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# Identification of predominant bacteria and controlling of microbial population in vase solution of cut orchid flowers

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ABSTRACT

Microbial and specially Bacterial growth in holding solutions of cut flowers can cause blockage in xylem and later will resulted to impair water uptake and faster wilting. In this experiment, after harvesting and transporting the cut orchid flowers to the Lab., they were put in the bottles containing distilled water which treated with 0, 1.3, 2.6, 3.9 and 5.2 mg/L aqueous ozone and during 24 hrs each 6 hours, bottles solutions were sampled and cultured in different media and the predominant bacterial type was determined. Result revealed: sequence of the strain of studied bacteria is 88, 87 and 86% sequence similarity to the sequence of the strains *Burkholderia cenocepacia* and *Burkholderia dolosa*. Moreover, aqueous ozone at 1.3 and 2.6 mg/L did not significantly decreased bacteria population compared to distilled water, while at 3.9 and 5.2 mg/L, the populations were significantly ($P \leq 0.01$) reduced.

**Keywords:** Bacteria, microbial population, aqueous ozone

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## 1 Introduction

One of the major causes for vase life reduction of many cut flowers is the presence of microorganisms in the holding solutions. These microbes are mostly bacteria, fungi or yeast blockage in xylem conduits during vase life of cut flowers may limit water uptake. These blockages might due to microbial growth, production of tyloses, sediment of materials in the lumen of xylem vessels and the formation of air emboli in the vascular system (Twumasi et al., 2005; van Doorn, 1997). These disrupt water uptake and affect the vase-life of cut flowers (Robinson et al., 2009). A high number of bacteria in the vase solution usually cause the vascular blockage (Kazemi and Ameri, 2012; Robinson et al., 2009). To inhibit microbial growth in the vase solutions of cut flowers, many compounds and chemicals have been used such as silver nitrate (Torre and Fjeld, 2001), silver thiosulphate (van Doorn, 1998), aluminum sulphate, hydroxyquinoline sulphate (Liao et al., 2001), hydroxyquinoline citrate (van Doom and Perik, 1990; van Doorn, 1998), sodium hypoclorite (Torre and Fjeld, 2001; van Doom and Perik, 1990) and aqueous ozone (Admane et al., 2018; Kumar and Ravishankar, 2019; Robinson et al., 2009). Nowadays, ozone is known as an antimicrobial agent and can be used for storing, washing and processing vegetables and fruits. Sarron et al. (2021) stated that the use of ozone, either as a gas form or as a solution in water, is possible to preserve fresh vegetables because of its ability to prevent spoilage caused by the growth of pathogenic microorganisms. It also preserves the quality characteristics

of these products. This molecule has antimicrobial activity due to its oxidative capacity against proteins, lipids, enzymes, nucleic acids, membranes and other cellular components. However, the performance that can be achieved during the use of ozone is highly dependent on the general conditions of the product and the environment.

The selected cut orchid hybrids particularly Dendrobium 'Darren Glory' (DDG) have short vase life and high occurrence of florets epinasty. One possible reason for these low postharvest qualities might be due to xylem blockage and stress in their water conductivity. The objectives of this experiment were to determine the predominant bacteria in vase solution and, to study the effects of aqueous ozone on microbial population in vase solution.

# 2 Materials and Methods

## 2.1 Materials and treatment

Twenty five stems of Dendrobium 'Darren Glory' (DDG) were purchased from a commercial farm in Bukit Changgang, Banting, Selangor, Malaysia. After reaching the lab, the stems were trimmed to 12 cm from the first open floret. Then each stem was olaced in a 4 L clean plastic beaker containing distilled water. Sixteen liters of distilled water was ozonized to produce 4 L of each concentration of 1.3, 2.6, 3.9 and 5.2 mg/L aqueous ozone with an ozone generator (Okamizu Food Detoxifier V.2.). The ozone concentration was determined using a low concentration ozone monitor (Model Q45H, USA). Two hundred mL of each aqueous ozone concentration was filled into each 330 mL sterile glass bottle. Then, one inflorescence was placed into each of the bottle, with five replications of inflorescence per . The inflorescences were placed in the postharvest Lab. under 25±2 °C, 78±2% humidity and 6.57 µmol/$m^2$/s light intensity, respectively. The vase water of 1.3, 2.6, 3.9 and 5.2 mg/L aqueous ozone treatments were renewed daily at 8 a.m.

## 2.2 Microbial culture

During 24 hours in vase water, 300 µL from vase water of each treatment (0, 1.3, 2.6, 3.9 and 5.2 mg/L) were pooled in a 2 mL autoclaved centrifuge tube, separately at 6 hours intervals. Then each tube was vortexed for 5 seconds, by a vortex mixer (Barnstead Thermolyne Maxi Mix II Type 37900 Model Mixer M37615, Singapore). Then 1 mL of these mixed waters were diluted 3 time (1:9) with autoclaved distilled water, separately. Three aliquots (150 µL) of each mixture were plated on nutrient agar (NA), potato dextros agar (PDA) and yeast extract agar (YEA) and spread using a sterile glass rod, with three replications per media. The inoculated plates were then sealed using parafilm and were placed in the growth chamber at 30 °C for 48 hours. Therefore CFU of bacteria was recorded. The graph of bacterial growth (log10 CFU/mL) for each treatment was plotted using Robinson et al. (2009) method with some modifications. This mean number of bacterial colony was determined for each treatment and using Log for each treatment in Microsoft Office Excel 2010 and the maximum number of bacteria (CFU/mL) was calculated. These microbial counts were repeated for day three and five.

## 2.3 Identification of the predominant bacteria

### 2.3.1 Sample isolation

For identification of the bacteria in storage water after treatments with aqueous ozone, the cut orchid stems were placed in individual glass bottles containing 250 mL of distilled water. Then after 48 hours, 200 µL of vase water (control) were removed from 5 separate bottles and pooled in an autoclaved 2 mL microcentrifuge tube. The mixture after 3 times dilution with sterilized distilled water (1:9) was plated on NA in Petri plates (150 µL). Plates were incubated for 48 hours at 30 °C and the predominating colony with white color and round shape, were re-streaked on NA to obtain a pure single colony. A pure culture of this bacterium was used for identification.

### 2.3.2 DNA extraction using QIAamp DNA mini Kit

The pure colony of bacteria was streaked onto the NA medium (Mortensen et al., 1994). About 109 CFU/mL of the bacterial cells was poured in a microcentrifuge tube, and was centrifuged for 10 minutes at 7500 rpm and then the supernatant was discarded. The pellet was re-suspended in 180 µl Buffer ATL and 20 µl of proteinase K was added, homogenized by vortexing and incubated at 56 °C in a shaking water bath for 30 minutes. The tube was vortexed for 15 second, and 200 µL buffer AL was added to the sample, and mixed thoroughly by vortexing. Then 200 µL of ethanol 96% was added and mixed again thoroughly by vortexing. The mixture was then placed in a 2 mL collection tube containing DNeasy membrane, centrifuged at 8000 rpm for 1 minute and flow–through and collection tube was discarded. The membrane was placed into a new 2 mL collection tube. 500 µl Buffer AW1 was added and centrifuged at 8000 rpm for 1 minute and Flow-through and collection tube was then discarded and the membrane was again placed in a new 2 mL collection tube, followed by an addition of 500µl Buffer AW2, centrifuged at 14000 rpm for 3 min to dry the DNeasy membrane, flow–through and collection tube was discarded. DNeasy membrane was then placed in a clean 2 mL tube and 200 µl buffer

AE was added and incubated at room temperature for 1 minute and then centrifuged again at 8000 rpm for 1 minute and the purified DNA is then kept until further used.

#### 2.3.3 Amplification of the intergenic region of the 16S-23S rDNA

The extracted DNA from the isolate was used as templates in the PCR amplifications using the XOR-F (5′-GCATGACGTCATCGTCCTG-3′) and XOR-R2 (5′-TCGGAGCTATATGCCGTG C-3′) primer pairs for amplifying the intergenic region 16S-23S rDNA of the Xoobacteria (Adachi and Oku, 2000). PCR reaction was carried out in a 25 µL PCR mixture tube. The PCR reaction contained 1 µL of DNA template, 0.4 µL of 10 mM dNTP, 0.6 µLof 25 mM MgCl2, 2 µL of 10X Taq polymerase buffer, 1µL each of the primer (10 pmols each) and 0.3 µL (0.5 units) Taq DNA polymerase (Fermentas, Inc). The sample was amplified through 30 cycles, each consisting of 30 second at 95 °C, 30 second at 63 °C, 1 minute at 72 °C, with initial denaturation of 2 minute at 95 °C and final extension of 7 minute at 72 °C. Five µL of each amplified PCR product was fractionated on 1.5% Agarose gel in 1X TBE buffer. Gel was stained with ethidium bromide and photographed under UV light (312 nm). The generated PCR products were then purified using Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany) and sent for sequencing.

#### 2.3.4 Sequencing of the intergenic region of the 16S-23S rDNA and the insertion sequence element, IS1113

The purified PCR product was sent for sequencing commercially (First BASE Laboratories Sdn Bhd). The sequence amplified from the isolate by the primer pairs XOR-F/XOR-R2 was blasted in the National Center for Biotechnological Information (NCBI) database using Basic Local Alignment Search Tool (Blast) (Altschul et al., 1990).

#### 2.3.5 Experimental design and data analysis

The experiment was conducted using a completely random design (CRD) with one orchid hybrid (DDG) and five concentrations of aqueous ozone (0, 1.3, 2.6, 3.9 and 5.2 mg/L). The analysis of data was done using the analysis of variance (ANOVA). Treatment means were compared by Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$. Data analysis was performed by using SAS, version 9.1, software.

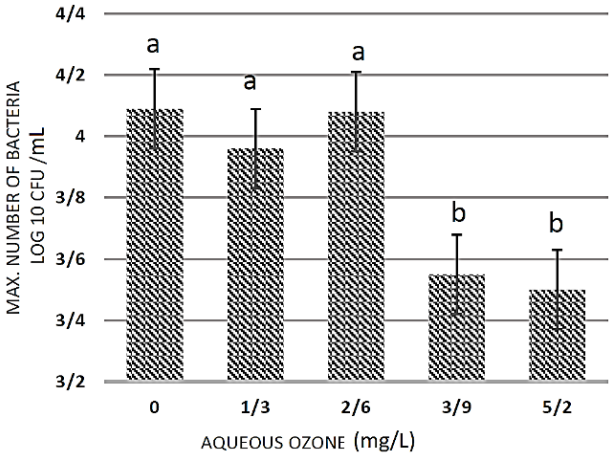


**Figure 1.** Effect of aqueous ozone (0, 1.3, 2.6, 3.9 and 5.2 mg/L) in vase water of Dendrodium 'Darren Glory' on maximum number of bacteria (log10 CFU/mL) during 24 hours. Different letters above bars indicate significant mean differences by Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$.

## 3 Results and Discussion

### 3.1 Number of colony for bacteria, fungi and yeast

Preliminary observation was carried out to determine the effect of various concentrations of aqueous ozone on the microbial growth in cut orchids vase water compared to distilled water after 24 hours. Aqueous ozone at 1.3 and 2.6 mg/L did not significantly decreased bacteria population compared to distilled water, while at 3.9 and 5.2 mg/L, the populations were significantly ($P \leq 0.01$) reduced (Fig. 1 and Fig. 2). It shows that efficacy of aqueous ozone to reduce the number of bacterial population depends on concentration. Thereby, it can be concluded that less than 3.9 mg/L aqueous ozone cannot control the number of bacteria. Ozone molecules react with many compounds of bacterial cell walls such as unsaturated fatty acid being exposed to bacteria and oxide them. High levels of oxidation can conduct to serious cell damage including membrane rupture and cell death. Additionally, the higher concentration of ozone leads in higher number of ruptures. Over this process, bacteria would be injured or died. These results were in agreement with results of Wang et al. (2017) and Robinson et al. (2009). Robinson et al. (2009) who reported that 5.5 ppm aqueous ozone could control the bacteria population in vase water of cut roses.

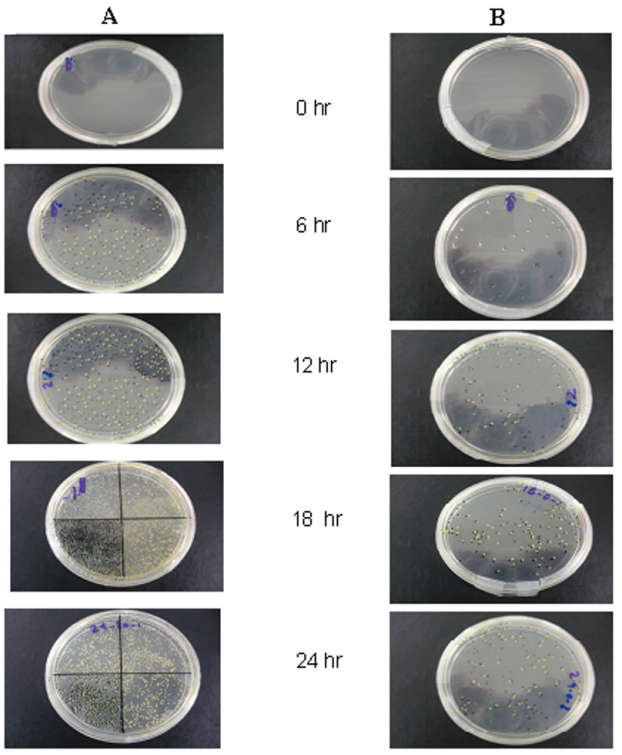


**Figure 2.** Effect of (A) distilled water and (B) 3.9 mg/L aqueous ozone on number of bacteria colony at 0, 6, 12, 18 and 24 hours in vase water of Dendrodium 'Darren Glory'.

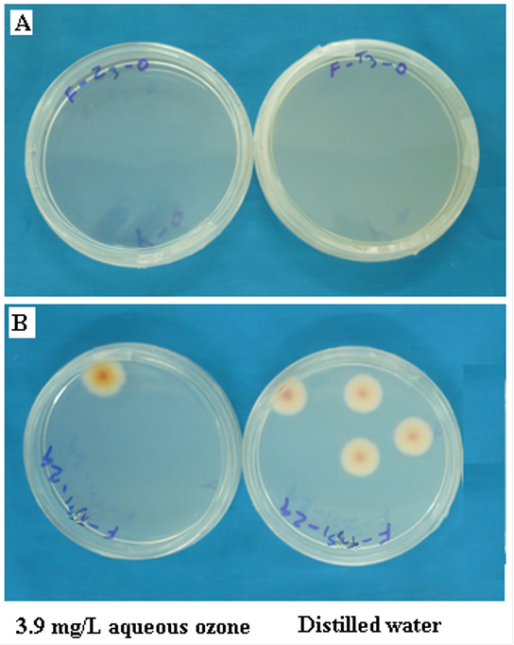


**Figure 3.** Effect of 3.9 mg/L aqueous ozone (left) or distilled water (right) in vase water for Dendrodium 'Darren Glory' cut inflorescence on number of fungi colony at 0 (A) and after 24 (B) hours of holding in the vase water.

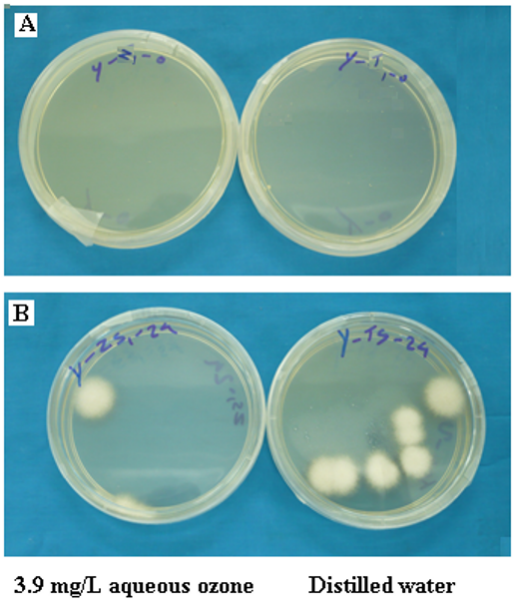


**Figure 4.** Effect of 3.9 mg/L aqueous ozone (left) or distilled water (right) in vase water for Dendrodium 'Darren Glory' cut inflorescence on number of yeast colony at 0 (A) and after 24 (B) hours of holding in the vase water.

Number of colonies for fungi and yeast were less than ten in control (distilled water), but with treatments of 3.9 and 5.2 mg/L, their colonies were remarkably reduced (both less than two) (Fig. 3 and Fig. 4). These results were consistent with the findings of Kim et al. (2003) on effect of aqueous ozone on disinfection of mold, and yeast and Najafi and Khodaparast (2009) on effect of gaseous and aqueous ozone in reduction of microbial populations in date fruits. However the result showed that daily use of 5.2 mg/L aqueous ozone as vase water did not show any symptoms of toxicity for the cut flowers and effectively can reduce the number of microbes, thus 5.2 mg/L aqueous ozone and renewed daily was chosen for the main experiment.

## 3.2 Detection of Burkholderia sp. in vase water of cut orchid hybrid (DDG)

The primer pair XOR-F/XOR-R2 produced the PCR products about 80 bp in size (Fig. 5) when the DNA templates used originated from the collected predominant bacteria in vase water. Sequence of the strain of studied bacteria showed 88, 87 and 86% sequence similarity to the sequence of the strains *Burkholderia cenocepacia* (AAKX01000076.1), *Burkholderia ambifaria* (ABLK01000026.1) and *Burkholderia dolosa* (AAKY01000044.1). The genous *Burkholderia* are very high genetic diverse and found in different environments in different environments (Coenye and Vandamme, 2003), including soil (Janssen, 2006) and hospital (Coenye and Vandamme, 2003) environments.

Many members of the genus can cause infections in humans and animals (Valvano et al., 2005). In spite of that, in recent years, a growing number of *Burkholderia* strains and species have also been reported as plant-associated bacteria. Indeed, *Burkholderia* spp. can be free-living in the rhizosphere as well as epiphytic and endophytic, including obligate endosymbionts and phytopathogen (Coenye and Vandamme, 2003; Janssen, 2006).

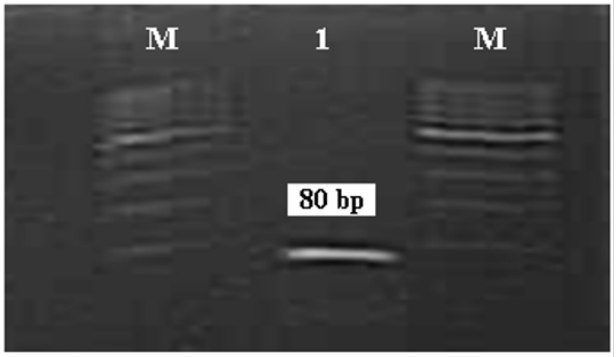


**Figure 5.** Agarose gel electrophoresis of the PCR products amplified by the primer pairs XOR-F/XOR (Lanes 1). Lane M: 100 bp DNA ladder (100-3000bp). Lane1: Xoo (80bp)

## 4 Conclusion

The predominant bacteria in the vase solution is related to the water and kind of cut flowers and in this experiment it was belong to *Burkholderia* genus. Optimum postharvest treatments for the cut orchid flowers depended on hybrids. Treatment of the cut orchid flowers with 5.2 mg/L aqueous ozone as the vase solution could be recommended as the optimum postharvest treatments to maintain quality and extend vase life of the DDG orchid hybrid. Finally, aqueous ozone can inhibit microbial growth in vase solution markedly.

## Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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