THE EFFECT OF LOW LEVEL OF OZONE ON GROWTH AND DEVELOPMENT OF *RHIZOPUS* SP. IN VITRO

(Pengaruh Paparan Ozon Level Rendah Terhadap Pertumbuhan dan Perkembangan *Rhizopus* sp. *In Vitro*)

Hartati Oktarina[1], Ian Singleton[2], Sanjay Anthony-Babu[3]
[1]Staff Pengajar Program Studi Agtoteknologi Fakultas Pertanian Universitas Syiah Kuala, [2,3]New Castle University, United Kingdom
E-mail: oktarina.hartati@gmail.com

Abstract

Postharvest diseases caused by microbial pathogens account for a great loss every year. Fungicides have been used for decade to control spoilage in conventional 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 diseases development in storage.

Ozone has been considered by researchers as an effective alternative to the use of traditional pesticides in food preservation because it leaves no residue on fresh produce so that safer to consumed.

This work determined the effect of low level ozone exposure (180 ppb) on colony development and spores production of *Rhizopus oryzae, R. stolonifer* and *R. microsporus var.chinensis* in vitro. Two types of inocula were used, mycelial and spore, as both are responsible for spread of spoilage when stored with uncontaminated food. The tested fungi were stored in an ozone chamber at 7-8 ^{o}C for 14 days. Colony development was studied by measuring the colony diameter while spores production by time fungi was assed on the 14th day of the incubation period. Ozone exposure at 180 ppb has varied result on colony development and spore production on each species examined.

Overall the work suggests that continuous low level of ozone exposure at 180 ppb for 14 days has different effect on different species depend on the ability each microorganism to counteract with ozone exposure.

Key words: *low level ozone, growth and development, In Vitro*

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 berdapampak buruk terhadap lingkungan dan menyebabkan resistensi. Meningkatnya kepedulian masyarakat terhadap dampak penggunaan pestisida sintetik terhadap kesehatan mendorong para ahli untuk melakukan penelitian untuk menemukan pengendalian yang ramah lingkungan yang dapat menekan perkembangan penyakit pada penyimpanan.

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 level rendah (180 ppb) pada perkembangan koloni dan spora yang dihasilkan oleh *Rhizopus oryzae, R. stolonifer* dan *R. microsporus var.chinensis* secara in vitro. Dua tipe inokulum digunakan pada penelitian ini, miselia dan spora, karena keduanya merupakan penyebab kerusakan pada bahan simpanan. Jamur yang di uji 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.

Dari hasil penelitian diketahui bahwa pemaparan ozon pada level 180 ppb selama 14 hari memberikan dampak yang berbeda terhadap perkembangan koloni dan jumlah spora yang dihasilkan oleh *R. oryzae, R. stolonifer* dan *R. microsporus var.chinensis* tergantung pada kemampuan setiap mikroorganisme tersebut untuk menetralkan paparan ozon.

Kata Kunci: *Ozon level rendah, Pertumbuhan dan Perkembangan, In Vitro*

INTRODUCTION

Infection caused by microorganisms, 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, R. stolonifer, Penicillium digitatum* and *P. italicum* are common fungi that occurred on fresh produce in storage (Golan, 2001).

Fungicides have been used for decades to control spoilage in conventional 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 *R. stolonifer* (Sarig, et al. 1996), *B. 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 odour in storage rooms (Suslow, 1998).

Even though it is known that ozone sanitises 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.

This review focuses on fungal postharvest disease, control of spoilage and the potential use of ozone to reduce spoilage in storage room.

METHOD

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 subcultured to fresh Rose Bengal plates.

Identification of isolates

Fungal genomic DNA was extracted according to GeneElute DNA kit (Sigma) manufacturer that kindly conducted by Insititute 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 28ºC. 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 was begun 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 analysed for Analysis of Variance (ANOVA) on Minitab 15. Significant differences between mean values were determined using the LSD (P = 0.05).

RESULT AND DISCUSSION

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 *R. oryzae*, *R. microsporus var.chinensis* and *R. stolonifer* with 100% 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 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. Ozone-enrichment at low level (180 ppb) increased the colony development on three *Rhizopus* isolates tested significantly (P value < 0.05). Examination of macroscopic visual showed that colony growth of *R. oryzae* (fig.1), and *R. stolonifer* (fig. 2) was faster than growth on *R. microsporus var.chinensis* (fig. 3), 8 mm per day and 2 mm/day respectively. After 14 days of observation, the colony of *R. oryzae*, and *R. stolonifer* covered almost entire plate while on *R. microsporus var.chinensis*, the growth of the colony was no more than 15 mm at the end of study. From visual examination also known that the colony area of the isolates are wider when they are exposed to ozone compared to control (fig.4).

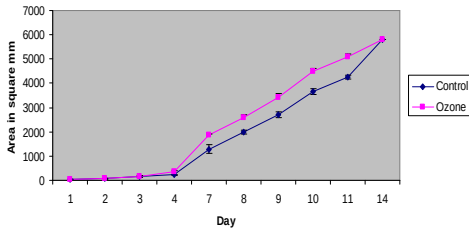

Figure 1 . The effect of ozone at 180 ppb on *R. oryzae* growth over 14 days with mycelial inocula. Both control and treated fungi were stored in room with same temperature 7-8 ^{o}C.

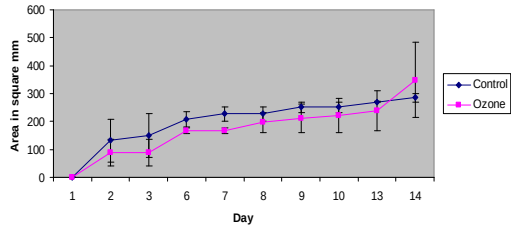

Figure 2. The effect of ozone at 180 ppb on *R. stolonifer* growth over 14 days period with mycelial inocula. Both control and treated fungi were stored in room with same temperature 7-8 ºC.

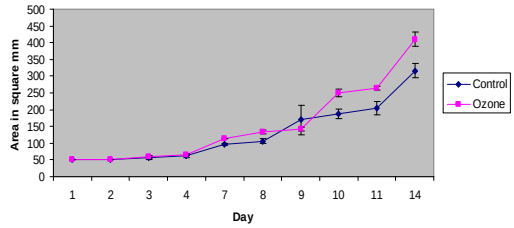

Figure 3. The effect of ozone at 180 ppb on *R. microsporus var.chinensis* growth over 14 days with mycelial inocula. Both control and treated fungi were stored in room with same temperature 7-8 ºC.

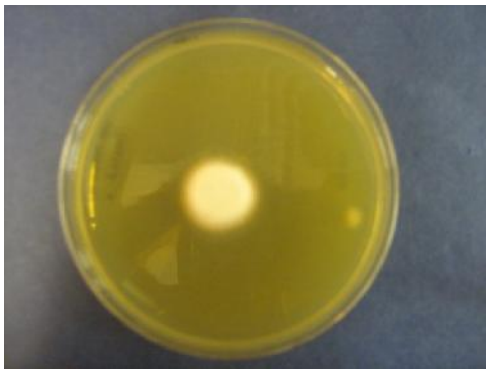

Figure 4. Area colony of treated and *R. microsporus var.chinensis* at the last day of study

***Effect of ozone on radial colony growth: spore inoculum*:** Ozone-enrichment resulted in the increase of *R. stolonifer* colony growth (fig. 5) significantly (P value < 0.05), has insignificant impact on reducing the colony growth of *R. microsporus var.chinensis* (fig. 6), and significantly decreased the colony growth on *R. oryzae* (fig. 7).

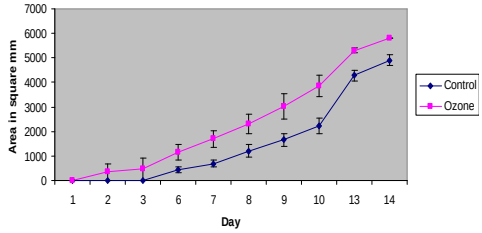

Figure 5. The effect of ozone at 180 ppb on *R. stolonifer* growth over 14 days with spores inocula. Both control and treated fungi were stored in room with same temperature 7-8 ºC.

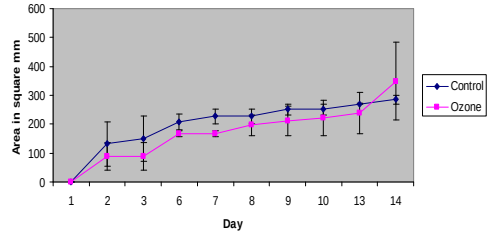

Figure 6. The effect of ozone at 180 ppb on *R. microsporus var.chinensis* growth over 14 days with spores inocula. Both control and treated fungi were stored in room with same temperature 7-8 ºC.

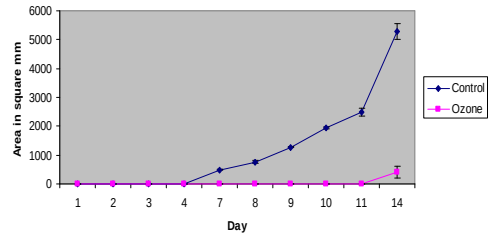

Figure 7. The effect of ozone at 180 ppb on *R. oryzae* growth over 14 days with spores inocula. Both control and treated fungi were stored in room with same temperature 7-8 ºC.

Effect of ozone on spore production in vitro

The numbers of spores produced by cultures with mycelial and spores inoculum are shown in figs. 8 & 9. 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 all species examined up to 98% and 75% with mycelial and spore inocula respectively.

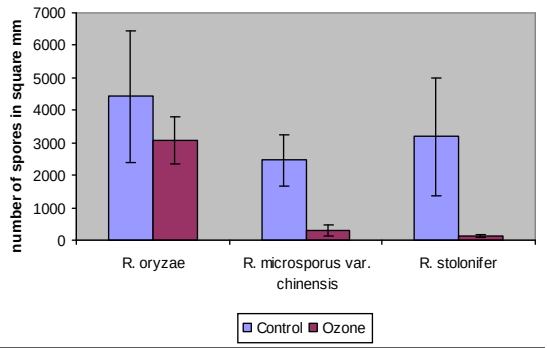

Figure 8. Ozone-enrichment (180 ppb) decreased spore production with mycelian inocula on *R. oryzae* (31%), *R. microsporus var.chinensis* (84%), and *R. stolonifer* (98%), respectively in period of 14 days. Bar chart shows the number of spores per square mm of the colonies.

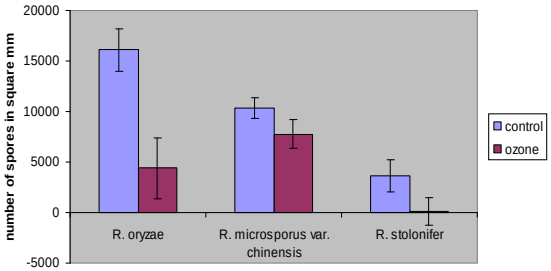

Figure 9. The effect of ozone-enrichment (180 ppb) on spore production with spore inocula on *R. oryzae*, *R. microsporus var.chinensis*, and *R. stolonifer*, respectively in period of 14 days. Bar chart shows the number of spores per square mm of the colonies. The error bar denotes standard deviation.

Discussion

Isolate Identification

R.stolonifer and *R. oryzae* cause of rhizopus rot, one of the most serious problems in storage that reduces post harvest quality. The pathogen is widely distributed in the soil and the atmosphere. Barkai-Golan & Kopeliovitch (1981) reported, the spores germination of the pathogen occurs in warm, moist environment and penetration takes place via injures or damages tissue. The infected area appears water-soaked through the distended skin.

R. microsporus var.chinensis is the later name for *R. chinensis*. In Indonesia along with *R. microsporus* varieties *microsporus*, *oligosporus*, and *rhizopodiformis* the species is well known for tempe making. There is lack of publication about the pathogen infected fresh produce in storage room. Further study is required to reveal the importance of the pathogen as postharvest disease.

The impact of ozone on *R. oryzae*, *R. microsporus var.chinensis*, and *R. stolonifer*

Rhizopus 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 *Rhizopus* significantly. In fact, the colony growth was wider when the isolates were exposed to ozone. This result 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*.

The effect of ozone-enrichment at 180 ppb also insignificantly reduced the number of spores on tested fungi. From visual examined under microscope it was known that *Rhyzopus* exposed to ozone did not produce sporangium as the one that untreated. Instead they grow wider as branches. Perhaps, this is the reason their colonies seen grow wider than control. Mitcham et al. (2009) claimed that this fungus will not grow at temperatures below 5°C (41°F). According to Antony-Babu & Singleton (2009), the number of spores that germinated forming a germ tube after treatment with high level of ozone (200,000 ppb) was reduced drastically to less than 50%. However, under sustained low level exposure (200 ppb) spore production in *Aspergillus ochraceus* was reduced by 94%.

Nevertheless, different finding was reported by Sarig, et. al., (1996) that ozone treatments at 8 mg min^{-1} in an air flow of 500 ml min^{-1} (16,000 ppb) significantly reduced decay in berries caused by *Rhizopus stolonifer*. The different result might be caused by different level of ozone and method applied. The experiment carried out by Sarig et. al., (1996) was begun with sterilize the

fresh product followed by ozone exposure. 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) 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 showed 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

Conclusion

Continuous ozone-enrichment at 180 ppb for 14 day did not reduce the colony growth of all species examined 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 sterilising before ozone treatment could offer a better result. From previous studies it is known that higher level of ozone (more than 500 ppb) able to reduce pathogen development. The ability of ozone to control pathogen was higher when fresh produce or media sterilising was applied before ozone treatment. Another reasonable factor for the differences gained by researchers probably due to the ability of each microorganism to counteract with ozone exposure was different.

The results from the current study suggesting a further research to explore what level of ozone that able to break the cell of species and also to test whether sterilising could improve the ability of ozone to control *R. oryzae, R. stolonifer* and *R. microsporus var.chinensis* on the storage room as postharvest treatment. Furthermore, the mechanism of fungi resistance to ozone is also important to reveal.

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