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Control of *Aspergillus* postharvest rot on plum tomato by potassium metabisulfite and potassium sorbate

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Abstract

In this study, fungal contamination of plum tomato fruits (*Solanum lycopersicum*) was preliminarily examined by the dilution plating method. The tomato samples were highly contaminated by molds and yeasts ($1.0x10^5$ - $1.4x10^8$ CFU/g), including *Aspergillus*, *Geotrichum*, *Penicillium*, *Rhizopus*, *Candida*, *Cryptococcus* and *Rhodotorula*. The mold strains that contaminated the tomatoes to the greatest extent were *Penicillium* and *Rhizopus*. Three fungal isolates (*Aspergillus niger* T6D5, *Candida guilliermondii* T7D4 and *Rhodotorula mucilaginosa* T7D5) were selected for further *in vitro* study. Then, the antifungal activity of organic acids (citric and tartaric acids) and salts (potassium acetate, potassium metabisulfite and potassium sorbate) against molds and yeasts was investigated. Salt solutions showed higher inhibitory effects than did acid solutions. Interestingly, *A. niger* T6D5 showed the highest susceptibility to potassium sorbate and potassium metabisulfite at 0.01-1% (w/v) MIC, respectively, compared to other fungi. In yeasts, potassium metabisulfite effectively inhibited the growth of *C. guilliermondii* T7D4 and *R. mucilaginosa* T7D5 at 0.25% (w/v) MIC. The use of organic acids and salts to control the growth of *A. niger* T6D5 on tomatoes was investigated. Among all the salts and acids tested, 1% (w/v) potassium metabisulfite and 3% (w/v) potassium sorbate completely inhibited the growth of *A. niger* T6D5 on tomatoes.

Keywords: *Aspergillus*, potassium metabisulfite, potassium sorbate, GRAS salts

1. Introduction

Plum tomatoes (*Solanum lycopersicum*) have been widely consumed for a long time ago. The fleshy tomato fruits are susceptible to fungal infections, leading to postharvest loss. In previous research, tomatoes were found to be contaminated with a wide variety of fungi including *Aspergillus niger*, *Alternaria* sp., *Botrytis cinerea*, *Helminthosporium solani*, *Mucor piriformis*, *Penicillium digitatum*, *Phytophthora infestans* and *Rhizopus stolonifer* [1-4]. Moreover, the yeasts *Candida* spp., *Rhodotorula* spp. and *Cryptococcus* spp. were also found to have infected tomatoes [5,6].

In recent times, a number of strategies have been used to prevent postharvest decay in tomato fruits. One is the control of postharvest diseases in fruit by treatment with chemical fungicides such as fenhexamid, pyraclostrobin and boscalid [7]. However, the continuous use of such fungicides leads to significant problems. One drawback is their toxicity to humans and other organisms. Another is that their use may leave behind chemical residues in the environment. Yet another is the emergence of fungicide-resistant biotypes. These factors amongst others have led to pressure from consumers to lower the use of fungicides and find alternatives that are less toxic to mammals and have low impact on the environment [8,9]. These alternative substances should be generally recognized as safe (GRAS) compounds such as synthetic inorganic or organic salts including acetates, benzoates, carbonates, sorbic acid and sorbates, and potassium metabisulfite [10]. In addition, acid salts have been widely tested against spoilage fungi in cherry tomato fruit [11].

The GRAS salts can be used as aqueous solutions for dipping or spraying or can be used as ingredients in edible coatings of fresh fruits. Over the last decade, the antifungal activities of acetate, carbonate, benzoate, propionate, sorbate and metabisulfite salts against some fungal strains including *Alternaria alternata*, *Botrytis cinerea*, *Fusarium sambucinum*, *Lasiodiplodia theobromae* and *Pythium sulcatum* have been reported [11-13]. The effect of GRAS additives on postharvest treatments of cherry tomatoes and citrus fruits has also been investigated [11, 14, 15]. Thus, the aims of the present study were to evaluate fungal contamination in fresh plum tomatoes in order to isolate and identify the fungi present, and to study the efficacy of selected acids and salts against some fungal types in artificially inoculated plum tomatoes.

2. Materials and methods

2.1 Analysis of total yeasts and molds in tomato

Ten samples of fresh plum tomatoes (*Solanum lycopersicum*) were purchased at local markets in Bangkok, Thailand. The pH and water activity were performed using a pH meter (Testo 205, Testo AG, Germany) and an AquaLab Series 3TE water activity meter (Decagon Devices, Inc., USA), respectively. Then, the fungal contamination of all samples was analyzed by the dilution plating method onto acidified Potato Dextrose Agar (PDA) and Dichloran Rose Bengal Chloramphenicol Agar (DRBC) [16].

2.2 Isolation and identification of fungal isolates

After determining the total counts of yeast and molds, the morphological characteristics of the samples were observed, and pure cultures were prepared by streak inoculation onto Czapek Yeast Extract Agar (CYA) and Malt Extract Agar (MEA). After incubation at 25°C for 5 days, the purity of each fungal isolate was microscopically checked. Once the pure cultures were prepared, the identification process was carried out. Briefly, all mold isolates were observed using a stereomicroscope and the direct microscopic method as described by Samson et al. [17]. Then, molecular identification of the molds selected from the pure cultures was carried out. In addition, yeast identification was performed according to the method described by Barnett et al. [18]. The identification included study of cell morphology, film formation at broth surface, asexual reproduction in 2% glucose yeast extract peptone agar, the formation of pseudomycelia, true mycelia and ballistospore formation, sexual reproduction on Gorodkowa agar and McClary's acetate agar, carbohydrate fermentation, growth on 50% glucose yeast extract agar, growth on MEA at 37°C and on MEA with 1% glacial acetic acid, and other identifications using the API 20 C AUX system (Biomerieux, France).

2.3 Strain identification by ITS1-ITS4 sequencing analysis

The genomic DNA of one selected mold isolate (T6D5) was extracted by using the FavorPrep™ Fungi/Yeast genomic DNA extraction mini kit (Flavorgen, Taiwan). The genomic DNA was used as template DNA for polymerase chain reaction (PCR) amplification of the ITS fragment using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [19]. The PCR reaction mixture with a total volume of 50 µL contained 1x KAPA *Taq* ready mix (Sigma, USA), 50 ng of genomic DNA, 0.25 µM ITS1 and 0.25 µM ITS4. The PCR amplification was performed in a thermal cycler with the following cycling conditions: an initial denaturation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, primer extension at 72°C for 90 sec, and final extension at 72°C for 10 min. The amplified product was purified by using a PCR cleanup kit (Geneaid, Taiwan). Then, the sequencing of the ITS1-ITS4 fragment of purified amplified PCR product was performed by U2Bio company (Thailand). The obtained nucleotide sequence was compared with ITS sequence data from strains available in the Genbank databases by using the BLAST (Basic local alignment search tool) program [20]. The sequence was aligned using the CLUSTAL program [21]. Phylogenetic and molecular evolutionary relationship analyses of the ITS1-ITS4 fragments among available fungal strains was conducted by using MEGA11 (Molecular Evolutionary Genetics Analysis) software [22] with bootstrap value calculated from 1,000 replicates.

2.4 Inhibitory effect of organic acids and their salts against some fungi

The inhibitory effects of organic acids and their salts against some selected fungi were investigated by determining the minimum inhibitory concentrations (MIC) of three salts (potassium acetate, potassium metabisulfite, and potassium sorbate) and two acids (citric and tartaric) using the agar dilution method as described by Collins et al. [23]. A total of six fungal strains were tested. Three strains, *Rhizopus stolonifer* TISTR 3144, *Candida lipolytica* TISTR 5655, and *Rhodotorula glutinis* TISTR 515 were obtained from the Microbiological Resources Centre for Southeast Asian Region (Bangkok MIRCEN, Thailand), and the other three strains

Aspergillus niger T6D5, *Candida guilliermondii* T7D4, *Rhodotorula mucilaginosa* T7D5 were as isolated from plum tomatoes by us (See results of Section 2.2) [17,18]. Briefly, a sterile stock solution of each acid and salt solution (50-400 mg/ml) was mixed with molten PDA for molds or with Yeast Malt Agar (YMA) for yeasts to provide final concentrations ranging from 0.05-8.0% (w/v). Then, for the molds, a mycelium plug (5 mm diameter) was placed at the center of each PDA agar plate containing a salt or an acid, which was then incubated at 25°C for 7 days [15]. For the yeasts, streak inoculation was performed at the surface of each YMA agar plate containing a salt or an acid, which was then incubated at 25°C for 3 days. Then, the presence or absence of a colony was checked at each concentration of tested acid and salt, and the MIC value was reported as the lowest concentration of each acid or salt solution that was able to inhibit the growth of tested fungi. Three replications of all tests were performed.

2.5 Application of acids and salts on controlling of fungi in tomato

2.5.1 Tomato preparation

Plum tomato fruits were washed with tap water and surfaced sterilized with 0.5% sodium hypochlorite for 5 min, rinsed with sterile water for 30 sec, and allowed to air dry [13].

2.5.2 Tomato treatments

Tomato samples were treated with selected acids and salts as described by Kolaei et al. [13]. Three wounds (1 cm deep) were created on each sterile tomato surface using a 5 mm diameter sterile cork borer. Then, a mycelium plug of the tested fungal isolate (T6D5) was placed in each wound. Potassium acetate, potassium metabisulfite, potassium sorbate, citric acid and tartaric acid (all at 1% and 3% w/v) (filter-sterilized through 0.45 μm cellulose acetate membrane, Filtrex, USA) were sprayed onto the surface of each tomato fruit. Tomatoes sprayed with sterile distilled water were used as a negative control. Each tomato sample was packed in a sterile polyethylene plastic bag, placed on a sterile tray, and incubated at 25°C for 7 days. After storage, mold growth was observed visually. The diameter of the fungal colony was measured. The experiment was performed in three replications.

3. Results and discussion

3.1 Fungal contamination

The tomato samples were highly contaminated with yeasts and molds (2.0×10^5 - 1.4×10^8 CFU/g on acidified PDA). These samples had an average pH of 4.15 and a_w of 0.98. The most common mold and yeast isolates were *Penicillium* spp. (35.3%) and *Rhizopus* spp. (23.5%). Other fungi present included *Aspergillus* spp. (5.9%), *Candida gulliemondii* (5.9%), *Cryptococcus laurentil* (5.9%), *Geotrichum* spp. (11.7%), and *Rhodotorula mucilaginosa* (11.8%). *Penicillium* was the most common mold isolated from the rotten tomatoes. This result was in agreement with a previous report that revealed that *Penicillium digitatum* was abundantly present (22.22%) in tomatoes sold in the Onuiyi market in Enugu, Nigeria [1]. *Penicillium* can grow in the pH range of 3-8 and water activity of less than 0.85 [24]. Similarly, Mbajiuka et al. found *Rhodotorula* and *Saccharomyces* contamination in tomatoes from the Umuahia market in Abia, Nigeria [25].

One interesting isolate found on the tomato samples was isolate T6D5, which was selected for further study. The colonies of the T6D5 mold isolate grown on PDA at 30°C attained an average diameter of 7.8 cm within 7 days (Figure 1A). Each colony consisted of a dense layer of dark brown conidiophores with globose conidial heads (Figure 1B). The conidia were ornamented with irregular warts, spines and ridges (Figure 1C). The genomic DNA of the T6D5 mold isolate was extracted and used for PCR amplification of the ITS1-ITS4 fragment. The PCR product of approximately 500 bp-size was amplified and sequenced (Figure 2A). The nucleotide sequence was deposited into GenBank under accession number OR618283. It showed the highest similarity (98-100 %) to the ITS1-ITS4 of *Aspergillus* sp., with 100% similarity to that of *Aspergillus niger*. By phylogenetic tree analysis using the Maximum likelihood (ML) method, the ITS1-ITS4 of the T6D5 mold isolate was clustered into the same group as *Aspergillus niger* ATCC 16888 (Figure 2B). Therefore, the T6D5 mold isolate was classified as *Aspergillus niger* T6D5. *Aspergillus* belongs to the Order Eurotiales, which contains a large number of species. This mold can be found in soil and vegetation [26] and is involved in the postharvest decay of fruits.

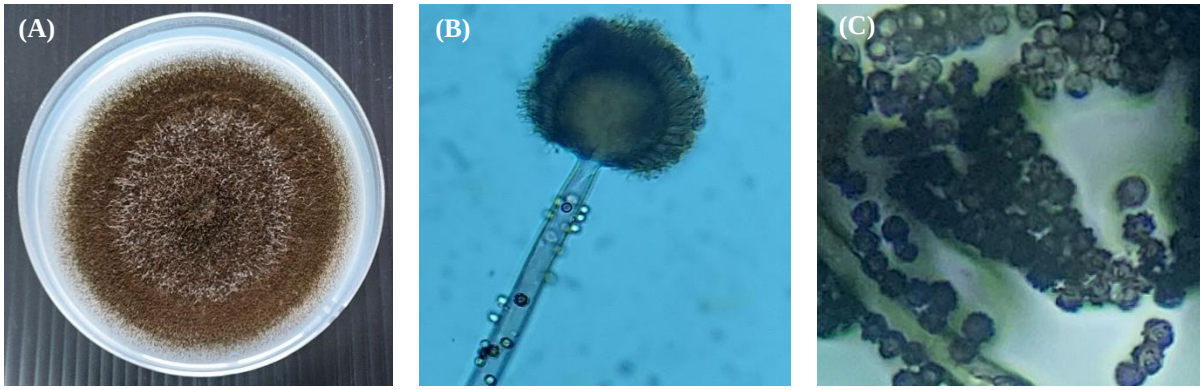

Figure 1 Morphological characteristics of the T6D5 mold isolate: (A) Colony of the T6D5 isolate on PDA at 30°C, 7 days; (B) Microscopic observation (400X), conidial head; (C) Microscopic observation, conidiospores with irregular warts, spines and ridges (400X, enlarged image).

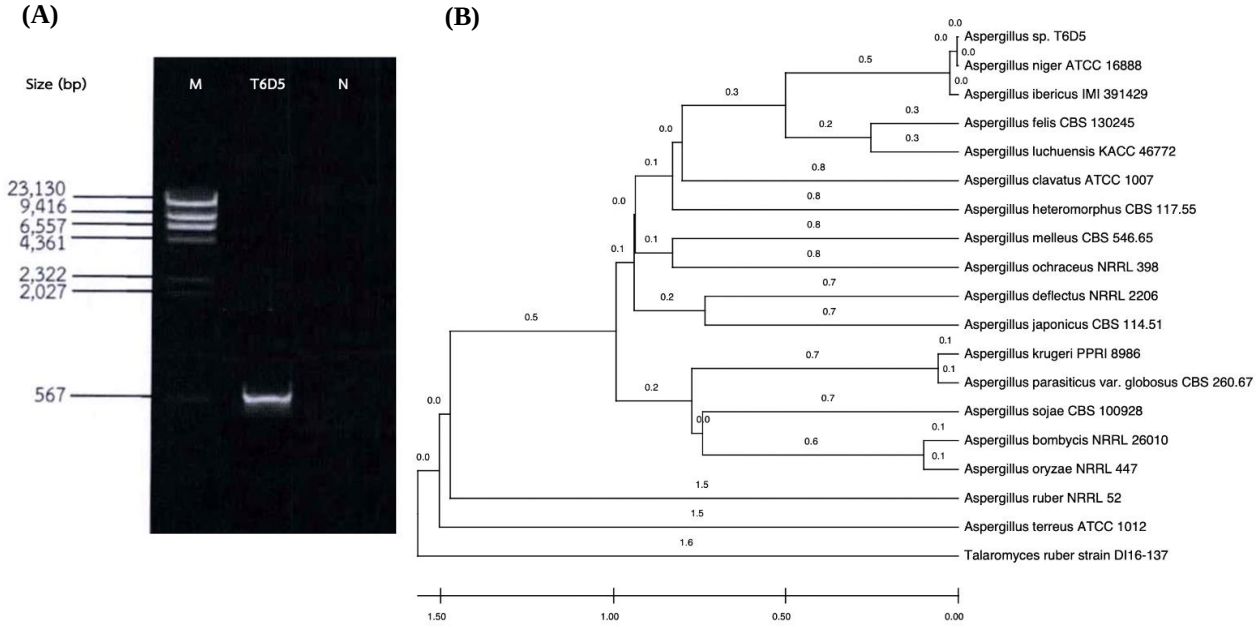

Figure 2 PCR amplification and sequence analysis of the ITS1-ITS4 fragment of the T6D5 mold isolate: (A) Agarose gel electrophoresis of PCR amplified product of ITS1-ITS4 fragment using universal primers ITS1 and ITS4, Lane 1 DNA marker λ digested by restriction enzyme *Hin*dIII, Lane 2 the amplified PCR product, and Lane 3 the negative control; (B) Phylogenetic tree analysis by comparison of ITS1-ITS4 sequences using Maximum likelihood method with 1,000 Bootstraps.

3.2 Antifungal activity of organic acids and salts

In this study, potassium metabisulfite and potassium sorbate better inhibited the growth of fungi than did potassium acetate, citric acid, and tartaric acid. Among all the fungal strains, potassium sorbate possessed the strongest antifungal activity against *A. niger* T6D5 isolated from tomato with 0.01% (w/v) MIC, but potassium metabisulfite strongly inhibited the growth of yeast strains tested with 0.25-0.5% (w/v) MIC. Potassium acetate effectively inhibited the growth of *R. stolonifer* TISTR 3144 with 2% (w/v) MIC. Tartaric acid displayed the strongest antifungal activity against *R. mucilaginosa* T7D5 with 2% (w/v) MIC, compared to other fungal strains (Table 1).

Table 1 Antifungal activity of acid and acid salts against some fungal strains.

Fungal strains	Minimum Inhibitory Concentration				
	Acid salts			Acids	
	Potassium acetate	Potassium metabisulfite	Potassium sorbate	Citric acid	Tartaric acid
Molds					
Rhizopus stolonifer TISTR 3144	2	1	0.05	>8	8
Aspergillus niger T6D5	>8	1	0.01	>8	>8
Yeasts					
Candida guilliermondii T7D4	>8	0.25	>8	>8	>8
Candida lipolytica TISTR 5655	>8	0.5	8	8	4
Rhodotorula glutinis TISTR 515	>8	0.5	1	4	4
Rhodotorula mucilaginosa T7D5	>8	0.25	1	8	2

In the current study, potassium metabisulfite and potassium sorbate exhibited strong antifungal activity. The results were in agreement with those of other researchers [12, 13, 27-29]. Moreover, others reported that potassium metabisulfite inhibited the growth of *Alternaria solani*, *Botrytis cinerea*, *Fusarium sambucinum*, *Pythium sulcatum* and *Rhizopus stolonifer* at 10 mM MIC [13], while 2% (w/v) potassium sorbate in coating composite was reported to have reduced disease in citrus fruits by 50% [12]. Sulfites are sulfur dioxide releasing agents. Potassium metabisulfite has been extensively used as a food preservative. The salts of metabisulfite, bisulfite and sulfite, at low pH level, dissociate into sulfurous acid, which inhibits microbial enzymes by breaking disulfide bonds [30]. Natskoulis et al. [31] reported that *Penicillium* was more susceptible to metabisulfites than was *Aspergillus*. Sulfite compounds inhibit microbial growth by disruption of cytoplasmic membranes, inactivation of DNA replication, inhibition of protein synthesis, and inactivation of membrane-bound enzymes [32]. Sorbic acid is commonly used in the form of its salt and is used as an antifungal agent in foods such as bakery products, cheese, salad dressing, and beverages [33]. Sorbates are the most common preservatives that have been used in various kinds of food. The most sensitive inhibition usually occurs in the final stage of spore germination which releases the spore wall into the vegetative cells. Moreover, the antimicrobial effects of sorbates occur through the inhibition of some enzymes and partial inhibition of the citric acid cycle, as well as through the disruption of cell walls, proteins, and RNA and DNA synthesis [34]. However, potassium acetate was not as effective as the other tested acids and salts. Kaiser et al. [35] and Pundir and Jain [36] reported similar results. This may have been due to the pKa of acetic acid (4.76). Potassium acetate inhibits fungal growth effectively only at pH of equal to or less than 4.5 [37].

3.3 Antifungal effect of acids and salts on plum tomatoes

3.3.1 Control of Aspergillus niger T6D5 on plum tomatoes

Potassium metabisulfite and potassium sorbate showed highly effective control of *A. niger* T6D5 on fresh tomatoes compared to potassium acetate, and citric and tartaric acids. Of all, potassium metabisulfite displayed the strongest antifungal activity on tomatoes against *A. niger* T6D5 with 100% inhibition at its lowest concentration (1% (w/v)) (Figure 3A), while potassium sorbate at 1% (w/v) could not completely inhibited the growth of this mold on tomatoes after storage at 25°C for 7 days (Figure 3B). However, potassium acetate, citric acid, and tartaric acid at 1-3% (w/v) could not inhibit the growth of the mold on tomatoes (Table 2).

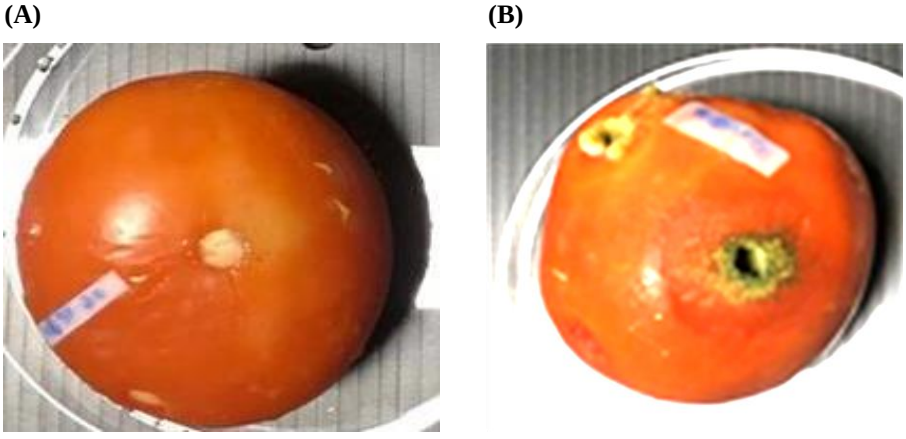

Figure 3 Effects of potassium metabisulfite (A) and potassium sorbate (B) at 1% on the control of *Aspergillus niger* T6D5 on tomato fruits at 25°C for 7 days.

Table 2 Antifungal effect of salts and acids on the control of *Aspergillus niger* T6D5 on tomato fruit after incubation at 25°C for 7 days.

Types of salts and acids	Degree of mold growth	
	1% (w/v) concentration	3% (w/v) concentration
Potassium acetate	+ + + +	+ + + +
Potassium metabisulfite	-	-
Potassium sorbate	+	-
Citric acid	+ + + +	+ + + +
Tartaric acid	+ + + +	+ + + +

-, no growth of mold surrounding each wound; +, 0.55-1 cm diameter of mold growth surrounding each wound; ++, 1-3 cm diameter of mold growth surrounding each wound; +++, 3-5 cm diameter of mold growth surrounding each wound; + + + + mold growth covered overall fruit.

In the current study, 1% (w/v) potassium metabisulfite and 3% (w/v) potassium sorbate strongly inhibited mold growth on tomatoes. Metabisulfite has been reported to liberate sulfur dioxide, which interferes with the cellular components of the mold. Its mode of action involves inhibition of cellular metabolism, protein and membrane synthesis and DNA replication [27, 32]. Moreover, the toxic action of anions and cations and pH alterations may produce inhibitory mechanisms. Thus, growth inhibition of *A. niger* T6D5 on plum tomatoes by these salts may be attributed to some of these mechanisms.

4. Conclusion

In this study, we confirmed the strong antifungal activity of potassium metabisulfite and potassium sorbate at 0.01-1% (w/v) MIC. Spraying with potassium metabisulfite at 1% and potassium sorbate at 3% effectively prevented postharvest decay by *A. niger* T6D5 on plum tomatoes. To enhance safety and reduce postharvest loss, future research should focus on spraying treatment of these salt solutions in combination with application of other natural antimicrobial agents such as plant extracts or essential oils.

5. Acknowledgements

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