EFFECT OF LOW LEVEL OF OZONE ON GROWTH AND DEVELOPMENT OF *BOTRYTIS ACLADA* IN VITRO

Pengaruh Ozon Terhadap Pertumbuhan dan Perkembangan Botrytis aclada in Vitro

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ABSTRAK

Penyakit pasca panen yang disebabkan oleh mikroba menyebabkan kerugian yang sangat besar setiap tahunnya. Pada pertanian konvensional, fungisida sudah digunakan selama bertahun-tahun untuk mengendalikan patogen pada tanaman. Sebagaimana telah diketahui, penggunaan bahan kimia secara terus menerus dapat berdampak buruk terhadap lingkungan dan menyebabkan resistensi. Ozon telah dianjurkan oleh para ahli sebagai alternatif pengendalian penggunaan bahan sintetik pada pengawetan bahan makanan karena ozon tidak meninggalkan residu pada bahan simpanan sehingga lebih aman untuk dikonsumsi. Penelitian ini bertujuan ini untuk mengetahui pengaruh ozon pada konsentrasi rendah (180 ppb) terhadap perkembangan koloni dan spora yang dihasilkan oleh *Botrytis aclada* secara in vitro. Dua tipe inokulum digunakan pada penelitian ini adalah miselia dan spora. Keduanya merupakan penyebab kerusakan pada bahan simpanan. Jamur yang diuji disimpan di dalam kotak yang dialiri ozon dengan suhu 7-8^{o}C selama 14 hari. Pengamatan terhadap perkembangan koloni dilakukan dengan mengukur diameter koloni, sementara spora yang dihasilkan diamati pada hari ke 14 masa inkubasi. Hasil penelitian menunjukkan bahwa pemaparan ozon pada konsentrasi 180 ppb selama 14 hari tidak menurunkan perkembangan koloni *B. aclada*, tetapi efektif mengurangi jumlah spora yang dihasilkan.

Kata kunci: ozon, botrytis aclada, in vitro

INTRODUCTION

Infection caused by micro-organisms, fungi and/or bacteria, on fruits and vegetables occur both before and after harvest. The harvested product may be infected by a pathogen under field conditions or during transit and storage. Jobling (2000) claimed that losses during the latter reach 20% of the total crop. *Botrytis cinerea, Rhizopus stolonifer*, *Penicillium digitatum* and *Penicilium italicum* are common fungi that occurred on fresh produce in storage (Golan, 2001).

Fungicides have been used for decades to control spoilage in con-ventional agriculture. It is generally known that long term use of chemical would harm the environment and bring resistance to organisms. Increasing public concern over the use of conventional fungicide due to health issues has prompted investigations to find alternative environmentally friendly control agent that might be used to suppress disease development on the storage.

Studies about the use of ozone to control microorganisms in the storage and/or transit have been conducted by researchers. Researchers with similar findings reported that ozone treatments significantly reduced the extent of berry decay caused by *Rhizopus stolonifer* (Sarig, et al. 1996), *Botrytis cinerea* on

strawberries (Perez et al. 1999), *Lasiodiplodia* sp. and *Cladosporium* sp. on longan fruit (Whangchai et al. 2005), and black spot (*Alternaria alternate)* and anthracnose rot (*Colletotrichum coccodes)* of tomato **(**Tzortzakis, et al. 2008). Advantages are gained by employing ozone in storage to eliminate fungi spoilage on the products. Unlike chemicals, ozone treatment has no residue remain on the products. In addition, ozone eliminates odor in storage rooms (Suslow, 1998).

Even though it is known that ozone sanitizes the storage environment to prevent microbial spoilage at certain level, the mode of action of ozone on fungi is not certain. However, Hibben and Stotzky (1969) suggested that spore morphology, moisture content and substrate play an important role. In addition, Krause & Weidensaul (1978) stated that "Since ozone attacks cellular membranes of higher plants, perhaps fungal membranes could be similarly affected". To understand the effect of ozone on growth of food spoilage fungi, further research is needed.

MATERIALS AND METHODS

Isolation of mould from garden strawberries

Mouldy garden strawberries (*Fragaria ananassa*) were used as source of food spoilage moulds in this study. The isolation procedure was carried out by removing the diseased tissue along with the moulds and transferring tissue to Rose Bengal Agar. The plates were incubated at 28 ºC for 7 days. Any contamination with yeasts was monitored through the incubation period and in such cases the moulds were sub cultured to fresh Rose Bengal plates.

Identification of isolates

Fungal genomic DNA was extracted according to GeneElute DNA kit (Sigma) manufacturer that kindly conducted by Institute for Research on the Environment and Sustainability, University of Newcastle, The UK. The sequence was edited and homology searches were performed using BLAST (Altschul et al., 1997) at the National Centre for Biotechnology Information (NCBI) via http://www.ncbi.nml.nih.gov/BLAST.

Effect of ozone on colony development in vitro

There were two methods applied to understand the effect of ozone on pathogen development; introduction of mycelial and spores extract since both spores and mycelia can act as inocula and has responsible for spread of spoilage when stored with uncontaminated food (Adams and Moss, 2008). On the first experiment, the fungi were grown on the surface of malt extract agar by inoculation with spores followed by incubation for 7 days at 28^{o}C. A mycelial plug of the isolates was introduced to the centre of plates containing Malt Extract Agar (three replicate plates per treatment). Following inoculation, control plates were stored at 7^{o}C room and the treated plates were stored in the ozone chamber with 180 ppb level of ozone at 7^{o}C. The colony growth on both control and treated plates (colony diameter in mm) were measured over a period of 14 days.

The second method started with extracting the spores by adding 10 ml ¼ Ringer Solutions to the surface of a colony and gently scraping to dislodge spores. Afterward the solution was filtered with cotton in a syringe and transferred into fresh eppendorf tube. Spore suspensions at 30 µL were then introduced to the centre of the Malt Extract Agar plate. Once the solution dries in the cabinet, the control plates were stored at 7^{o}C room and the treated plates were stored in the ozone chamber with 180 ppb level of ozone at same temperature. The colony growth on both control and treated plates (colony diameter in mm) were measured over a period of 14 days.

Effect of ozone on spore production in vitro

On the 14th day, a plug of new mycelia from both experiments, mycelial and spores extract was removed from each plate, from the edge of colony for the mycelial experiment and from the middle of colony for spore extract experiment. The aim of the technique used was to avoid examining the old mycelial from the plate. The plug was transferred into 1000 µL 1% of SDS followed by centrifuging at 10,000 rpm for 10 minutes and adding another 200 µL 1% of SDS into the tube followed by shaking the tubes on vortex machine. To find out the number of spores on each isolate, 4 µL of solution was dropped on the centre of Haemacytometer which was then covered by cover slip and observed under electronic microscope. Spores from five random squares were counted as the sample.

Statistical analyses

The data on growth rate and number of spores in each strain were statistically analyzed by Analysis of Variance (ANOVA) on Minitab 15. Significant differences between mean values were determined using the LSD ($P = 0.05$).

RESULTS

Isolate Identification

PCR of 28S rRNA was carried out to identify the suspected spoilage. The identification using the Basic Local Alligment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI) revealed that the isolate was *Botrytis aclada* with 94% of certainty.

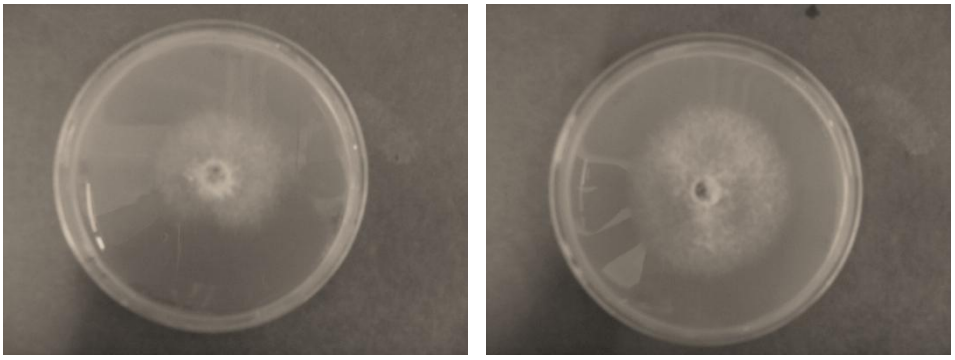
Impact of ozone on colony development in vitro

Variation between different inoculum types, mycelial and spore inocula

Visual examination of the culture plates showed that the initial growth of colony was generally faster when inoculated with mycelial rather than spore. Growth from mycelial inocula was observed on the second day of the observation, while from spore inocula the growth was detected on the 7th day. However, the growth patterns were not found to be different between the inocula used.

Effect of ozone on radial colony growth: mycelial inoculum.

Fig. 1 shows ozone-enrichment at low level (180 ppb) increased the colony development on *B. aclada* isolate significantly (P value < 0.05). Examination of macroscopic visual showed that colony growth was 8 mm per day. After 14 days of observation, the colony covered almost entire plate. From visual examination, it showed that the colony area of the isolates were wider when they were exposed to ozone compared to control (Fig. 2).



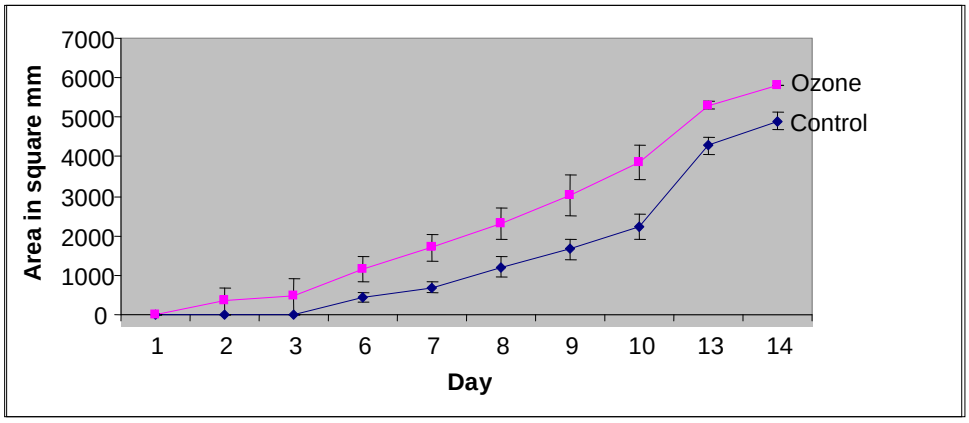


Figure 1. Colony area on control (A) and ozone treated (B) of *Botrytis aclada* on 7th day of observation

Figure 2. The effect of ozone at 180 ppb on *Botrytis aclada* growth over 14 days with mycelial inocula. Both control and treated fungi were stored in room with same temperature 7-8°C.

Effect of ozone on radial colony growth: spore inoculum

The colony growth on *B. aclada*, (Fig. 3) both not exposed and exposed to ozone increased gradually, until the 14th day. However, on the last day of monitoring, the colony growth of control was quadruple the ozone treated plates.

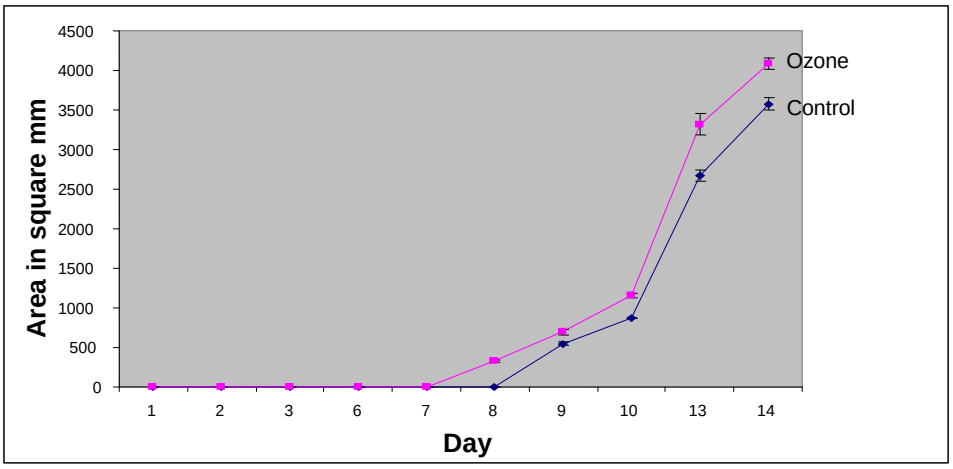


Figure 3. The effect of ozone at 180 ppb on *Botrytis aclada* growth over 14 days with spores inocula. Both control and treated fungi were stored in room with same temperature 7-8°C.

Effect of ozone on spore production in vitro

The numbers of spores produced by cultures with mycelial and spores inoculums are shown in Figure 4. These numbers are expressed as number of spores/mm^2 surface area of the culture. Ozone exposure at 180 ppb decreased the number of spore produced by isolate examined up to 96% and 49% with mycelial and spore inocula respectively.

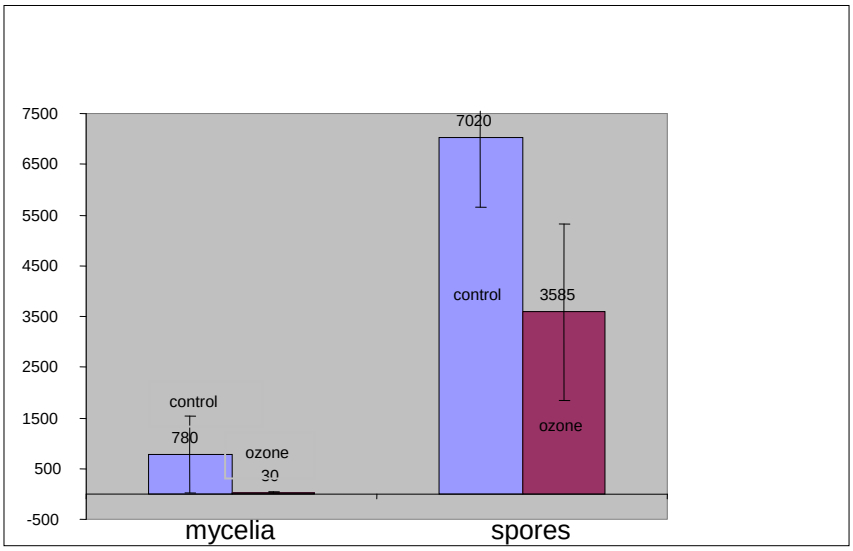


Figure 4. Ozone-enrichment (180 ppb) decreased spore production on *Botrytis aclada* 69% and 49% with mycelial and spore inocula, respectively, in period of 14 days. Bar chart shows the number of spores per square mm of the colonies.

DISCUSSION

Isolate Identification

Botrytis neck rot caused by *Botrytis aclada* is often a serious postharvest disease of onion while infection of the pathogen on strawberries not well documented. According to Kader (2002), the disease usually develops from the cut leaves into the inner bulb scales, which become water-soaked and light brown to dark brown. In contrast with *B. cinerea, B. aclada* is other species of Botrytis that relatively little known. Shirane et al. (1989) noted significant differences in nuclear number per conidium between the species. Mean value for nuclear number per conidium for *B. aclada* is 1.3-1.5 that similar to *B. cinerea* with values of 4.0-5.1.

The impact of ozone on *Botrytis aclada*

B. aclada has very small and light spores that can be easily carried by air flow to infest fresh fruit in the storage. Ozone enrichment has been suggested by the experts to reduce damage on storage products. However, the effects of ozone on growth of food spoilage fungi are not well documented.

The study revealed that continuous low-level atmospheric ozone-enrichment (180 ppb) did not reduce the colony growth of *B. aclada* significantly. In fact, the colony growth was wider when the isolates were exposed to ozone. This result is in line with those reported by Tzortzakis et al. (2007) that ozone-enrichment at 0.1 μmol mol^{-1} (100 ppb) resulted in no significant changes in colony diameter of *B. cinerea* exposed to ozone *in vitro*.

A higher level of ozone was tested to *B.cinerea* on grapes by Palou et al. in 2002. The result was that ozone exposure at 0.3 ppm (300 ppb) did not reduce gray mold incidence during cold storage of table grapes. Ozone exposure also reported did not control gray mold caused by *B. cinerea* on grapes (Spalding, 1968). According to Shimizu et al. (1982), ozone was incapable to control wound infection of *B. cinerea* on 'Kyoho' grapes exposed to gaseous ozone at 500 ppm (500,000 ppb)

Nevertheless, different finding was reported by Krause & Weidensaul (1978) that ozone treatments at 0.30 ppm (300 ppb) for two 6-hr periods in vitro significantly reduced germination of

conidia, germ tube length, pathogenicity, and/or virulence *of Botrytis cinere*. The different result might be caused by different level of ozone and method applied. The experiment carried out by Krause & Weidensaul (1978) was begun with sterile, deionized-distilated water. These findings indicated that, in addition to its sterilizing effect, ozone also induced resistance to post-harvest decay development.

The effect of ozone on different fungal development (*Alternaria alternata* and *Colletotrichum coccodes*) on agar (measured by colony diameter) was also studied by Tzortzakis et al. in 2008. They found that the fungal development was unaffected by ozone exposure although colony morphology did appear to be slightly affected. The reduction in pathogen development was shown when fruit pre-exposed to ozone were subsequently inoculated with *A. alternata*, even when the fruit were subjected to 0.05 μmol mol^{-1} (50 ppb).

Another considerable factor that leads to different results from previous studies is the level of ozone exposed to the pathogen. According to Suslow (1998) ozone is a highly effective sanitizer at concentrations of 0.5 to 2 ppm (500- 2000 ppb). From the previous researches can be understood that sterilizing helps the reduction of fungal development in ozone storage room even though the level of ozone exposed was low.

The cause of the different result gained although the ozone level at similar level probably due to each microorganism have evolved their own mechanism to counteract cellular reactive oxidative species (Aguirre et al., 2006) and so the ozone resistance mechanisms of each organisms could be different one to another.

The mode of action of ozone on fungi remains uncertain. However, Hibben & Stotzky (1969) suggested that spore morphology, moisture content, and substrate play an important role. In addition, Krause & Weidensaul (1978) stated that "Since ozone attacks cellular membranes of higher plants, perhaps fungal membranes could be similarly affected".

CONCLUSION

Continuous ozone-enrichment at 180 ppb for 14 day did not reduce the colony growth of *B. aclada* with both mycelial and spores inocula. However, ozone exposure effectively reduces the number of spore produced.

FUTURE WORK

Perhaps, a higher level of ozone and sterilizing before ozone treatment could offer a better result. From previous studies it is known that higher level of ozone (more than 500 ppb) was able to reduce pathogen development. The ability of ozone to control pathogen was higher when fresh produce or media sterilizing was applied before ozone treatment. Another reasonable factor for the differences gained by researchers is probably due to the different ability of each microorganism to counteract with ozone exposure.

The results from the current study suggest a further research to explore what level of ozone that is able to break the cell of *B. aclada* and also to test whether sterilizing could improve the ability of ozone to control the pathogen. Furthermore, the mechanism of fungi resistance to ozone is also important to reveal.

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