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THE EFFECTS OF ANTIBIOTICS, NEEM OIL AND *TRICHODERMA* ON SPOILAGE BACTERIA AND FUNGI OF CHERRY TOMATOES

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Abstract

This study investigated the effects of antibiotics, neem oil and *Trichoderma* on spoilage bacteria and fungi of cherry tomatoes. The bacterial and fungal spores and biochemical reactions of the bacterial strains (*Rahnella aquatilis, Microbacterium oxydans, Pseudomonas panacis, Gordonia sputa, and Escherichia coli*) from cherry tomatoes were measured. In addition, the bacterial response to antibiotics and the antifungal indices of neem (*Azadirachta indica*) essential oil and *Trichoderma* were analyzed in vitro.

The number of bacteria and fungus were 208.40 (x 10 colony/mL) and 6.40 (x 10 spores/mL), respectively. In the biochemical reactions (beta-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urea hydrolysis, and gelatinase), the selected bacterial strains were positive for the substrates of ONPG (ortho-Nitrophenyl-β-galactoside), arginine, lysine, ornithine, urea, and charcoal gelatin. In contrast, in these substrates (Na thiosulfate, tryptophan, Na pyruvate, inositol, rhamnose, sucrose, and melibiose), the biochemical reactions (H_2S production, deaminas, indole production, acetoin production, and fermentation/oxidation) were negative for the selected bacterial strains. Cefatazidime (30 µg), Ciprofloxacin (CIP, 5 µg) and octafloxin (OFX, 5 µg) displayed the highest sensitivity against the tested bacterial strains. The neem oil and *Trichoderma* displayed the highest inhibition for *Aspergillus niger*.

Based on these results, antibiotics and neem oil can be useful for reducing the spoilage bacterial and fungal spores of cherry tomatoes.

Key words: *Aspergillus niger, Biochemical reactions, Solanum lycopersicum, Vancomycin.*

1. Introduction

The tomato is one of the most widely consumed fresh fruits across the world since it supports the human body with a well-balanced diet and possesses nutritional compounds [1]. Tomato fruits are consumed not only as food but also as a nutrient supplement, flavoring ingredient, medicine, detoxificant and human system cleanser [2, 3]. The postharvest quality of tomatoes primarily depends on microbial activity. The quality deterioration of tomatoes starts after harvesting. It is essential to know the bacteria and fungi that are involved in spoiling the quality of tomatoes. In order to sustain the original taste and increase the shelf life of tomatoes, we need to prevent or reduce the bacteria and fungi activity.

Microorganisms are related to the decay of tomato fruits [4]. Microbial contaminations are a risk for animals and people due to its toxicity. It can result in: diarrhea, gastroenteritis, meningitis, and respiratory tract infections [5].

Microorganisms can produce mycotoxins which are naturally occurring chemical toxins that cause food poisoning [6, 7]. Microorganisms are responsible for the quality deterioration of tomato fruits [8]. *Aspergillus niger, Pseudomonas solanacearum, Sclerotium rolfsii* and *Fusarium oxysporum* were found in tomatoes in the forest and savanna ecologies of Nigeria [7]. These contaminating pathogens are a serious problem for food safety [9]. The microbial infections of fruits can occur during: growth, harvest, postharvest processing, storage, conveyance, packaging and loading and off-loading; whereas, bacteria and fungi are prevalent via various channels and selling outlets [5, 10, and 11].

Tomatoes exist across the globe and are used in stews and soups and consumed raw in salads. There is insufficient research on the bacterial and fungal spores and the biochemical reactions of bacterial strains (*Rahnella aquatilis, Microbacterium oxydans, Pseudomonas panacis, Gordonia sputa,* and *Escherichia coli*) from cherry tomatoes, bacterial response to antibiotics, and antifungal indices of neem (*Azadirachta indica*) essential oil *in vitro* culture of cherry tomatoes. The frequency, isolation, and characterization of microorganisms associated with tomatoes were noted and a microbial control technique was applied to avoid tomato spoilage. This study was conducted to determine the effects of antibiotics, neem oil and *Trichoderma* on spoilage bacteria and fungi of cherry tomatoes.

2. Materials and Methods

2.1 Tomatoes and treatments

Summer grown light red maturity stage of cherry tomatoes (*Solanum lycopersicum* cv. 'Unicorn') were collected from the local market at the Gangwon Province in the Korea Republic to conduct this experiment. The bacterial colony and fungal spores and the biochemical reactions of the bacterial strains (*Rahnella aquatilis, Microbacterium oxydans, Pseudomonas panacis, Gordonia sputa,* and *Escherichia coli*) from cherry tomatoes were analyzed. In addition, the bacterial response to antibiotics, antifungal indices of neem (*Azadirachta indica*) essential oil and *Trichoderma* were also analyzed *in vitro*.

2.2 Count of bacterial colony and fungal spores

Bacterial colony and fungal spores count was conducted according to Islam *et al.,* [12]. In briefly, a chilled (4 ^{0}C) cherry tomato slice (3 cm^2) was poured in 10 mL of 0.1% peptone for sterilization and shaken. The nutrient agar (NA) and potato dextrose agar (PDA) were used for bacteria and fungi accordingly. The plates were incubated for two days at 37 ^{0}C for bacteria and five days at 25 ^{0}C for fungi. After incubation, the bacteria and fungi were identified based on the colony characterization and microscopic methods.

2.3 Biochemical activities

Gram stain, oxidases, and catalases reactions were performed to identify the bacterial species. The biochemical properties such as: ß-galactosidase, H_2S production, urease, and indole production were determined using a biochemical test kit API 20NE strip (BioMerieux Inc., Durham, N. C.) to confirm the bacteria identification [13].

2.4 Antibiotic susceptibility

A standardized disc diffusion method was used by applying the zone size to evaluate the bacteria's sensitivity to the selected antibiotics. The susceptibility of the bacterial strains was isolated from the tomato fruits (*Solanum lycopersicum* L.). Luria-Bertani Agar medium (LB) plates were used to determine the performance of: neomycin (30 µg), octafloxin (5 µg), ciprofloxacin (5µg), ceftazidime (30 µg), vancomycin (30 µg), amikacin (30 µg), and gentamicin (10 µg) [14]. Antibiotic discs (BD BBL™ Sensi-Disc™ Sparks, MD 21152 USA) were positioned aseptically on the surface of the agar plates using sterilized forceps and thereafter incubated at 37 ^{0}C for 24 hours, while the inhibited zones were measured and classified as either sensitive or resistant.

2.5 Neem antifungal activity

The neem oil was amended in a PDA to create 2.5% and 5%, concentrations in the Petri plates [15]. The solidified agar plates were inoculated at the center with a 5mm-diameter mycelial disc of a pathogen and incubated at 27 ^{0}C for 10 days. The plates without neem oil served as controls. The inhibition percentage was calculated using the formula:

$$\text{Inhibition percentage (\%)} = A_1\text{-}[A_2/A_1] \times 100$$

Where, A_1 is the colony area of uninhibited fungus in the control group, and A_2 is the colony area of fungus in the dual culture.

2.6 Antagonistic activity of *Trichoderma*

The antagonistic fungi combinations were examined on 20 mL of PDA in 9-cm Petri plates. A mycelial plug (0.5 cm in diameter) was taken from an actively growing 3-day old culture of *Aspergillus niger, Botrytis cineria, Cladosporium* sp., *Fusarium* sp., and *Penicillium* sp. *Trichoderma* isolates were placed 8 cm apart from each other on the PDA. The plates were incubated at 28 ^{0}C [16]. The observations of the antagonistic activities of *Trichoderma* isolates on *Penicillium* sp. were recorded after 5 days. The inhibition percentage was calculated using the formula for neem antifungal activity.

2.7 Statistical analysis

The differences in the mean values were analyzed by Duncan's multiple range test (DMRT) of one-way analysis of variance (ANOVA) using the Statistical Package for the Social Science, version 16 (SPSS Inc., Chicago, IL, USA) software.

3. Results and Discussion

3.1 Count of bacterial colony and fungal spores

The bacterial colony and fungal spores were 208.4 (x 10 colony/mL) and 6.4 (x 10 spores/mL), respectively (Table 1).

Table 1. Count of bacteria and fungi which spoilage cherry tomatoes

Bacteria (x 10 colony/mL)	Fungi (x 10 spores/mL)
208.4 ± 3.67	6.4 ± 0.51

Each data point is the mean of five replicates ± standard error.

The density of the fungal spores was comparatively lower than the bacterial colony. Wogu and Ofuase [17], reported that the mean microbial count ranges were 2 x 10^4 - 35 x 10^4 for the New Benin market; 1 x 10^4 - 25 x 10^4 for the Vegetable market; 2 x 10^4 - 23 x 10^4 for the Oba market and 1.1 x 10^4 - 9.3 x 10^4 for the Santana market. Fungal and bacterial contamination may occur due to the large amount of water content in tomatoes and cause tomatoes to lose their fitness for consumption. Trias *et al.*, [18], and Ogunbanwo *et al.*, [7], reported that fungal and bacterial contamination increases due to the high carbohydrate and poor protein content of tomatoes.

3.2 Biochemical tests

The biochemical reactions of the selected bacterial strains were positive for: beta-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urea hydrolysis, and gelatinase in the substrates of Na thiosulfate, tryptophan, Na pyruvate, inositol, rhamnose, sucrose, and melibiose (Table 2).

H_2S production, deaminas, indole production, acetoin production, and fermentation/oxidation biochemical reactions demonstrated negative results in the substrates of: Na thiosulfate, tryptophan, Na pyruvate, inositol, rhamnose, sucrose, and melibiose. The variation of the microbial contamination depended on the geographic location, seasonal changes, and agronomic practices (cultivation, harvesting, handling and packaging) [19].

Table 2. The biochemical reactions of the selected bacterial strains from tomato fruit samples

Biochemical reaction	Substrate	*Rahnella aquatilis*	*Microbacterium oxydans*	*Pseudomonas panacis*	*Gordonia sputi*	*Escherichia coli*
Beta-galactosidase	ONPG	+	+	+	+	+
Arginine dihydrolase	arginine	+	+	+	+	+
Lysine decarboxylase	lysine	+	+	+	+	+
Ornithine decarboxylase	ornithine	+	+	+	+	+
Citrate utilization	citrate	+	-	+	+	+
H_2S production	Na thiosulfate	-	-	-	-	-
Urea hydrolysis	urea	+	+	+	+	+
Deaminase	tryptophane	-	-	-	-	-
Indole production	tryptophane	-	-	-	-	-
Acetoin production	Na pyruvate	-	-	-	-	-
Gelatinase	Charcoal gelatin	+	+	+	+	+
Fermentation/oxidation	glucose	+	-	-	-	-
Fermentation/oxidation	manitol	+	-	-	-	-
Fermentation/oxidation	inositol	-	-	-	-	-
Fermentation/oxidation	sorbitol	+	-	-	-	-
Fermentation/oxidation	rhamnose	-	-	-	-	-
Fermentation/oxidation	sucrose	-	-	-	-	-
Fermentation/oxidation	melibiose	-	-	-	-	-
Fermentation/oxidation	amygdalin	+	-	-	-	-
Fermentation/oxidation	arabinose	+	-	-	-	-

3.3 Antibiotics effect

The highest sensitivity against the bacterial strains occurred in the cefatazidime (30 µg), ciprofloxacin (CIP, 5 µg) and octafloxin (OFX, 5 µg) antibiotics (Table 3).

In addition, the maximum resistant ability against all of the bacterial strains except for Gordonia sputa was exhibited in vancomycin (30 µg). Wogu & Ofuase [17], reported on the antibiotic sensitivities and resistances to the bacteria that exists on a spoiled tomato that are a high risk for consumers' health. When antibiotics are applied, sensitive bacteria die and resistant bacteria survive. Antibiotics target specific bacterial cell walls, cell membranes, proteins, RNA, DNA, and folate synthesis [20, 21].

3.4 Neem oil as antifungal agent

The neem oil showed the highest antifungal activity against *Aspergillus niger*, and the lowest in *Penicillium* sp. (Table 4).

Moderate antifungal activity was recorded against *Cladosporium* sp., *Fusarium* sp., and *Botrytis cinerea*. Mandal *et al.*, [22] reported that neem extracts protect crops against fungal infestation. Mahmoud *et al.*, [23], demonstrated similar results and found that the application time and concentration of the neem oil facilitates the attack on the fungal pathogen's cell wall. Since neem oil contains desactylimbin, quercetin and sitosterol [24], it can potentially fight against fungal pathogens.

3.5 Antagonistic activity of *Trichoderma*

The *Trichoderma* demonstrated the highest antifungal activity against *Aspergillus niger,* followed by *Cladosporium* sp., *Fusarium* sp., *Botrytis cineria,* and *Penicillium* sp. (Table 5).

Trichoderma contain lytic enzymes that hydrolyze the phytopathogenic fungal cell wall [25]. Moreover, *Trichoderma harzianum, Fusarium oxysporum*, and *A. pullulans* produce volatile antifungal substances [26] that potentially can be used as a biofumigant and to control postharvest diseases of fruits [27]. Both the inhibition percentage and inhibition zone had maximum values against the *Aspergillus niger of Trichoderma* treatment.

Table 3. Response of the tomato fruits associated strains to the antibiotic actions

	Antibiotics influence						
Bacterial strains	Amikacin (30 µg)	Cefatazidime (30 µg)	Ciprofloxacin (5 µg)	Gentamicin (10 µg)	Neomycin (30 µg)	Ofloxacin (5 µg)	Vancomycin (30 µg)
R. aquatilis	S	S	S	R	R	S	R
M. oxydans	R	S	S	I	S	S	R
P. panacis	R	S	R	S	R	S	R
G. sputi	I	I	S	S	S	R	S
E. coli	I	S	S	R	R	S	R

S = sensitivity, R = resistant, I = indifferent.

Table 4. Antifungal indices of neem (*Azadirachta indica*) essential oil

Neem oil dosage (%)	Antifungal index				
	Aspergillus niger	*Botrytis cineria*	*Cladosporium* sp.	*Fusarium* sp.	*Penicillium* sp.
0.0	0c[z]	0c	0c	0c	0c
2.5	12b	10b	15b	8b	6b
5.0	32a	22a	25a	23a	14a
P value	***	***	***	***	***

[z]Mean separation of columns by Duncan's multiple range tests (DMRT) (n=3). ***; significant at p≤ 0.001.

Table 5. *In vitro* antagonistic activity of *Trichoderma* sp. against fungal species

Fungal species	Inhibition (%)	Inhibition zone (cm)
Aspergillus niger	33.97a[z]	0.63a
Botrytis cineria	25.17bc	0.33ab
Cladosporium sp.	30.50ab	0.57ab
Fusarium sp.	25.80bc	0.37ab
Penicillium sp.	21.77c	0.23b
P value	***	***

[z]Mean separation of columns by Duncan's multiple range tests (DMRT) (n = 3). ***; significant at p ≤ 0.001.

4. Conclusions

- The effects of antibiotics, neem oil and *Trichoderma* on spoilage bacteria and fungi of cherry tomatoes were examined in this study.

- The highest sensitivity against the tested bacterial strains were displayed by cefatazidime (30 µg), ciprofloxacin (CIP, 5 µg) and octafloxin (OFX, 5 µg).

- The neem oil and *Trichoderma* showed the highest antifungal activity against *Aspergillus niger.*

- Based on the above results, antibiotics, neem oil and *Trichoderma* can be useful in reducing the spoilage bacterial and fungal spores of cherry tomatoes.

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