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Full Paper

Control of *Fusarium* sp. on pineapple by megasonic cleaning with electrolysed oxidising water

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Abstract: The effects of megasonication (MS) with electrolysed oxidising (EO) water (MS/EO treatment) on *Fusarium* sp. which causes postharvest decay of pineapple cv. Phu Lae were investigated. Spore suspensions containing 10^5 conidia mL^{-1} and 1 cm mycelium discs of *Fusarium* sp. were subjected to MS (1 MHz) in EO water with available free chlorine at 100 ppm for 10 min., and compared with non-treated control samples. The MS/EO treatment completely inhibited spore germination and mycelial growth for 3 days and reduced fruit decay. Development of *Fusarium* sp. on de-crowned pineapple fruit was also investigated by scanning electron microscopy. The fungal growth was restricted on de-crowned pineapple fruit for 72 hr, following MS/EO treatment. The MS/EO treatment also enhanced the activity of two enzymes: phenylalanine ammonia lyase (PAL) and peroxidase (POD), which play important roles in plant defense responses.

Keywords: megasonication, electrolysed oxidising water, pineapple, *Fusarium* sp., pineapple, fruit decay, crown rot

INTRODUCTION

Pineapple is a commercially important crop in Thailand. A high incidence of postharvest diseases in pineapple is primarily caused by the fungi *Ceratocystis paradoxa* and *Fusarium* sp. The fungi preferentially penetrate wounds caused by de-crowning during postharvest handling, prior to export [1]. Several fungicides are presently used as postharvest treatments to control fruit-decay-causing fungi. Fungal decay of pineapple fruit may be controlled by dipping in Triabendazole or Benomyl and methyl bromide fumigation. However, these chemicals may also leave toxic residues and cause environmental pollution. Advanced oxidation techniques have been used as alternatives

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to conventional fungicides for controlling plant diseases [2-4]. In a similar manner, our present study aims at testing a combination of megasonication (MS) by high-frequency ultrasound and electrolysed oxidising (EO) water to control postharvest fungal decay in de-crowned pineapple fruit.

Ultrasound refers to sound wave with frequency above those audible to humans [5]. It causes chemical and physical changes to biological structures (in a liquid medium) owing to the rapid formation and destruction of cavitation bubbles [6]. The potential use of ultrasound in food industry has been recognised since the 1970s [7]. Chemat et al. [8] stated that it can be used in food processing, preservation and extraction at 100-1000 kHz. In addition, using MS at 1 MHz can also reduce chlorpyrifos residue on bird's chili [9]. Ultrasound treatment significantly reduces spoilage and pathogenic microorganisms in fruits and vegetables [6]. Consequently, it has been used in postharvest treatments to reduce decay and to maintain the quality of fruits and vegetables.

EO water, an alternative to chlorinated water, was developed in Japan [10]. It has been studied for use in aquaculture [11], agricultural and food industrial processes [12, 13], as well as in postharvest disease control [14-16]. It is produced by electrolysing a dilute solution of sodium chloride, using an ion exchange membrane to separate the anode from the cathode. Water collected at the anode has unique oxidising properties due to its hypochlorous acid (HOCl) content and low pH [10].

Plants' defence-related enzymes such as phenylalanine ammonia lyase (PAL) and peroxidase (POD) are also involved in defence against pathogenic microorganisms. PAL, a key enzyme in the phenylpropanoid pathway, induces the synthesis of various fungitoxic phytoalexins [17]. POD is involved in the cross-linking of cell-wall components and the generation of antifungal metabolites [18]. These enzymes induce active defence mechanisms against pathogens [17]. In this study, which is a continuation of our previous experiments [19], the effects of MS and EO water on decay and elicitation of defence enzymes is also investigated.

MATERIALS AND METHODS

Fungal Culture

Fusarium sp. was obtained from the culture stock of the Department of Biology, Faculty of Science, Chiang Mai University. The fungi were cultured on potato dextrose agar (PDA) for 7 days at 25°C. A spore suspension was prepared by adding 10 mL of sterile distilled water to the *Fusarium* sp. cultured on a petri dish and the spores were transferred to 100 mL of sterile distilled water in a 250-mL conical flask. The suspension was shaken for 10 min. on an orbital shaker at 25° and filtered through two layers of sterile muslin. The spore suspension was diluted with sterile distilled water to a concentration of 10^5 spores mL^{-1} as determined by the haemacytometer.

Generation of EO Water and Megasonic Treatment

The conditions used in our previous work [19] were employed. To mention briefly, EO water was generated by electrolysis in a chamber with positively and negatively charged titanium electrodes coated with TiO_2 and separated by a polypropylene membrane. Water (12 litres) containing 5% NaCl was introduced into the system. The electrodes were then subjected to direct current of 8 A and 8 V from a DC power source. The pH was recorded with a pH/ion meter and the oxidation-reduction potential (ORP) was measured by a pH/ORP meter. The amount of free-chlorine concentration was determined by the N,N-diethyl-*p*-phenylenediamine test [20]. The EO water, with an initial free-chlorine concentration of 650 ppm, was diluted with distilled water to 100 ppm and used for the microbiological study within 5 hr.

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For fungal study, spore suspensions or mycelium discs were sonicated in a megasonic polyethylene cylinder reactor, 10 cm in diameter, with an input power of 3W and a frequency of 1 MHz (Honda Electronics Co., Japan).

For fruit treatment, the fruits (up to 10 kg) were subjected to megasonic waves of 3W at a constant frequency of 1 MHz. The experiments were carried out in an ultrasonic 50-L water bath, dimensions: 44.5 × 51.5 × 35 cm (Honda Electronics Co., Japan).

Spore Survival and Mycelial Growth

One mL of a $1x10^5$ spore suspension or 1 cm of mycelium disc was placed in the megasonic chamber containing 9 mL of EO water (freshly prepared) with a free-chlorine concentration of 100 ppm and subjected to continuous MS at 1 MHz for 10 min. at room temperature. Subsequently, 0.1 mL of the treated spore suspension in EO water was added to 0.9 mL of 0.1N sodium thiosulfate. After well mixing, the spread-plate technique was applied; 0.1 mL of the solution was spread on a PDA plate. A mycelial disc was placed onto a PDA plate, which was then incubated at 27°. The fungal survival was expressed as the number of colony-forming units (cfu mL^{-1}) after incubation for 48 hr and the mycelial growth was recorded daily. Each treatment consisted of 9 replicates and the experiment was repeated twice independently. The control treatment consisted of a 1-mL spore suspension or 1 cm of mycelium disc in 9 mL of distilled water in place of EO water.

Disease Incidence and Quality Changes

The prepared spore suspension (0.1 mL) was artificially inoculated on the centre of de-crowned pineapple fruit. All treated fruits—two groups of 36 fruits each—were incubated at room temperature for 3 hr. The first group was immersed in the MS chamber with the dimension of 44.5 × 51.5× 35 cm, containing EO water with a free-chlorine concentration of 100 ppm, and subjected to continuous MS at 24W and a constant frequency of 1 MHz. The second group of pineapple fruits washed with distilled water were used as control. The treated fruits were then placed in a basket and air-dried. After that the fruits were covered with plastic bags and maintained at 13° for 20 days. Samples were taken at 5-day intervals during storage for decay monitoring. To determine changes in fruit quality, weight loss and pH were measured. Total soluble solids was determined with a digital refractometer (Atago, Model PAL-a, Japan) using 2-3 drops of juice obtained by squeezing the fruits [21]. Titratable acidity was assessed as outlined by AOAC [22]. Ascorbic acid was determined by dichloroindophenol method [23]. The experiment was repeated twice.

Disease Development

De-crowned pineapple fruits were inoculated with a $1x10^5$ spore suspension of *Fusarium* sp. and subjected to MS with EO water following the procedure mentioned above. De-crowned fruits treated with distilled water were used as control. To observe the varying stages of fungal penetration, fruit samples were taken at 12, 24, 48 and 72 hr after treatment. The samples were preserved in formaldehyde-acetic acid-alcohol solution. Pieces of 3-10 mm were cut out of the samples. After three rinses of 5 minutes each in sterile distilled water, the samples were mounted with carbon adhesion tape on a specimen holder and examined under a scanning electron microscope (KEYENCE VE-9800, Japan) at an acceleration voltage of 5 kV.

Plant's Defense-Related Enzymes

De-crowned pineapple fruits were inoculated with *Fusarium* sp. and subjected to MS/EO-water treatment as described above. On every five days of storage, healthy, non-infected areas of

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the sampled fruit were separated, cut into small pieces, frozen in liquid nitrogen, freeze-dried and stored at -21° for enzyme assays. The sampling was done in triplicate. De-crowned fruits treated with distilled water were used as control.

For PAL assay, freeze-dried samples of de-crowned pineapple fruit (1.0 g) were homogenised with 10 mL of 150mM Tris buffer (pH 8.5). The homogenate was centrifuged at 12000 g for 30 min. at 4°. The supernatant was analysed for enzyme activity. The PAL assay system routinely consisted of the supernatant (1 mL), 50 mM L-phenylalanine (1 mL) and buffer (1 mL). The change in absorbance at 290 nm was monitored after 1 hr of reaction at 40°. Under these conditions, a change in absorbance of 0.01 was found to be equivalent to the production of 3.09 nmoles of cinnamic acid [17]. One unit of enzyme activity was defined as the production of 1 nmol cinnamic acid per hr.

For POD assay, the freeze-dried samples of de-crowned pineapple fruit (0.5 g) were homogenised with 20 mL of 0.10M sodium phosphate buffer (pH 7.0) containing 3mM ethylenediaminetetraacetic acid and 0.1 g of polyvinyl pyrrolidone. The homogenate was centrifuged at 12000 g for 20 min. at 4°. The supernatant was analysed for POD activity, which was determined spectrophotometrically at 470 nm [24]. The reaction medium contained 2.855 mL sodium phosphate buffer (0.10M, pH 7.0), 45 µL guaiacol (1%), 40 µL H_2O_2 (0.3%) and 60 µL supernatant. The absorbance was recorded at 470 nm and one unit of enzyme activity was defined as the amount causing an absorbance change of 0.01 per min.

The specific activity of the enzymes was expressed as units/ mg protein. Soluble protein content was determined using bovine serum albumin as standard [25].

RESULTS AND DISCUSSION

Spore Survival and Mycelial Growth

Spores treated with MS/EO water were completely inactivated (spore survival population = 0 log cfu mL^{-1}). In the control group spore survival was 7.88 log cfu mL^{-1}. The concentration of free chlorine in EO water was sufficient to reduce fungal growth. Of the chlorine compounds, hypochlorous acid is the most effective for disinfestation. It damages the microbial cell by oxidising nucleic acids and proteins, causing lethal damage [23]. The low pH in EO water sensitises the outer membrane of the cell, thereby allowing hypochlorous acid to enter the cell more efficiently. Similar results have been achieved with *Penicillium digitatum* after 1 min. exposure [27]. Other researchers also reported that it takes about 30 sec. or less to inhibit thin-walled fungi and 2 min. or more to inhibit thicker-walled species [3]. The reduction of spore survival by MS, on the other hand, is mainly due to free-radical attack resulting in physically disrupted cell membranes [28]. The effects of the combined treatment on mycelial growth are shown in Figure 1. Mycelial growth was delayed for 3 days, but after 5 days of storage the mycelium started to regrow. The mycelium structure was composed of a compact mass of hyphae and the ultrasonic wave might not have penetrated the inner mycelial matrix, leaving some parts protected from the high pressure and temperature effects of MS and from EO-water disinfection.

Disease Incidence and Quality Changes

The MS/EO-water treatment significantly inhibited decay incidence during the first 5 days ($p < 0.05$), and by the 10^{th} day the average disease incidence was 8.33%, compared with 66.67% for control (Figure 2). The combined treatment seems to effectively disinfect the fruit from pathogenic fungi through mechanical disruption of the microbial cells and disinfection by EO water. This result

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is similar to those by other researchers. Ultrasound combined with EO water resulted in a greater reduction of the bacterial contamination of broccoli [29]. A combination of ultrasound and sanitisation enhanced removal of *Salmonella* and *Escherichia coli* O157:H7 on apple and lettuce [30]. The compression pressure generated by ultrasound might have facilitated the penetration of chemical oxidants through cellular membranes and the cavitation might have assisted in the disaggregation of the microorganisms, resulting in increased efficiency of the sanitisation treatment [31]. In our study MS with EO water seems to be applicable to pineapple handling systems due to its marked effect against fungal decay without any effect on fruit quality, as revealed by the results on weight loss, total soluble solids, titratable acidity, pH and ascorbic acid content (data not shown).

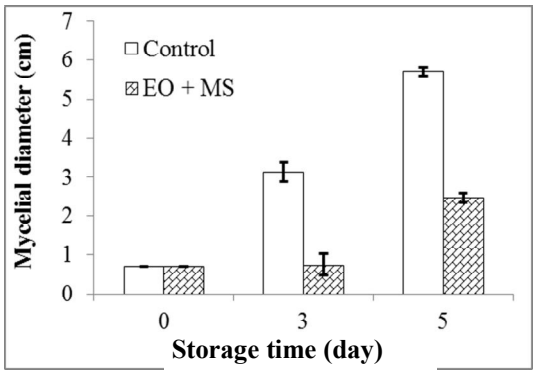

Figure 1. Mycelial disc growth diameter of *Fusarium* sp. on PDA plates, after being treated with MS/EO water, then incubated at 27 °C for 5 days

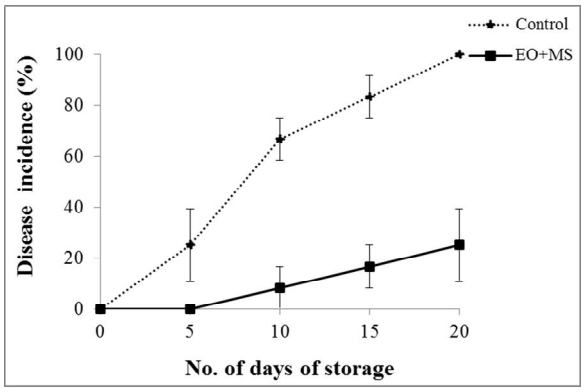

Figure 2. Effect of MS with EO water on disease incidence (% of infected fruits) of de-crowned pineapple during low-temperature storage for 20 days

Disease Development

The scanning electron micrograph of de-crowned pineapple in the control group showed germination of *Fusarium* sp. after 72-hr incubation. The germination of fungal spores was first observed in the control after 24 hr of storage whilst the MS/EO-water-treated fruits showed no sign of disease incidence (results not shown). After 72-hr incubation the treated samples still remained clear of fungal growth (Figure 3B) whilst the control group supported a high mass of fungal mycelia (Figure 3A). The combined treatment seems to effectively inhibit spore germination on de-crowned pineapple.

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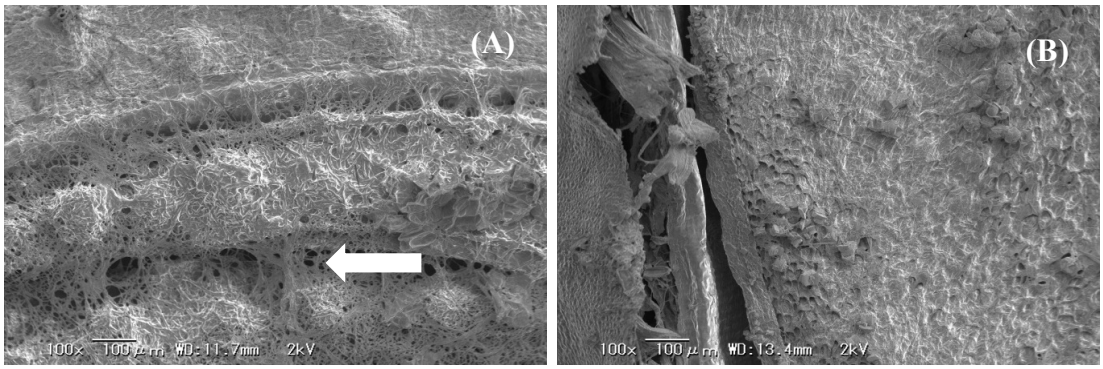

Figure 3. Scanning electron micrographs of de-crowned pineapple samples (4000 × magnification, 8-nm resolution) after 72-hr storage at 25° and 75-80% relative humidity: (A) development of *Fusarium* sp. in control sample (arrow indicates *Fusarium* sp. mycelia); (B) sample treated with MS/EO water

Plant Defence-Related Enzymes

The PAL level was found to increase rapidly and reach a peak after 10 days and then it gradually declined (Figure 4A). The PAL activity in the treated pineapple sample increased about 1.8-fold above that recorded for the control. The MS/EO-water treatment also increased the POD activity during storage as shown in Figure 4B. The mode of action of EO water and MS in inducing plant pathogen resistance is not yet fully understood. However, EO water has been reported to stress plants [32-34]. In lettuce, higher PAL levels were induced by stress from wounding plus ethylene [35]. In the present study we found that PAL and POD activities were induced by the MS/EO-water treatment, which may also induce enzyme responses to other kinds of stress including wounding and inoculation with *Fusarium* sp. as indicated in the control graphs in Figure 4.

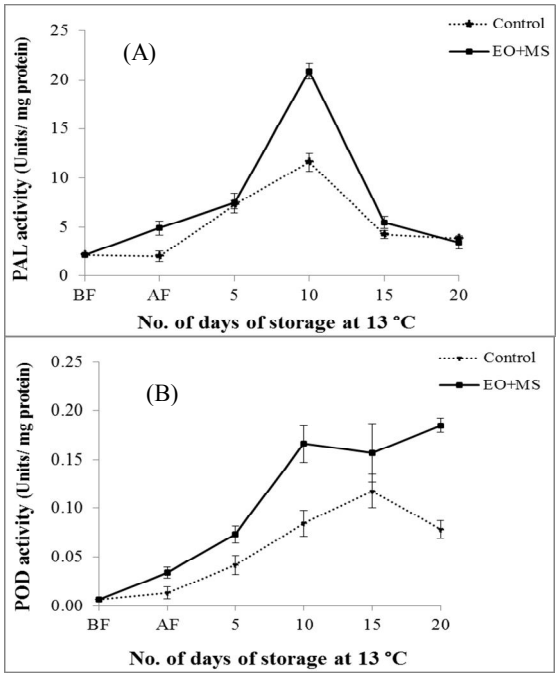

Figure 4. Effect of MS and EO water on activity (units/ mg protein) of PAL (A) and POD (B) in de-crowned pineapple fruit during storage at 13°C for 20 days **(**BF = before MS/EO-water treatment; AF = after MS/EO-water treatment)

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CONCLUSIONS

The use of MS combined with EO water is recommended as a postharvest treatment of pineapple. The treatment significantly inhibits spore survival of *Fusarium* sp., prolonging the shelf life of pineapple by up to 20 days without affecting the fruit's quality. The antifungal effect of the treatment may also be due to the triggering activity of the plant's defence-related enzymes.

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