to RT15 and RT24B (figure 3).

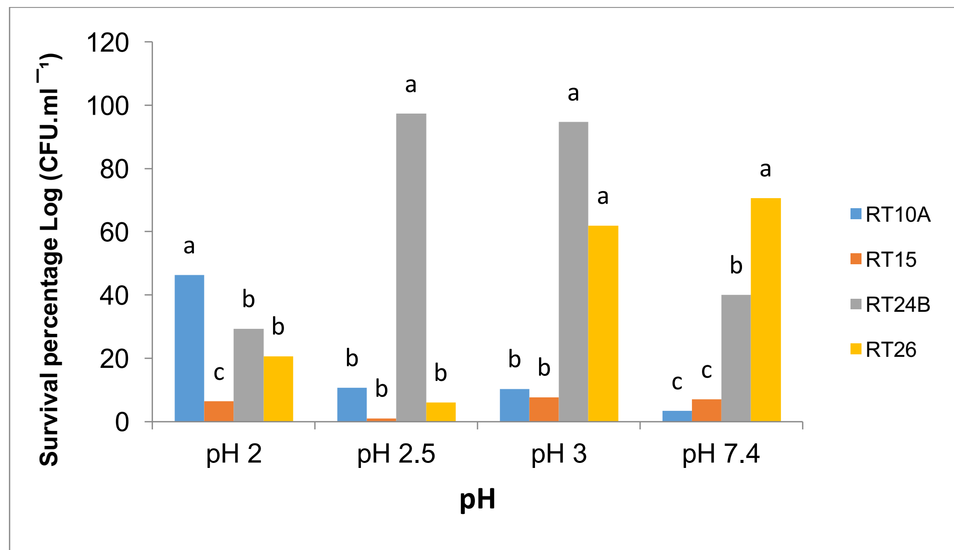

Figure 4: Adaptation of fungal isolates to different acid levels

The human stomach pH is 2-3. Therefore, the RT10A could only survive in pH 2, and RT24B could only survive significantly ($P < 0.05$) in other concentrations except pH 2, RT26 was able to significantly grow in pH 3 and pH 7.4 as compared to other acid levels whereas there was no significant growth in RT15 for all the acid levels (figure 4).

Table 5: Antifungal activity of fungal isolates of Tomato. Values are means of triplicate determinations with standard deviations (Mean ± SD). Values are given as means SD from three independent experiments. Values not sharing common superscript differ significantly at P < 0 05 (Fisher's test)

Fungal isolates	Antifungal drugs						
	Zone of inhibition in mm						
	Amphotericin B	Clotrimazole	Fluconazole	Ketoconazole	Mecillinam	Nystatin	Penicillin G
RT10A	9.3 ± 1.5	8.7 ± 0.6	0.0 ± 0.0	4.3 ± 0.6	0.0 ± 0.0	17 ± 2	0.0 ± 0.0
RT15	10 ± 0.0	4 ± 1	4.7 ± 0.6	0.0 ± 0.0	9 ± 1	4.7 ± 0.6	6.7 ± 0.6
RT24B	0.0 ± 0.0	10.3 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	8.67 ± 0.6	0.0 ± 0.0	5.67 ± 0.6
RT26	10 ± 0.0	3.33 ± 0.6	4.33 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	6 ± 0.0	4.67 ± 0.6

This antagonistic isolates should not confer resistance to many antifungal drugs. RT15 was only significant to Ketoconazole drug, RT 26 was resistant to Ketoconazole and Mecillinam, followed by RT10A which was resistant to Fluconazole, Mecillinam and Penicillin G, whereas the RT24B was resistant to 4 of the 7 antifungal drugs namely Amphotericin B, Fluconazole, Ketoconazole and Nystatin.

Storage trials

The 4 fungal species namely RT15, RT24B, RT10A, RT26 were sprayed on tomato fruits harvested at ARC-VOPI and stored for fifteen (15) days. The treatments were conducted at two different storage temperatures (8°C cold room and room temperature), where they were sprayed with isolated fungal antagonistic bio-preservative.

The RT15 was able to reduce the weight loss and decreased the spoilage percentage of tomato fruits significantly, through both the 8°C cold room and room temperature storages, as shown in Figure 5a, 5b and table 6.

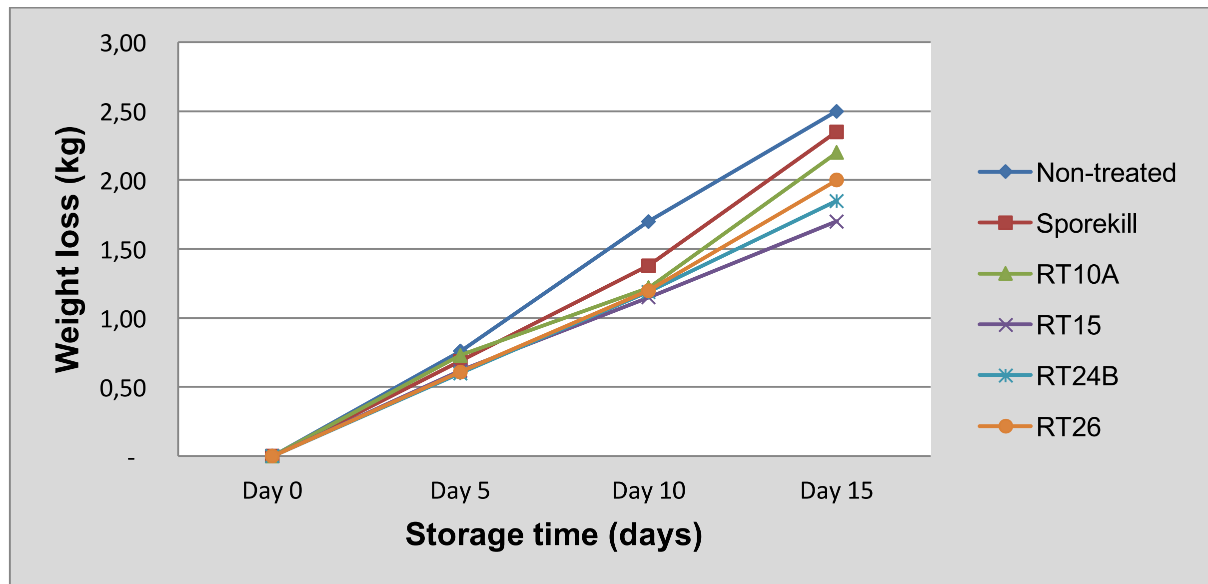

Figure 5a: Effect of postharvest spraying with different treatments on weight loss of tomato fruits stored at 8°C cold room storage.

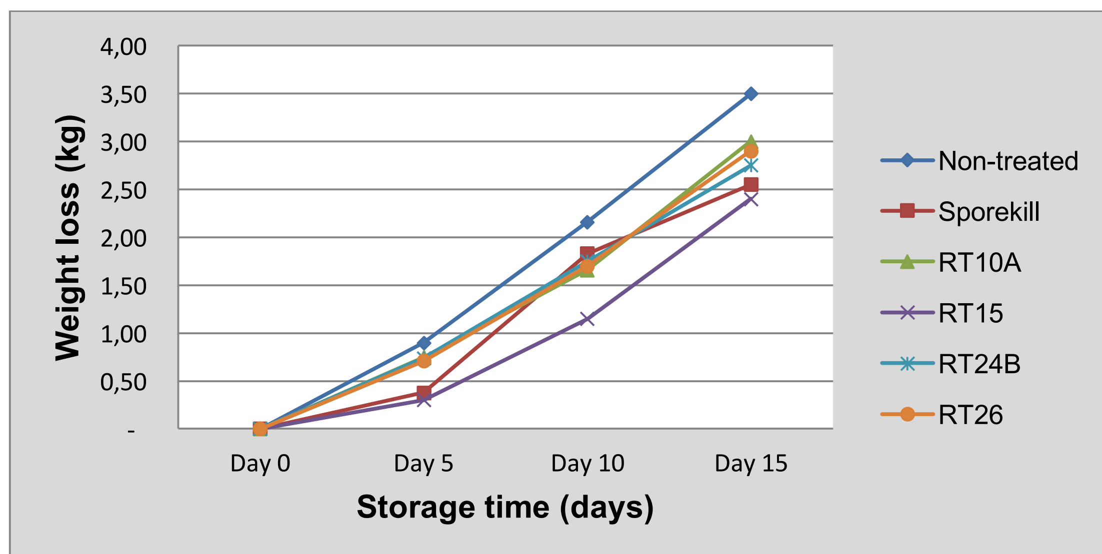

Figure 5b: Effect of postharvest spraying with different treatments on weight loss of tomato fruits stored at uncontrolled room temperature storage

Table 6: Day 0, day 5, DAY 10 and day 15 data for all sets of treatments under different storage temperatures

Parameter	Days	% Spoilage of all sets					
		A RT10A	**B RT15**	**C RT24B**	**D RT26**	**E Non-treated**	**F Sporekill**
Controlled temperature	0	0%	0%	0%	0%	0%	0%
	5	7%	3%	3%	7%	13%	0%
	10	19%	7%	10%	20%	25%	7%
	15	30%	20%	20%	40%	47%	20%
Uncontrolled temperature	0	0%	0%	0%	0%	0%	0%
	5	23%	17%	10%	30%	39%	3%
	10	34%	23%	24%	47%	52%	10%
	15	50%	27%	37%	53%	60%	23%

CONCLUSION

Spraying tomato fruits during storage with RT15 is effective in controlling postharvest decay by extension of shelf-life. This fungal specie is capable of inhibiting the growth of fungal pathogens that cause tomato spoilage. It extends the shelf life of tomato fruits during post-harvest processes and storage for extra days. The technology hence reduces the perishability of tomato fruits, from the time of harvest until the time of ingestion for few more days. Therefore, a cheaper, healthier and more affordable biocontrol agent for the control of post-harvest rots in tomato fruits has been identified, this is expected to give more economic power to the smallholder farmers.

The project has been implemented with absolute focus on the addition of post-harvest values on tomato as grown by resource poor farmers, in order to retain harvests with maximum profit gained in views. The project is met with various challenges that have opened our eyes to new lessons, we have tried as much as possible to incorporate some of these into the project as we progressed.

The overall objective has been met in terms of isolating, identifying, and characterizing antagonistic fungal strains that will be available to rural and small-scale farmers for tomato post-harvest management and to further determine the probiotic properties of these antagonistic strains. This is evidenced by the results and data generated from this research.

We shall make every effort that the product is registered with relevant authorities, following which commercial production and sale of the product can commence. In addition, the product development team will continue to improve on the product and determining how best to store and preserve it, in order to reach more resource poor farmers who are supposed to be the primary beneficiary of the outcome of the research.

Finally, ARC and the research team would like to take this opportunity to thank the IDC Agro Processing Competitiveness Fund for the financial support provided. This support has significantly enabled to develop this product with very great potential for commercialization, thus facilitating access local skills

development and employment opportunities. The project has trained one Masters Student and two research technicians.

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