Response of stored grain insect pests and barley to ozone treatment

By

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

signature: Xue Dong Date: 19-03-2022

Acknowledgments

Time flies, I have already spent more than 3 years at Murdoch University and my student career has drawn to a close. A 3-year life is very short, but it is very important in my whole life. The PhD journey was like a rollercoaster journey, I went through the daily ups and downs. Although this is a hard period, I learnt a lot throughout my PhD journey, including personal communication, research processing, self-motivation.

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Attribution Statements

The content in this thesis was developed by the Candidate with advice from their supervisory panel.

The following individuals contributed to the thesis.

Contributor	Contribution (%)	Concept Development	Data Collection	Data Analyses	Drafting of Chapters
Name 1 Xue Dong	75	X	X	X	X
Name 2 Yonglin Ren	10	X		X	
Name 3 Xiangyang Yu	5	X		X	
Name 4 Manjree Agarwal	5	X		X	
Name 5 Garth Maker	5	X		X	

Contribution indicates the total involvement the Candidate has had in the creation of the thesis. Placing an 'X' in the remaining boxes indicates what aspect(s) of the thesis each individual engaged in.

By signing this document, the Candidate and Principal Supervisor acknowledge that the above information is accurate and has been agreed to by all other contributors.

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Candidate **Principal Supervisor**

Abstract

Ozone (O_3) has distinct advantages over other stored grain pest treatments, such a toxicity to a broad range of organisms and rapid auto-decomposition to oxygen without leaving residues, making it a promising fumigant to protect stored grains.

This research project focused on understanding how stored grain insects and barley respond to ozone. Several studies have assessed the efficacy of O_3 against pests in stored maize and wheat grain, but little is known about the toxicity of O_3 on stored grain pests in barley and its effects on barley quality and germination. My results indicated that more than 2 days of ozone treatment should be considered as the endpoint to evaluate insect mortality rate due to delayed toxicity of O_3. C×t product 36 mg h/L (700ppm × 24 h) offered complete mortality for all stages of the two species tested insects *R. dominica* and *T. castaneum*, without affecting barley commercial quality.

As barley is to be used for malting purposes, it is important to understand that effect of ozone on barley germination, whether positive or negative. Relevant short period treatment (20 min) with ozone at 700ppm can facilitate barley germination, but it was adversely impacted for longer O_3 exposure times, such as 24 h.

Ozone treated barley seed released a greater number and quantity of volatile organic compounds (VOCs). However, alcohols and hydrocarbons gradually decreased, whereas aldehydes and organic acids markedly increased with increasing time of ozone treatment. Acetic acid was identified as a potential ozone stress-specific marker. Furthermore, the dosage-dependent function of acetic acid on barley germination was verified and results indicated that low dosage (0.05 - 0.5 mg/g of barley) of acetic acid could lead to increasing germination rate.

Potential chemical biomarkers from barley seeds during germination were identified using gas chromatography mass spectrometry (GC/MS). Statistical assessment of the data via principal component analysis demonstrated that the metabolic changes during germination were reflected by time-dependent shifts. Alcohols, fatty acids and ketones were the major contributors to the time-driven changes during germination. In addition, ozone induced an increase in fatty acids at the early stage of barley germination and probably enhanced germination by supplying carbon skeletons and energy for germination via the TCA cycle.

My results indicate that ozone could be utilized to improve the quality of malting barley on enhancing germination rate. The key finding is that acetic acid could be used as a regulator to control germination. Moreover, this PhD study is the first-time explored effect of O_3 on metabolite profiles of barley germination, which could lead to identifying the factors might impact barley germination or malt quality.

Publications

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Xue Dong, Manjree Agarwal, Yu Xiao, Yonglin Ren, Garth Maker and Xiangyang Yu. (2022). Ozone Efficiency on Two Coleopteran Insect Pests and Its Effect on Quality and Germination of Barley. *Insects*, 13(4), 318.

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List of abbreviations

ABA	Abscisic Acid
Acetyl-CoA	Acetyl Coenzyme A
AMU	Atomic Mass Units
ANOVA	Analysis of variance
AR	After-ripening
BP	Between-paper
CAR	Carboxen
CW	Carbowax
DAT	Day after treatment
DI-SPME	Direct immersion Solid-Phase Microextraction
DPIRD	Department of Primary Industries and Regional Development
DVB	Divinylbenzene
DVB/CAR/PDMS	Divinylbenzene/Carboxen/Polydimethylsiloxane
EI	Electron Ionisation
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FDR	False Discovery Rate
GA	Gibberellin
GC-MS	Gas Chromatography-Mass Spectrometry
HLT	Half-Life Time
HP-5MS	5%-phenyl-methylpolysiloxane stationary phase
HSD	Honestly Significant Difference
HS-SPME	Headspace Solid-Phase Microextraction
ISTA	International Seed Testing Association
LC-MS	Liquid Chromatography-Mass Spectrometry
LLE	Liquid-liquid extraction
LT	Lethal Time
MC	Moisture Content
MSD	Mass Selective Detector
NIST	National Institutes of Standards and Technology
NMR	Nuclear Magnetic Resonance
PA	Polyacrylate
PCA	Principal Component Analysis

PCs	Principal Components
PDMS	Polydimethylsiloxane
PhD	Doctor of Philosophy
PLS-DA	Partial Least Squares Discriminant Analysis
RI	Retention Indices
ROS	Reactive Oxygen Species
SBSE	Stir Bar Sorptive Extraction
SE	Standard Error
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SVI	Seedling Vigour Index
TCA	Tricarboxylic Acid
TIC	Total Ion Chromatogram
TW	Thousand Weight
UHP	Ultra High Purity
UV	Ultraviolet Light
VIP	Variable Importance in Projection
VOCs	Volatile Organic Compounds

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Chapter One

Literature Review

1.1. Barley

1.1.1. World barley production and trade

Barley (*Hordeum vulgare* L.) is a widely cultivated and highly adaptable crop. In terms of global production, maize is ranked first accounting for 38.1%, followed by wheat, rice and barley representing 25.9%, 25.8% and 4.95%, respectively (Figure 1.1A). According to FAO, in the past two decades, the area harvested for barley production has decreased from >58 to ~52 million ha (Figure 1.2A), whereas production increased from 128 to ~160 million tonnes (Figure 1.2B).

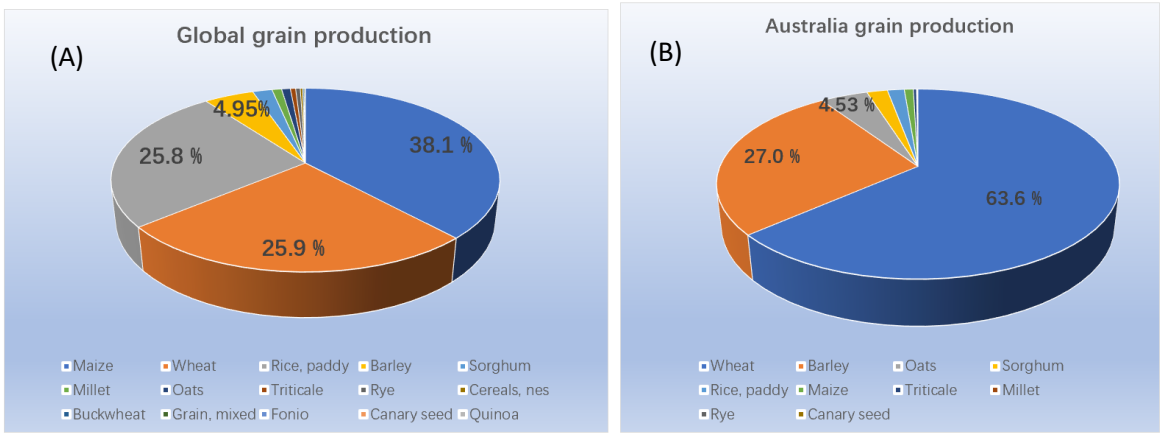


Figure 1.1. Global and Australian grain production (FAO, 2017)

In Australia, wheat and barley are the most important grain crops, accounting for 63.6% and 27.0% of production (Figure 1.1B). As the second-largest crop, barley is grown across a large geographic area with almost four million hectares from Western Australia to the eastern states (GRDC, 2017). From 1999 to 2019, the harvested area gradually increased from 2.59 to 4.43 million ha (1.2A), with production increasing from 5.04 to 9.25 million tonnes (Figure 1.2B). Australia is ranked second barley production, making up 9.16% of global production, following Russia (Table 1.1) (FAOSTAT, 2019).

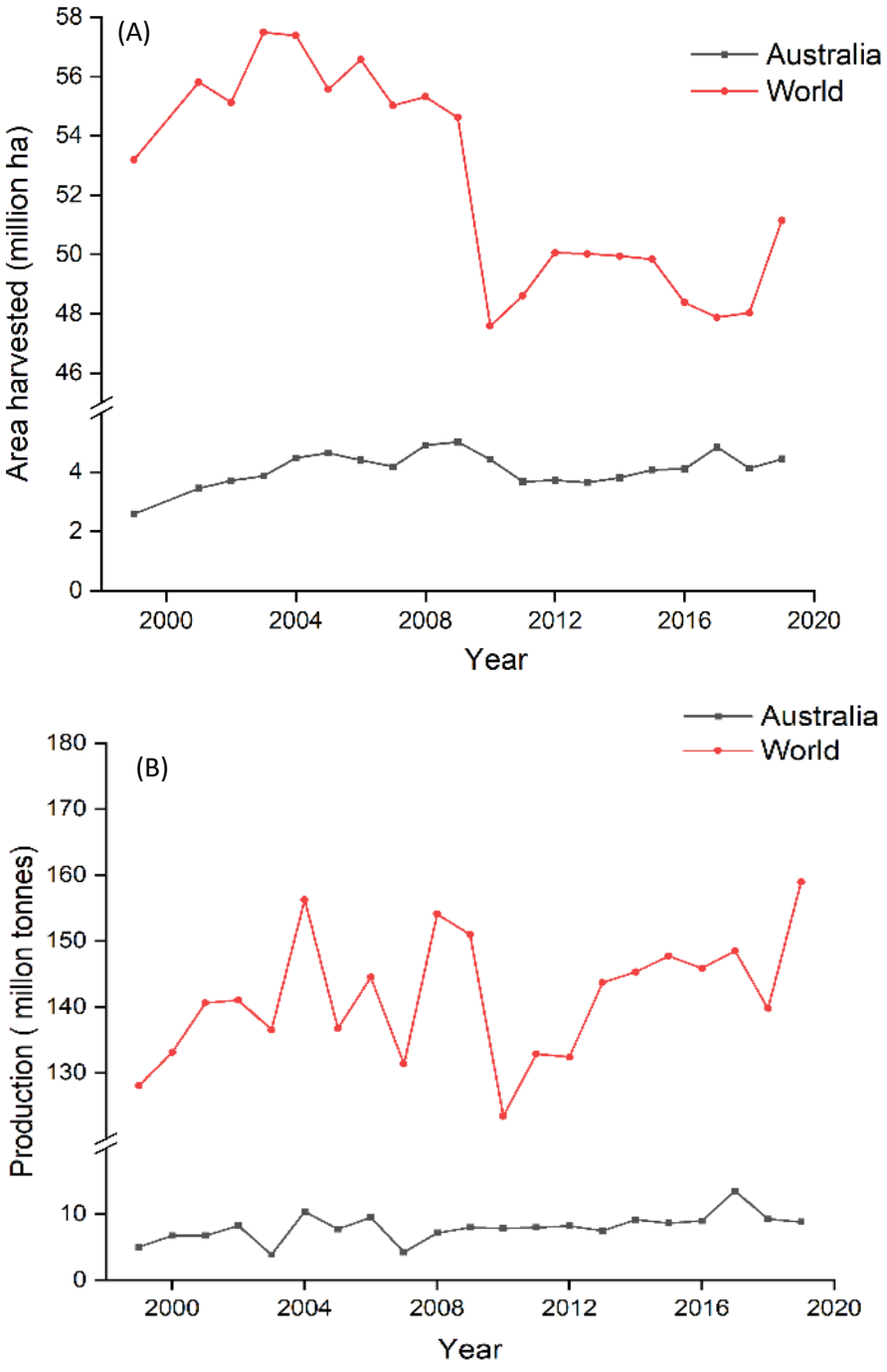


Figure 1.2. Global barley harvested area and production (FAOSTAT, 2019).

Table 1.1. Top producing countries of barley (FAOSTAT, 2019).

Rank	Countries	Production (tonnes)	Proportion of total world production (%)
	World	147404262	-
1	Russia	20598807	14
2	Australia	13505990	9.16
3	Germany	10853400	7.36
4	France	10545427	7.15
5	Ukraine	8284890	5.62
6	Canada	7891300	5.35
7	United Kingdom	7169000	4.86
8	Turkey	7100000	4.82
9	Spain	5785944	3.93
10	Denmark	3992300	2.71

The total global barley trade, including exportation and importation, increased from 42 to more than 76 million tonnes in the past 20 years. Australian barley trade showed the same trend, increase from 3.78 to 6.12 billion tonnes (Figure 1.3). The major exporters are EU countries, Australia, Canada and the USA. In 2019, Australia ranked third in the global exportation accounted for around 9.24% of the world's total barley export market, valued at US$0.72 billion (Table 1.2). The major markets for Australian grain are the Middle East and Asia, with China, Saudi Arabia and Iran currently the major barley importers, occupying 40% of total global importation. Saudi Arabia was the leading importer from 1999 to 2011. Since that time, the trade market has been boosted by a massive increase in imports by China, which comprises more than 20% of the total barley imports.

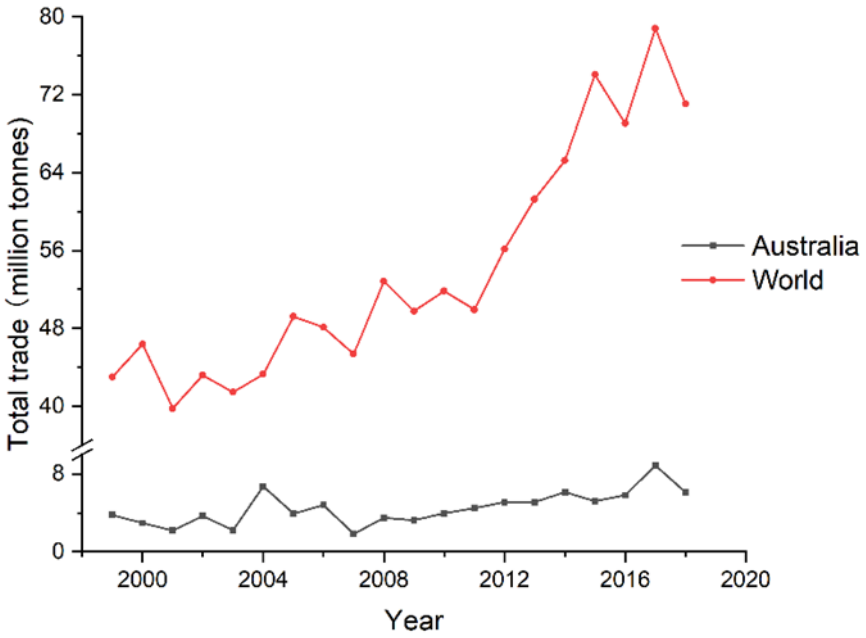


Figure 1.3. Global and Australian barley trade (FAOSTAT, 2019)

Table 1.2. Top importing and exporting countries of barley (FAOSTAT, 2019)

	Importation			Exportation			
Rank	Countries	Importation (million tonnes)	Proportion of total (%)	Countries	Exportation (million tonnes)	Export value (million US$)	Proportion of total (%)
	World	31.33024	-	World	31.061984	6.532088	-
1	China	5.928778	18.92	France	7.171937	1.494534	22.88
2	Saudi Arabia	3.905759	12.47	Russia	3.940653	0.763616	11.69
3	Iran	3.287269	10.49	Australia	2.872279	0.724937	11.10
4	Netherlands	2.156518	6.88	Argentina	2.517497	0.587557	8.99
5	Belgium	2.011363	6.42	Ukraine	2.348829	0.527417	8.07
6	Germany	1.268081	4.05	Canada	2.183447	0.44958	6.88
7	Spain	1.221055	3.90	UK	1.666221	0.328138	5.02
8	Japan	1.147828	3.66	Kazakhstan	1.640083	0.307581	4.71
9	Jordan	0.860236	2.75	Germany	1.580572	0.299458	4.58
10	Brazil	0.671337	2.14	Romania	1.123914	0.205472	3.15

1.2. Economic uses of barley

Besides a small amount of barley used as seeds for the next sowing season, most of the rest is used for brewing or feed. Australia has an excellent reputation for producing high-quality barley, which is well-suited for malting, mainly used for beer production and distillation of spirits, such as malt whiskey and Japanese spirits. Most farmers grow barley for sale as malting barley, but when it cannot meet malting quality, it is sold as feed barley (Kumar et al., 2013). Australia supplies around 2.3 million tonnes of malting barley and 6 million tonnes of feed barley annually, accounting for 30–40% of the world's exported malting barley and 20% of global feed barley.

Malt is produced from a cereal grain (such as barley) that has been allowed to germinate for a limited time prior to the kiln. The high malting quality barley with a high germination rate and uniform malting characteristics can achieve malting grade and brewing purpose. Bright colour is also required for producing high-quality malt and brewed products, but unfavourable growing conditions and pathogen infection can lead to stained barley. Barley with fungal staining resulted in low malt extract, poor flavour, over foaming, and reduced shelf life of beer (Oliveira et al., 2012). Protein is also an essential factor for malting barley quality. The ideal protein range is between 9.5-12.5%. Barley with either higher or lower protein levels will cause fewer malt extracts. Low moisture content is also crucial for malting barley. More than 12.5% of moisture content will promote fungal growth. Grain plumpness and weight are also crucial parameters of malting barley.

Unmalted barley can also be utilized for human consumption such as malt beverages, baking, confectionery, and breakfast cereals with many benefits. Barley has attracted attention from health professionals for its fibre content, especially β-glucan, which can assist with losing weight, decreasing blood pressure, blood cholesterol, and blood glucose in Type 2 diabetes, as well as preventing colon cancer (Martínez et al., 2018). The US Food and Drug Administration (FDA) claimed that β-glucan soluble fibre could

reduce plasma cholesterol levels and the risk of heart disease (Ames and Rhymer, 2008). Thus, barley is a promising product that could partly replace wheat flour, providing soluble dietary fibre to benefit human health.

Barley that does not meet the quality requirements for malting or human food could be used for stock feeding as a major source of energy, protein and fibre. Approximately 40% of Australian feed barley was fed to feedlot cattle, 34% to dairy cows, 20% to pigs and 6% to grazing ruminants, with less than 1% was used for poultry (Zhou, 2009). The two most important factors considered by feed barley buyers are grain colour and moisture content (Bleidere and Gaile, 2012). Grain brightness is an attractor for purchasers for feeding purposes. The dark colour of barley is imparted by tannins (Evers et al., 1999), which contribute to an astringent flavour that is disliked by most domestic animals. Thus, barley grain with low tannin content presenting in bright colour is much more suitable for stockfeed. Moisture content is also an essential trait for storage purposes. Excessive moisture content could promote microorganism growth leading to quality loss (Magan and Aldred, 2007).

1.2.1. Challenges related to grain supply chain

Both pre- and post-harvest treatments contribute to the final profits from barley production and the quality of the final products. In terms of pre-harvest losses, about 20%-40% is lost before harvesting due to diseases, pests, weeds, abiotic stress and harvest destruction (Figure 1.4). Ideally, it is better to export newly harvested gain to the global market, because it can be sold at a higher price and reduce storage costs. However, grain production in any country varies from year to year. Thus, it should be stored strategically from over-production years to compensate under-production years.

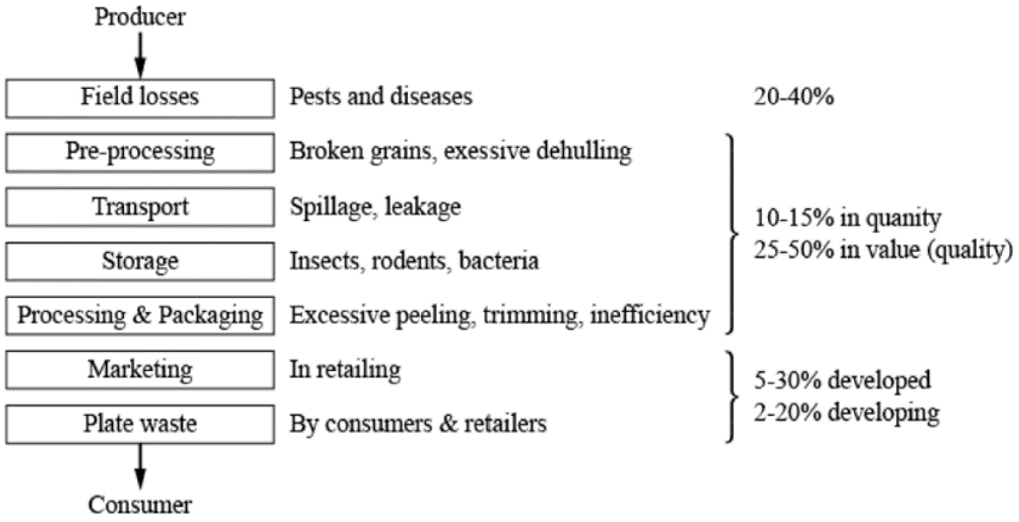


Figure 1.4. Losses along the food production chain (Mesterházy et al., 2020)

Furthermore, grains are stored for other reasons, such as when the production point is not the consumption point or production time is not the consumption time (Neethirajan et al., 2007). A large amount of grain losses have occurred after harvesting, including processing, transportation, storage and packing. Physical damage during post-harvest makes the grain more vulnerable to insect and pathogen attack. It has been estimated that one-quarter to one-third of the world's grain crop is lost every year during storage, with the majority attributed to insect and pathogen attack (Mesterházy et al., 2020). Insect and pathogen infestations in grain result in quantity and quality losses and decrease crop economic values.

1.2.2. Stored grain insects

Globally, the impact of insects on post-harvest losses is approximately 1.3 billion tonnes of food every year (FAO, 2010). Major insect pests of stored grains belong to three Orders: Coleoptera (beetles), Lepidoptera (moths), and Psocoptera (psocids). They are further classified into primary and secondary pests. Primary pests have the ability to attack whole grain and feed on the embryo, endosperm, or cotyledons and develop inside the grain. Secondary pests can only feed on broken grain that has already been damaged by the primary pests or as a result of harvesting and transporting. The

major primary grain insects are beetles of the genus *Sitophilus*, including the rice weevil *S. oryzae* (L.), the maize weevil *S. zeamais* Motschulsky and the granary weevil *S. granarius* (L.); the borers, including the lesser grain borer *Rhyzopertha dominica* (F.) , the larger grain borer *Prostephanus truncatus* (Horn), and the Angoumois grain moth *Sitotroga cerealella* (Olivier) (Athanassiou and Arthur, 2018). The major secondary pests include two species of *Tribolium*, the rust red flour beetle *T. castaneum* (Herbst) and *T. confusum* (Trematerra et al., 2000); a group of *Cryptolestes* species, the rusty grain beetle *C. ferrugineus* (Stephens) and flat grain beetles *C. pusillus* (Schönherr) and *C. pussilloides* (Steel and Howe) (Jagadeesan et al., 2013); two *Trogoderma* species, the Khapra beetle *T. granarium* Everts and the warehouse beetle *T. variabile* Ballion; the saw-toothed grain beetle *Oryzaephilus surinamensis*; the rice moth *Corcyra cephalonica* (Stainton); the Indian meal moth *Plodia interpunctella* (Hübner) and a group of *Liposcelis* psocid species, such as *L. bostrychophila* Badonnel, *L. entomophila* (Enderlien), and *L. paeta* Pearman (Nayak et al., 1998).

Besides consuming grain, the insect has multiple effects on grain quality and quantity, including contamination from dead insect bodies, waste products, faeces, and dust from insect activities (Fleurat-Lessard, 2002). Additionally, high quantities of uric acid produced by insects result in grain rancidity (Mason and McDonough, 2012).

1.3. Management for the disinfestation of stored products

1.3.1. Fumigation status

Consumer preference is for safe and clean food free from insects, moulds and chemical residues. Therefore, insects and pathogens must be controlled at various stages like on-farm storage, bulk storage, and during transport, to maintain the quality of grains. Currently, fumigation is a common method to eliminate grain storage pests, but it has several drawbacks. Methyl bromide is a broad-spectrum and fast-acting fumigant used to control pests in agriculture and shipping, but it causes depletion of the ozone layer. Thus, it was phased out in developed countries by 2005 (UNEP, 2002) and globally by

2015 with important exceptions for critical uses, quarantine and pre-shipment (Secretariat, 2006). Phosphine (PH_3) became the most widely used fumigant after the phase out of methyl bromide, however, it is a slow-acting fumigant and requires more than seven days for effective treatment (Rajendran and Sriranjini, 2007). Additionally, continuous use has led to the evolution of resistant populations and environmental contamination (Nayak et al., 2020). Ethyl formate is a promising option, occurring naturally in orange juice, honey, apples, pears and wine with a pleasant aroma. It is therefore used as a synthetic flavouring agent and fragrance. Ethyl formate has high water solubility; therefore it could be rapidly absorbed by higher moisture content commodities. It has been registered as a fumigant for treatment of dried fruit in Australia as ERANOL®, but not for grain processing (Finkelman et al., 2010).

Fumigation with essential oils is an alternative method for stored grain products. 1,8-Cineole is the most toxic fumigant compound in rosemary and Eucalyptus essential oils and exhibits high toxicity against PH_3-resistant *T. castaneum* relative to the susceptible insects (Lee et al., 2002). However, essential oil fumigation is costly, and it has short persistence due to high volatility.

1.3.2. Non-chemical methods

Entomopathogenic fungi (EF) are considered the most promising biocontrol agents, which infect insects through mycelium penetrating into insects cuticle and then growing in the haemocoel causing insects death (Batta and Kavallieratos, 2018). However, there is no commercial bio-pesticides based on EF bioagents are registered against the stored-grain insects (Batta and Kavallieratos, 2018). The main limitations of application of EF in insects control are high moisture requirement for conidial germination and subsequent sporulation, more time consuming to kill insects and potential risk to immunodepressive or immunocompromised people (Rumbos and Athanassiou, 2017; Singh et al., 2017).

Other non-chemical methods such as cold plasma, irradiation and controlled atmosphere have also been reported to eliminate insects in stored grain. (Navarro, 2012; Carocho et al., 2014; Sutar et al., 2021). However, they are costly, lacking large-scale applicability. Therefore, it would be imperative to find alternative strategies to solve resistance problems to control grain storage pests and ensure food security and quality.

1.4. Ozone treatment

1.4.1. Properties of ozone

Ozone (O_3) occurs naturally in the Earth's upper atmosphere, more than 30 km above the Earth's surface, where it is formed from dioxygen by the action of ultraviolet light (UV) and electrical discharge. The largest ozone production occurs in the tropical stratosphere, which forms a protective layer to absorb the sun's UV radiation. In recent years, the increasing ground level of O_3 emitted from fossil fuel has caused serious biological and environmental problems. In terms of plants, ozone can suppress the growth of plants and decrease the yield of crops, which is related to photosynthesis reduction, plant tissue destruction, and premature senescence (Reich and Amundson, 1985; Darrall, 1989; Franzaring et al., 2000). The damage caused depends on the ozone concentration, length of exposure, tissue age, and genetic susceptibility of the plant (Samuel et al., 2000).

Ozone treatment is a green technology in various industrial processes, such as water treatment, surface disinfection, wood pulp bleaching, and chemical production via strong oxidation (Einaga and Futamura, 2004; Silva and Jardim, 2006). Oxygen is used as the precursor for industrial O_3 production. Singlet oxygen radicals combine with the oxygen molecule (O_2) to form O_3. Generation of the free oxygen radical occurs by breaking strong O-O bonds, which requires significant energy, for which UV radiation and corona discharge methods can be employed (Tiwari et al., 2010). UV creates ozone when a wavelength >254 nm hits oxygen. Corona discharge generates ozone by applying high voltage. Ozone is produced at the site of operation and cannot be stored

since it quickly decomposes to oxygen atoms. According to the literature, the half-life time (HLT) of O_3 in distilled water is 20-30 min at 20°C, while HLT of gaseous O_3 is more stable, at approximately 12 h in atmospheric air. Additionally, McClurkin suggested that temperature and pH also affect ozone decomposition in water. In terms of gaseous ozone, apart from temperature, the half-life of gas ozone is affected by airspeed and humidity (McClurkin and Maier, 2010). Sterilization with ozone would be more effective in still air at low temperature and humidity than in high temperature and humidity.

1.4.2. Utilization of ozone in grain processing

Ozone has been used in the food industry for more than 100 years, and it was first used as the food preservative of frozen meat in 1910 (Carletti et al., 2013). In recent years, growing emphasis has been placed on the positive influence of ozone application in agriculture, such as the utilization of ozone in food processing for inactivation of the pathogen, reduction of storage pests and degradation of various mycotoxins (Allen et al., 2003; Bonjour et al., 2011; Savi et al., 2014). All of these applications would mitigate post-harvest losses and increase food safety to a large degree (Tiwari et al., 2010). Compared with traditional preservation technologies, ozone displays many advantages. Ozone occurs naturally in the atmosphere and can be generated on-site without transportation problems. Ozone rapidly auto-decomposes to oxygen without leaving residues, and has a wide spectrum of activity against microorganisms, making it an attractive approach for medical sterilization, food processing, and grain storage.

Besides microbes, ozone is also effective at eliminating storage pests and degrading mycotoxins in grain. The efficacy of ozone against stored product insect pests including maize weevil, (*Sitophilus zeamais* (Motsch)), rice weevil (*Sitophilus oryzae* (L)), red flour beetle (*Tribolium castaneum* (Herbst)), confused flour beetle (*Tribolium confusum* (Jacqueline du Val)), less grain borer (*Rhyzopertha dominica* (Fabricius)), Indianmeal moth (*Plodia interpunctella* (Hübner)) and Mediterranean flour moth (*Ephestia kuehniella* (Zeller)), has been reviewed (Isikber and Athanassiou, 2015).

Ozone toxicity for insects does vary depending on the species and life stages (McDonough et al., 2011). It is worth mentioning that ozone is also effective for phosphine-resistant pests. Sousa et al. (2008) reported that 150ppm gaseous O_3 presented high efficacy against both susceptible and phosphine-resistant insects from 16 populations of *T. castaneum* and 11 populations of *R. dominica.* O_3 was also lethal to different stages of *R. dominica* and *T. castaneum.*

These advantages make ozone an attractive approach for food processing and grain storage. From environmental and economic perspectives, the practicability of using ozone for grain processing has been documented by previous studies (Kells et al., 2001; Allen et al., 2003; Wu et al., 2006). The US FDA approved the utilization of ozone in aqueous and gaseous phases as an antimicrobial agent for treatment, storage, and processing of foods (Rice and Graham, 2001).

1.4.3. Penetration of ozone through grain

Ozone has a different efficacy against insects inside or outside the grain, mainly because grains can increase the decomposition of O_3 (Işikber and Öztekin, 2009). The reaction of O_3 within a grain can be divided into two phases. The first phase is that O_3 interacts with active sites present in or on the kernel surface, causing the decomposition of O_3. The second phase is the free movement of O_3 through grain layers once reactive sites are saturated, with O_3 concentration gradually increasing to an effective dosage for target pests. The penetration of ozone into the bulk of grains also depends on gas diffusion, air speed within the grain layer, initial ozone concentration, bed thickness, temperature and adsorption by the grain surface (Pandiselvam et al., 2015; Subramanyam et al., 2017a).

Various grains have distinct effects on O_3 insecticidal efficacy, attributed to differences in surface and size of kernels. Ozone is also effective against pests in different grain commodities (Table 1.3). *T. castaneum, O. surinamensis, S. zeamais, S. oryzae* were

exposed to 200ppm ozone for 1, 2, 3, 5, 6, 8, 10 and 12 h with 0 and 10 g of wheat (Subramanyam and Li, 2017b). Five days after ozone treatment, 99% mortality for adults of *Sitophilus* spp., *O. surinamensis* and *T. castaneum* occurred at 2.00-5.56, 4.33-11.18 and 14.35-29.89 h, respectively. For controlling *R. dominica* in wheat, 2.5 g/m^3 of ozone for 8 h killed 97% adults, 100% of pupae and eggs, and 99% of larvae (Mishra et al., 2019). One-hundred percent and 95% of *T. confusum*, *T. castaneum*, *S. zeamais*, and *Plodia interpunctella* adults and larvae in maize were killed when subjected to 50ppm ozone for 3 days (Kells et al., 2001). Thus, the successful application of O_3 against stored grain insects requires sufficient concentration and exposure time, which may in turn affect grain quality.

Table 1.3. Effect of ozone fumigation on insects in stored grain

Commodity	Insects	Ozone treatment	Mortality	Reference
Wheat	*Tribolium castaneum,* *Oryzaephilus surinamensis* *Sitophilus zeamais* *Sitophilus oryzae*	200ppm for 1, 2, 3, 5, 6, 8, 10 and 12 hour assessed for 5 days	Adults: 90% Adults: 100%	Subramanyam and Li (2017b)
Wheat	*Rhyzopertha dominica*	2.5 g/m^3 of ozone concentration and 8 h of treatment	Adults: 97% Pupae: 100% larvae: 99% Egg: 100%	Mishra et al., (2019)
Wheat	*Ephestia kuehniella*	13.9 mg/L of ozone 2 h and at 30 min intervals for 5 h	Larvae: 100% Pupae: 95% Egg: 95% Adult: 100%	Işikber and Öztekin (2009)
Maize	*Tribolium confusum* *Tribolium castaneum* *Sitophilus zeamais* *Plodia interpunctella*	50ppm ozone for 3 d	Larvae: 83% Adults: 92% Adults: 100% Larvae: 95%	Kells et al., (2001)
Maize	*Tribolium castaneum*	50ppm	50% mortality (71.4 h) at 20°C 95% mortality (151.8 h) at 20°C	Pereira et al., (2008)

1.4.4. The effect of ozone treatment on grain quality

Grain quality is a complex concept, which generally refers to the physical condition such as colour, kernel size, test weight, damaged grains, moisture content, immature grain and chemical composition (starch, protein, fatty acid, fibre and mineral content) of the grain. Grain quality presents different definitions in the grain supply chain, including for growers, traders, store managers and consumers.

Colour plays a vital role in consumer acceptance of flour and its products, evaluated by three-dimensional values including lightness, redness and yellowness. The carotenoids and polyphenols in flour are the main factors affecting whiteness. Ozone can oxidize colorants such as carotenoid pigments and polyphenols, increasing whiteness while decreasing the redness and yellowness of flour, depending on the reaction conditions. Sui et al. (2016) found that 20 mg S/L of ozone treatment for 10 min increased the lightness of wheat flour from 93 to 97. Similar observations were recorded by other researchers on other grain flours. The lightness value of corn flour was significantly increased, and the redness and yellowness decreased when exposed to 100 mg/L ozone for 180 min (Qi et al., 2016). The lightness values of sorghum flour significantly increased while yellowness values significantly decreased at 0.06 L/min of ozone treatment for 45 min (Marston et al., 2015).

Starch is a major component of many grains contributing to final grain products. Starch is a complex branched glucose polymer that is comprised of amylose (Yu et al., 2018). The quality of products is determined by their starch properties, such as gelatinization and retrogradation (Zhu, 2018). For barley, kernels with a higher level of starch can produce more beer from a given weight of malt (Rani and Bhardwaj, 2021). Ozone oxidizes the hydroxyl group of starch to carbonyl and carboxyl groups and then depolymerizes starch molecules by cleaving glycosidic linkages (Sivaranjani et al., 2021). The effects of ozone on starch properties depend on the reaction conditions. Wheat kernels were treated with 60 μmol/mol ozone for up to 3 h before milling and

starch extraction, which increased the carboxyl content of starch without affecting the crystallinity and morphology (Savi et al., 2014). Yan et al. (2012) reported that sorghum flour treated with ozone at 0.02 and 0.06 L/min up to 30 min led to both polymerization and degradation of starch.

Fatty acid content is another principal attribute of grain quality. Palmitic acid (16:0) is a major saturated fatty acid , and linoleic acid (18:2) is a major unsaturated fatty acid in most cereals, except for brown rice and oats, which are rich in oleic acid (18:1). Millets are richer in stearic acid (18:0) than other cereals (Chung and Ohm, 2000). Ozone treatment directly influences fat composition, as ozone can react with the double bonds associated with unsaturated fatty acids. Obadi et al. (2018) found that ozone gas (5 g/h, up to 45 min) increased the acid value of lipids in wheat flour. However, there was no effect of ozone treatment on lipid content in wheat kernels (Dubois et al., 2006), as lipids in wheat kernels are much better protected by the grain structure than those in flour.

In addition to those parameters outlined above, grain quality parameters vary depending on the intended use, such as food, feed, and seed purposes. There is a large demand for malting barley, and the moisture content, protein content, and germination rate are important parameters to evaluate malting quality. Moisture content (MC) is crucial to maintain grain quality in storage. High moisture content contributes to increasing mould growth and deteriorating degradation process. Ideally, the MC of malting barley should be less than 13.5% (Fleurat-Lessard et al., 2005). If the MC of harvested malting barley is above 13.5%, it should be dried carefully and slowly to avoid heat damage. Ozone treatment reduces moisture content by oxidizing the free water of grain product (Qi et al., 2016).

Protein is a major component of grain and is determinative of the quality of the final products. For barley, the protein content affects the chemical composition and enzyme

activity of the final malt. Generally, the ideal protein content of malting barley is 11-12.5% (McMillan et al., 2020). Higher protein content can result in lower extracts for the malting barley. On the other hand, low protein content leads to a lack of necessary enzymes to modify the barley kernel and break down the starch during brewing (BMBRI, 2010). Most studies demonstrated that ozone treatment did not affect the protein content of wheat flour and barley flour (Li et al., 2015; Mei et al., 2016).

1.4.5. The effect of ozone treatment on seed germination

Germination ability is a crucial parameter to evaluate grain quality. If a seed loses the capability to germinate, it cannot be planted as a seed or used in malting. Therefore, enhancing seed germination plays a pivotal role in improving crop production. Quality of barley significantly influences its end utilization since the germination rate of barley seed must be more than 95% for the malting process (Shrestha and Lindsey, 2019). Ozone has been regarded as an alternative strategy to solve pest resistance problems to chemical fumigant and ensure food security and quality. It was concluded that 15 g/m^3 for 20 min ozone treatment decreased the incidence of *Aspergillus, Alternaria, Fusarium, Cercospora, Phomopsis fundi,* and *Penicillium* in soybean seeds without damaging the physiological quality (Rodrigues et al., 2019). Consistently, it has been shown that ozone significantly reduced E. *coli* population on alfalfa sprouts without affecting sprout quality (Sharma et al., 2003). It has also been reported that ozone was employed for wheat seed disinfection without altering the seed germination capacity (Marique et al., 2012) and had no adverse effect on the physical and biochemical properties of the seeds.

Studies have suggested that germination and growth parameters of lentil, tomato, corn, wheat, barley, lettuce seeds were enhanced under ozone treatment (Table 1.4). De Souza et al. (2020) demonstrated that the germination rate of lentil was enhanced to 90% after $1g/m^3$ ozone treatment for 3 and 10 min. Similarly, utilization of ozone enhanced the germination rates, as well as the germinating energy in winter wheat seeds (Avdeeva et al., 2018b). The optimal dosage of ozone to improve the germination of seeds and

energy of germination has been standardized as 14.0–17.0 g·s/m^3 doses for 14 days. Sudhakar et al. (2008) evaluated the effect of low concentration of ozone (0.1, 0.2, 0.3, 0.4ppm) on tomato seedling growth. Growth parameters of seedlings involving shoot length, root length, leaf area, dry weight were significantly enhanced when seeds were subjected to 0.2ppm of a short pulse of ozone exposure (2 min/day) for 10 days. Sudhakar et al. (2011) also showed that low ozone concentration (0.01 g/seed) for a moderate time (20 min) was beneficial for promoting the germination of tomato seeds. Consistently, a study indicated that 20 g/m^3 ozone had a positive effect on corn seed germination (Violleau et al., 2007). The potential reason might be attributed to reactive oxygen species (ROS), which are elicited by O_3. ROS production has been regarded as a detrimental factor for damaging cell structure. In contrast, some researched revealed favourable roles of ROS in seeds. The role of ROS in cell growth and plant development has been documented by previous studies, which indicated that ROS promote cell growth by stimulating cellulose synthases (Gapper and Dolan, 2006). Additionally, ROS, as the cellular signalling, break seed dormancy by regulating the hormone levels, particularly abscisic acid (ABA). Dormant barley seeds which were subjected to hydrogen peroxide presented the reduction of endogenous ABA (Wang et al., 1998b). In alpine plants, chronic exposure to O_3 (125ppm for 10 days) enhanced seed germination compared to the control, suggesting that ozone may induce antioxidant and DNA-repair mechanisms or dormancy-breaking effects in hydrated seeds (Abeli et al., 2017). Monroy Vazquez et al. (2017) investigated seed germination and seedling growth of scarified seeds in response to sublethal ozone doses. The result suggested that in *Opuntia* spp., O_3 can be used in sublethal doses to increase seed germination and seedling development.

Conversely, some work showed different results. A study analyzed morphological characteristic features and germination rate of soft spring seeds under ozone treatment. In spite of severe morphological alterations in the seedlings after ozone treatment, the germination of the seeds was not affected (Alexander et al., 2018). Consistently, it was

concluded that the sanitary treatment with ozone (25 g/m^3) for 120 min reduced the incidence of fungi and maintained the seed vigor and germination at the same level as control (Rodrigues et al., 2019).

Additionally, some reports suggested that the germination of wheat seeds had been significantly reduced after high concentration and/or prolonged exposure to ozone gas. A study investigated the effect of gaseous ozone as an anti-fungal fumigant for stored wheat. It suggested that applying different ozone doses at 0.016, 0.065, 0.16 and 0.33 mg/g wheat/min, no effect on wheat germination was observed even after 60 min of ozonation. However, when the concentration reached 0.98 mg/g wheat/min, the germination rate was reduced by ozonation for more than 20 min, resulting in losses in seeding (Wu et al., 2006). Thus, the disinfection of fungi could be achieved by applying ozone doses below the threshold. The work of Savi et al. (2014) also presented a similar result. High exposure to O_3 gas (180 min at 60 μmol/mol) impacted germination of wheat seed, reducing germination capacity by 12.5%. Overall, research indicated that mild and limited concentrations of ozone enhanced seed germination rate, while excess ozone could have adverse effects. For fumigation purposes, concentrations below that which would adversely affect the germination rate is enough for fungal disinfection. Nonetheless, many studies have demonstrated that ozone could promote seed germination to alleviate dormancy, whereas limited knowledge of metabolism alternation is still a major gap in our understanding of seeds' response to ozone treatment.

Table 1.4. Effect of ozone on seed germination and growth parameters

Plants	Concentration	Period	Effect	References
Tomato	0.2ppm	2 min/day, 10 days	Positive effect on growth parameters of seedling involving shoot length, root length, leaf area and dry weight	Sudhakar et al., (2008)
Corn	20 g/m^3	6.8-20.5 min	Increase seed germination rate	Violleau et al., (2008)
Barley	25 mg/cm^3	120 min	Promote seed germination	Ma et al., (2020)
Lentil	1 g/m^3	3 or 10 min	Increased the seed germination to 90%	De Souza et al., (2020)
Cantaloupe	4.3 mg/L	320 s	Non-significant reduction of germination and sprout vigour	Trinetta et al., (2011)
Opuntia spp.	0.58 mg/L	1 min	Improve the seedling development and seed germination rate irrespective of the species	Monroy-Vázquez et al., (2017)
Winter wheat	14.0–17.0 $g{\cdot}s/m^3$	14 days	Improving the germination, the germinating energy	Avdeeva et al., 2018
Alfalfa	5 mg/L	20 min	No negative effects on germination mass and color of alfalfa sprouts.	Mohammad et al., (2019)
Alfalfa	20 mg/mL	5 or 10 min	Sprout quality remains unaffected	Wade et al., (2003)
Wheat seeds	0.98 mg/g wheat	20 min	Germination was reduced to 85.4%	Wu, et al., (2006)
		30 min	Germination was reduced to 80.0%	
		45 min	Germination was reduced to 61.3%	

1.5. Metabolic profiling of grain

1.5.1. Primary metabolites in grain

Germination begins when the seed absorbs water and ends with the appearance of the radical, which can be classified into three phases: imbibition, activation, and visible germination. The most critical phase is activation because essential physiological and biochemical processes such as hydrolysis, macromolecules biosynthesis, respiration, subcellular structures, and cell elongation are reactivated for initiating germination (Bewley et al., 2012), accompanied by changes in hundreds of metabolites. As the end products of gene and protein regulatory processes, metabolites represent the ultimate response of biological systems to both genetic and environmental changes. Therefore, the final changes in metabolite levels are potentially more helpful in understanding the biochemical reaction of plants to environmental stress.

Dynamic changes in metabolites during germination have been reported to be associated with the mobilization of polysaccharides, proteins, organic acids, and lipids (Han et al., 2017). According to the literature, these seed reserve contents are associated with germination percentage (Soriano et al., 2014). A study investigating *Medicago truncatula* indicated that soluble sugar (such as glucose and sucrose) has a positive effect on germination percentage of seeds (Vandecasteele et al., 2011). Proteins, a storage reserve for nitrogen and carbon, are accumulated in developing seeds. These proteins are rapidly hydrolyzed to supply nitrogen during germination (Millerd, 1975). Wahid and Bounoua (2013) demonstrated that germination percentage of *Pinus pinaster* seed was promoted with increasing protein content. During seed germination, fatty acids released through lipid mobilization were degraded through the β-oxidation and glyoxylate cycles, and subsequently converted into sugars (Graham, 2008). It has been suggested that germination intensity was positively correlated with fatty acid content (Świeca et al., 2012). Starch, the most abundant carbohydrate reserve in plant seeds, serves as the primary source of carbohydrate during germination and seedling

growth. There was a close relationship between starch content and the germination time in watermelon seeds, but no correlation with the final germination percentage (Wang et al., 2011).

1.5.2. Secondary metabolites in grain seeds

Secondary metabolites such as flavonoids and volatile organic compounds (VOCs) are not directly involved in normal growth, development, and reproduction, but play an important role in corresponding with these processes. Functional VOCs not only contribute to the odour of food but have pivotal roles in plant communication, increasingly recognized as an essential factor in the ecology of natural and agricultural systems (Ninkovic et al., 2016). For example, some VOCs inhibit seed germination and seedling growth of neighbouring weedy plants to reduce competition (Romagni et al., 2000). Plant VOCs are also important in relieving both biotic and abiotic stresses, which can result in the production of ROS and cause cell damage. According to the literature, isoprene, monoterpenes, sesquiterpenes and green leaf volatiles were demonstrated to mitigate oxidative stress (Vickers et al., 2009; Stolterfoht et al., 2019). They have been reported to induce pl1ant defences to stress and allow plants to respond faster to subsequent stresses (Cofer et al., 2018). Certain VOCs can also modulate plant growth. Alcohols, aldehydes and volatile fatty acids can increase seed germination in red rice (Cohn and Hughes, 1981). Ethanol breaks dormancy by promoting the tricarboxylic acid cycle (TCA cycle) and glycolysis (MIYOSHI and SATO, 1997). Some ketones, such as 2-nonanone and 2-undecanone were reported to stimulate germination of *Lactuca sativa* seeds (Fincheira et al., 2017). Acetic acid, mainly produced through the catabolism of precursor compounds, promotes the germination ability of stored wheat seeds and facilitates seed development in maize (Dylan and Michael, 2020). Terpenes (alkene chemical class) are known to inhibit seed germination and growth of annual plants. Phenols were reported to inhibit legume seed germination by accumulating in the seed-coat during development and remain after dispersal or harvest (Smýkal et al., 2014).

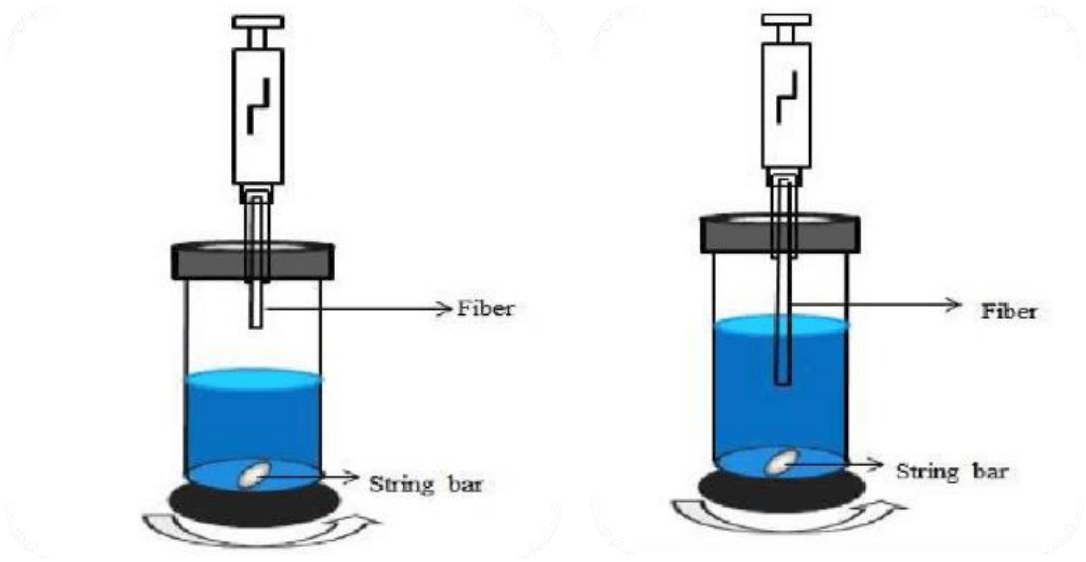
1.5.3. Method for barley metabolite profiling

Metabolite profiling assesses the biochemical composition of complex matrices based on extraction, detection, identification and quantification of a broad spectrum of metabolic compounds by gas chromatography-mass spectrometry (GC–MS), liquid chromatography-mass spectrometry (LC-MS), or nuclear magnetic resonance (NMR) spectroscopy. As a valuable tool to analyse substrates and products in various metabolic pathways, the metabolomic approach has also been utilized in grain research.

Metabolomic studies related to wheat and barley grains are summarized in Table 1.5. Some works focused on metabolic changes during the malting process of barley (Frank et al., 2011; Gorzolka et al., 2012), metabolite profiling of naturally contaminated barley grain (Perkowski et al., 2012), characterisation of volatile aroma compounds in different barley cultivars (Dong et al., 2015b), and linking genetic and environmental factors with the seed phenotype (Khakimov et al., 2017).

Regardless of whether the method involves LC or GC, metabolite analysis in complex barley matrix requires several clean-up steps before sample analysis. In the past, metabolic profiling of lipids and other volatile compounds largely depended on complicated solvent extraction techniques, like liquid-liquid extraction (LLE) (Lafarge and Cayot, 2019), stir bar sorptive extraction (SBSE) (Baltussen et al., 1999), and solid-phase extraction (SPE) (Thurman and Mills, 1998). However, these techniques have limitations, comprising harmful organic solvents and expensive devices with a limited lifetime, along with cross-contamination problems. Solid-phase microextraction (SPME), developed by Arthur and Pawliszyn et al. (1990), has been regarded as a rapid and simple technique in sample extraction and pre-concentration for further volatile analysis. Specifically, SPME integrates sampling, extraction, concentration, and injection into a single process, achieving high sample throughput (Gonçalves et al., 2014). The extraction of samples can be divided into two approaches (Figure 1.5). The first is headspace SPME (HS-SPME), introduced in 1993 (Zhang and Pawliszyn, 1993)

and preferred for volatile or semi-volatile compound analysis. As shown in Figure 1.5A, the polymeric film is exposed to the gas phase adsorbing volatiles and semi-volatiles in the headspace of the liquid, gas, or gaseous metabolites. Compounds that equilibrate between the sample and the headspace are trapped by the fibre coating, which lead to purer extracts and greater sensitivity. Additionally, the HS-SPME method significantly increases the life span of the sorbent since the fibre coating does not contact the sample matrix (Mills and Walker, 2000). For the extraction of molecules with low volatility, direct immersion SPME (DI-SPME) is a technique, in which the fibre is directly immersed into the sample matrix (shown in Figure 1.5B) (Al-Khshemawee et al., 2018). Whatever the technique, the performance of the SPME method is largely influenced by fibre coating, which is directly related to the extraction efficiency. These sorbents are suitable for applications of SPME in a wide range of non-polar and polar organic compounds.



(A) (B)

Figure 1.5. Modes of (A) headspace solid phase microextraction, (B) DI-SPME

The different fibre materials offer properties for absorbing compounds with different polarities. Currently, the different commercial coating used for SPME analyses include polydimethylsiloxane (PDMS), polyacrylate (PA), divinylbenzene (DVB), carboxen

(CAR), and carbowax (CW) (Jalili et al., 2020). The materials have also been combined, such as three phase (divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibres to give a broader range of polarities than the single one. After sample extraction, analysis is undertaken with LC- or GC-MS for compound separation and identification. Recently, SPME was performed to detect volatile compounds in combination with GC–MS to identify volatiles from food, environment, insects and plants (Jalili et al., 2020).

Table 1.5. Metabolomics used for grain analysis

Sample	Purpose of analysis	Type	Extraction and preparation	Separation-detection	Metabolites reported	Reference
Barley grain	Metabolite fingerprinting of barley whole seeds, endosperms, and embryos during industrial malting	Untargeted	Derivatization for GC-MS	GC-MS	73	Gorzolka et al., 2012
Barley grain	Differences in metabolomic profiles of the naturally contaminated grain	Untargeted	HS-SPME	GC-MS	46	Perkowski et al., 2012
Barley grain	Utilize GC-MS metabolomics to link genetic and environmental factors with the seed phenotype	Untargeted	Derivatization for GC-MS	GC-MS	104	Khakimov et al., 2016
Barley grain	Characterization of volatile aroma compounds in different barley cultivars	Untargeted	HS-SPME	GC-MS	41	Dong et al., 2014
Barley grain	Spatio-Temporal Metabolite of salt stressed barley seeds during germination	Untargeted and targeted	acetonitrile/water (75:25; v/v)	MALDI-MSI; LC-QToF-MS)	234	Gupta et al., 2019
Barley grain	Investigate time-dependent metabolic changes of the malting process of barley	Untargeted and targeted	water (75:25; v/v)	GC-MS/ GC-FID	173	Frank et al., 2011

1.6. Research Gaps

Ozone is a naturally occurring substance. As a disinfestation agent, it has distinct advantages, such as being highly toxic to a broad range of microorganisms and rapid auto-decomposition to oxygen without leaving residues, making it an attractive approach for medical sterilization, food processing and grain storage. There were discrepancies in the effect of O_3 on grain quality when the dosage of O_3 were sufficient for the eliminating insect pests in grain. These discrepancies might be mainly caused by the difference of dose and duration and grains. The knowledge gaps we will address are:

A. Two coleopteran insect pests *Rhyzopertha dominica* and *Tribolium castaneum* are major pests of stored grain in Australia. Current control measures including phosphine fumigation and non-chemical method have limitations. Phosphine is the widely used fumigant, however, continuous use has led to the evolution of resistant populations and environmental contamination. Non-chemical methods are generally costly for commercially treatment of large-scale bulk grain or need long-term treatment. The effects of O_3 application on these species is unknown for the barley storage present in Australia. Additionally, the effects of ozone application on barley quality and germination is unclear.

B. Enhancement of seed germination plays a pivotal role in improving crop production, along with shortening manufacturing costs of malting process. Ozone can promote seed germination, but how ozone affect barley metabolites profile to enhance germination is not clear.

C. VOCs have pivotal roles in cell signaling and seed germination. Despite numerous studies that have analyzed VOCs from barley seeds to elucidate its aroma components, the volatile metabolite profile of barley under ozone treatment is still unknown.

D. Researchers focused on protein, transcripts and primary during germination of barley, but the potential biomarkers of barley germination are still unknown. In

addition, metabolites changes of germinating seeds in response to ozone treatment remain elusive.

1.7. Research Aims

This research aims to investigate stored grain insects and barley responses to ozone exposure, offering new insights into utilizing ozone in grain storage and supplying information for grain processing. Specifically, the main goals are as follow:

- Provide an overview of ozone application, challenges of the grain supply chain, and the metabolic profiling of grain (Chapter 1).
- To understand ozone efficiency on *Rhyzopertha dominica* and *Tribolium castaneum* in barley and how ozone affects germination, physiology, and quality of barley (Chapter 2).
- To understand how barley VOCs responds to ozone treatment based on optimised solid-phase microextraction (SPME) combined with gas chromatography-mass spectrometry (GC–MS). VOCs that play a possible role in ozone-response will be highlighted. (Chapter 3)
- To identify potential biomarkers of seed germination through metabolic profiling and provide novel insights into the biochemical mechanisms of barley seed germination at the metabolic level, especially when subjected to ozone treatment. (Chapter 4)

Attribution Statements

The following chapter has been drafted in accordance with the "*Insects*".

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The following authors contributed to this manuscript as outlined below.

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Name 3 Xiangyang Yu	5	X		X	
Name 4 Garth Maker	5	X		X	
Name 5 Yu Xiao	5	X		X	
Name 6 Yonglin Ren	5	X		X	

Contribution indicates the total involvement the author has had in this project. Placing an 'X' in the remaining boxes indicates what aspect(s) of the project each author engaged in.

By signing this document, the Candidate and Principal Supervisor acknowledge that the above information is accurate and has been agreed to by all other authors.

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Candidate **Principal Supervisor**

Chapter Two

Efficiency of ozone on two coleopteran insect pests and its effect on quality and germination of barley

Abstract

Storage pests cause considerable grain losses. Ozone (O_3) is a potential fumigant to control pests in stored grain since it can safely and rapidly auto-decompose without leaving residues. In this study, an O_3 purging flow system was used to ensure that insects and barley were exposed to continuous and consistent ozone treatment. The efficacy of O_3 on all life stages of *Rhyzopertha dominica* (lesser grain borer) and *Tribolium castaneum* (red flour beetle) in barley and physiological effects on barley and its quality were investigated. Complete control of all life stages of *R. dominica* and *T. castaneum* in barley was obtained at 700ppm O_3, 24±1°C and 55-60% RH for 24 h exposure without negatively impacting soluble protein, moisture content, seed colour and hardness and the thousand weight of barley seeds. Eggs and pupae of these two insect species were more tolerant than larvae and adult stages. Prolonged exposure times (40 to 1440 min) and mortality assessment intervals (1, 2 and 7 days) increased O_3 efficacy due to the reaction characteristics and delayed toxicity. Aging barley seeds appeared more sensitive to prolonged ozone duration than new seeds. 20 and 40 min could promote the germination rate, while longer O_3 exposure (1440 min) was unfavourable for germination and seedling growth. Thus, it is imperative to select an optimal O_3 exposure time to transfer ozone into quality contributors of final products and achieve the desired outcomes.

2.1. Introduction

Postharvest loss has become a crucial issue of the grain supply chain. It has been estimated that one-quarter to one-third of the world's grain crop is lost every year during storage, with the majority attributed to insect attack (Mesterházy et al., 2020). The lesser grain borer, *Rhyzopertha dominica,* and the red flour beetle, *Tribolium castaneum,* are notorious global pests, destroying various stored grains, including barley, wheat, oats, maize and rice. Both species can cause severe economic loss by decreasing the quantity and quality of stored grain. As a primary grain insect, *R. dominica* has the ability to attack whole kernels, while *T. castaneum* is a secondary pest and can only attack damaged grain, dust and milled products (Shankar and Abrol, 2012). Fumigation is often the cheapest and most effective process available, playing a major role in preserving commodities (Banks, 1989), including elimination of grain storage insect pests. In actual practice, however, most gases have been eliminated owing to unfavourable properties, the most important being chemical instability and destructive effects on commodities (Bond and Monro, 1984). Methyl bromide is a fast-acting fumigant, but it has been removed from fumigant registration because it caused depletion of the ozone layer. Phosphine is a widely used fumigant, however, continuous use has led to the evolution

of resistant populations and environmental contamination (Nayak et al., 2020). *R. dominica* was the first pest with strong resistance to phosphine recorded in Australia (Collins, 2003). Nayak analyzed 20 years of resistance data in Australia, suggesting that resistance to phosphine had increased significantly over this period and is currently between 60–80% (depending on species). In North America, there was a significant increase in the frequency of resistance in both *T. castaneum* and *R. dominica* over two decades (Cato et al., 2017; Gautam et al., 2016). Opit et al. (2012) suggested that the most resistant *T. castaneum* population was 119-fold more resistant than the susceptible strain and the most resistant *R. dominica* population was over 1500-fold more resistant in North America. Strongly resistant populations of *T. castaneum* and *R. dominica* were also reported in Asia, including China (Huang et al., 2019; Song et al., 2011), Pakistan (Wakil et al., 2021), and India (Rajendran et al., 2001; Rajendran, 2018).Ethyl formate is an alternative fumigant, but it has high water solubility which could be rapidly absorbed by high moisture content commodities. It has been registered as a fumigant for treatment of dried fruit in Australia (Finkelman et al., 2010). Diatomaceous earths are used to control stored grain, causing insects death through absorbing the epicuticular lipids of the insect cuticle, but it has a negative effect on the physical properties of grains, particularly bulk density (Athanassiou et al., 2006; Korunic et al., 1998; Ziaee et al., 2021). Controlled atmosphere with inert gases such as CO_2 is an alternative non-chemical method to control grain pests, however, it requires long exposure times (more than 10 days) (Kumar et al., 2022). Entomopathogenic fungi (EF) are considered the most promising biocontrol agents, which infect insects through mycelium penetrating into the insect's cuticle and then growing in the haemocoel causing insects death (Batta and Kavallieratos, 2018). However, there are no commercial biopesticides based on EF bioagents that are registered against the stored-grain insects. The main limitations of the application of EF in insect control are the high moisture requirement for conidial germination and subsequent sporulation, more time consuming to kill insects, and the potential risk to immunodepressive or immunocompromised people (Singh et al., 2017; Rumbos and Athanassiou, 2017). Other non-chemical methods such as cold or low temperature treatment, irradiation, and controlled atmosphere have also been reported to eliminate insects in stored grain, however, they are costly for commercially treatment of large-scale bulk grain or require long-term treatment (Navarro, 2012; Carocho et al., 2014; Sutar et al., 2021). Therefore, alternative methods are needed to control grain storage pests.

Ozone (O_3) could overcome deficiencies of other fumigants. Ozone occurs naturally in the atmosphere and could be generated on-site without transportation problems. As described in Chapter 1, ozone has distinct advantages over conventional fumigants. There has been growing

emphasis on the application of O_3 to control *R. dominica* and *T. castaneum.* Sousa et al. (2008) reported that 150ppm gaseous O_3 presented high efficacy against both susceptible and phosphine-resistant insects from 16 populations of *T. castaneum* and 11 populations of *R. dominica.* Ozone was also lethal to different stages of *R. dominica* and *T. castaneum* (McDonough et al., 2011; Mishra et al., 2019). Ozone was effective against insects inside or outside the grain, mainly because grain surfaces and interiors promote increase the decomposition of O_3 (Isikber and Athanassiou, 2015). The reaction of O_3 within a grain can be divided into two phases. The first phase is that O_3 interacts with active sites present in or on the kernel surface, causing decomposition of O_3. The second phase is the free movement of O_3 through grain layers once reactive sites are saturated, with O_3 concentration gradually increasing to effective dosage for target pests. Various grains also have distinct effects on O_3 insecticidal efficacy, which is attributed to differences in surface morphology and size of kernels.

The successful application of O_3 against stored grain insects requires sufficient concentration and exposure time, however it may affect germination and other quality parameters. Zhu (2018) indicated that moderate ozone treatment could facilitate dough strength, and enhance storage stability and whiteness of wheat flour. Malting quality of barley, such as α-amylase, fine grind extract, dynamic viscosity, and soluble protein were not impacted by ozone treatment of 26 mg/g (Dodd et al., 2011). Conversely, as a strong oxidant, O_3 can react with some substrates in grains directly or via producing reactive oxygen species. Starch and lipid oxidation, protein modifications, grain discolouration, odour alteration and germination loss may result from excessive use of O_3 (Tiwari et al., 2010).

There were discrepancies in the effect of O_3 on grain quality when the dosage of O_3 was sufficient for eliminating insects pests in grain (Tiwari et al., 2010). These discrepancies might be mainly caused by the differences of dose, duration, and grains. While some studies have assessed the efficacy of O_3 against pests in maize and wheat, relatively few studies have focused on the toxicity of O_3 on stored grain pests with barley. Thus, this study aims to explore the susceptibility of all life stages of *R. dominica* and *T. castaneum* in barley seeds to different durations of gaseous O_3, and to investigate the effect of gaseous O_3 on germination ability, seedling growth, and quality of barley with different storage times. Additionally, the different response of old and new barley seeds that were subjected to ozone treatment was also examined.

2.2. Materials and methods

2.2.1. Insects

There were two insect species of stored product insects that were used for bioas-says, which were *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and *Rhyzop-ertha dominica* (F.) (Coleoptera: Bostrychidae). *T. castaneum* strain MUWTC-6000 and *R. dominica* strain MUWRD-7 were the phosphine susceptible, held at the Postharvest Biosecurity and Food Safety Laboratory, Murdoch University, Australia. The narrow aged insects (2-3 days) were obtained by incubating 3000 adult insects with 1000 g of food—broken wheat for *R. dominica* and wheat flour/yeast 12:1 ratio for *T. castaneum* in 2 L jars sealed with mesh lids. The parent insects were removed after 3 days and the remaining culture medium were incubated in 2 L glass jars at 28 ± 1°C and 70 ± 2% relative humidity (RH) with continuous darkness. The newly emerged adults were narrow aged and transferred to a 2-L jar containing fresh food. The insects used in the experiments were one month old.

2.2.2. Barley samples

Barley varieties Spartacus CL with 12.3% and 10.9% moisture content were harvested in 2013/2014 and 2019/2020 from Lake Grace, Western Australia. The sample that was harvested in 2013/2014 was stored at 4°C for 6 years. In the grain production business, the long-term storage of grain often occurs due to the relatively limited demand for consumption, cultivation, unexpected meteorological disasters, and also to prevent a change in specific characters. The moisture content of the barley was determined with a Graintec HE 50 electronic moisture meter (Graintec Pty Ltd, Toowoomba, Australia). The results obtained were calculated from four replicate measurements. The insect-free samples were stored at 4℃ and equilibrated to room temperature before germination tests and quality measurements were conducted to room temperature at 25 ± 1°C.

2.2.3. Fumigation procedures with ozone

A commercial O_3 generator (Model FH-CYJ1520A-20g/h, Shanghai Fenghua Optoelectronics Technology Co. Ltd., Shanghai, China) was utilised to treat barley seeds, continuously produceding 700ppm O_3 from atmospheric air. Ozonation of barley seeds was carried out in a 2.4 L and 16.6-cm diameter Hysil semi-batch reactor with a glass desiccation chamber fitted with a lid containing a 2-cm central hole for ozonation (Figure 2.1). Gas was introduced from the ozone generator directly to the bottom of the reactor containing 1 kg of barley and passed through the seeds using a 1-cm diameter plastic tubing. Prior to introduction of O_3, barley seeds were mixed with 3 g adults (approximately 2,000) and 7 g egg, pupae and larvae were placed

on a supporting metal mesh with 13-cm diameter, 5 cm from the bottom to enable gas circulation. The ozone concentration to and from the reactor was continuously measured by an ozone monitor (Shenzhen Yuan Technology Co., Ltd., Shanghai, China). Nine treatments were designed for this study, comprising control (fresh air passed through the glass chamber), 700ppm O_3 exposure for 0, 10, 20, 40, 120, 240, 480, 960 and 1440 min, with three replicates. All treatments were performed in ambient conditions at 20 ± 5℃ and 55 ± 3% RH.

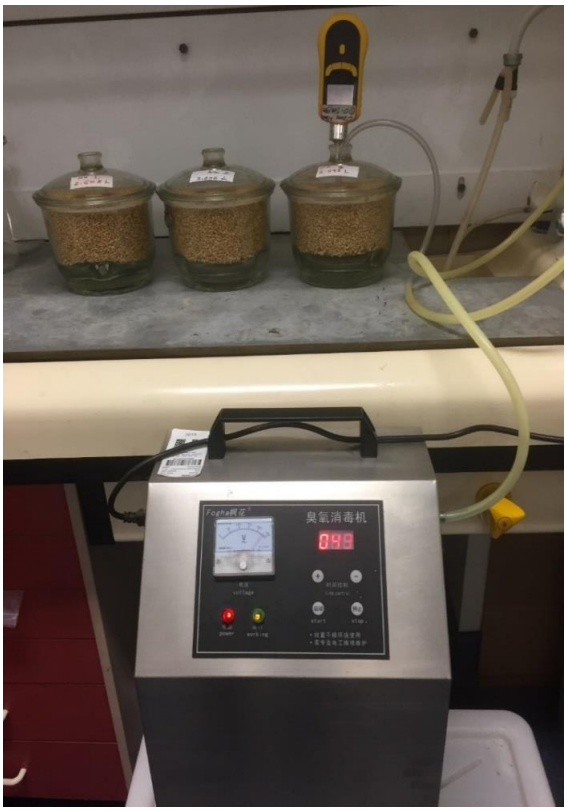

Figure 2.1. Fumigation procedures with ozone.

2.2.4. Assessment of insect mortality

Bioassay samples, all adults, larvae, pupae and eggs of treated and untreated control were retrived at the end of the fumigation period from the treated and untreated barley with 710 and 180 μm sieves. Adults were removed and placed into 250 mL vials with food and incubated at 28 ± 1°C and 70 ± 2% RH. The live and dead adult insects were counted at different assessment intervals, including immediately (0DAT), 1, 2 and 7 days after O_3 treatment (1DAT, 2DAT, 7DAT). The remaining mixed-age cultures were incubated at 28 ± 1°C and 70 ± 2% RH. Subsequent emerging adult insects were counted weekly for a period of 5 weeks, with live and dead adults removed at each count. The mortiality was calculated based on comparison of

emerging adults between treated and untreated control samples. Once all control insects emerged, the experiment was terminated.

2.2.5. Germination test

The germination test was carried out based on the between-paper (BP) method of the International Seed Testing Association methods (ISTA, 2006). Four hundred and twenty-four seeds (eight replicates of 53 seeds) in each harvest year were randomly selected. A large filter paper was saturated with 60 mL distilled water and folded in half. A steel template (290×580 mm) was placed over this wet paper, with holes in this stencil allowing barley seeds to be placed 30 mm apart. Once the seeds were positioned correctly, the template was removed and the upper half of the filter paper was folded over the seeded area. The folded paper containing the seeds was then loosely rolled from one side perpendicular to the base and tied with elastic bands. All treatments were stored individually in polythene zip-lock bags and incubated for 7 days at 25 ± 1°C in the dark.

The germination rate was detected 7 days after the start of the germination test. Morphological characteristics were tested, including the length of the shoot and root, and the ratio of root to shoot length on Day 7. Seedling vigour index was calculated using the below formula (Eq. 1) according to Islam et al. (Islam et al., 2009).

$$\mathbf{Y = (a + b)\, c/100} \qquad \textbf{Eq. 1.}$$

Where: Y is Seedling Vigour Index (SVI)

a is mean root length (cm)

b is mean shoot length (cm)

c is the percentage of germination rate (%)

2.2.6. Grain quality measurement

Duplicate barley samples were randomly taken from each treatment for quality measurement. A near infrared range (NIR) instrument (Infratec™ 1241 Grain Analyser, Foss, VIC, Australia) was utilized for analyzing grain quality. The functioning of this analyzer is based on the

measurement of transmission spectra of samples in the near-infrared region. The instrument had an extended wavelength range of 570–1100 nm. Ten subsamples of whole kernels were scanned for each grain sample. The parameters we assessed in this research including total soluble protein content, Moisture content (MC), colour, hard-ness and thousand weight (TW) of grain. Colour results were reported as lightness, which was determined by L* values (0 = black and 100 = white). TW was estimated by weighting 1000 seeds in gram.

2.2.7. Statistical analysis

Data from time-response bioassays were subjected to Probit analysis (IBM SPSS Statistics, version 25.0). Time-mortality curves and LT_{50} and LT_{95} were generated. A one-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test were employed to evaluate if there was a significant difference between the O_3 treatments and control. The results were considered significant only when $p \leq 0.05$. Origin 2019b was used for curve figures.

2.3. Results

2.3.1. Toxicity of ozone on *R. dominica* and *T. castaneum* adults at different assessment intervals

The mortality rate of both insect species increased with prolonged O_3 exposure time and assessment interval (Figure 2.2). For *R. dominica*, the mortality rate significantly increased with extended O_3 exposure time. Adults of *R. dominica* and *T. castaneum* in barley were knocked down imediately at 700ppm with 2 h exposure, but 90% of them recovered after treatment, however 1440 min O_3 treatment killed 92% of adults immediately, 97% at 1 DAT and 100% at 2 and 7 DAT (Figure 2.2a). *T. castaneum* displayed a similar tendency when subjected to different O_3 exposure times and assessed at intervals after O_3 treatment. The mortality rate was 81% at 0 DAT, and increased to 95% at 1DAT, and 100% at 2 and 7 DAT when subjected to 960-min treatment (Figure 2.2b). Time–mortality regression for the populations of *R. dominica* and *T. castaneum* exposed to O_3 are represented in Table 2.1. A total of 700 ppm ozone of each treatment time (0, 40, 120, 240, 480, 960, and 1440 min) for the two species was conducted. The Pearson goodness-of-fit Chi-square (χ^2) test showed that the probit model fit to data was significant ($p<0.05$), thus, a heterogeneity factor (χ^2/df) was used in the calculation of confidence limits. At 700ppm O_3, the median lethal time (LT_{50}) of *R. dominica* on 0 DAT was 472.91 (343.04-653.55) min and that of *T. castaneum* on 0DAT was 453.07 (228.14-769.72) min. The LT_{90} values for *R. dominica* and *T. castaneum* on 0DAT were 2419.93 (1465.95-6336.68) and 1823.12 (972.31-27304.4) min, respectively. The data

indicated that *R. dominica* was more tolerant to O_3 treatment. LT_{50} and LT_{90} of both insects at Additionally, the LT_{50} and LT_{90} of both insects at O_3 treatment dramatically decreased on 1DAT, 2DAT, and 7DAT compared with 0DAT. The LC_{50} of *R. dominica* and *T. castaneum* on 7DAT were the lowest, which were 213.50 and 63.60 min, respectively. The relative potency for *R. dominica* and *T. castaneum* was 2.22 and 7.12, respectively. Our results indicated the delayed toxicity of O_3 and that it is better to evaluate the O_3 efficacy against these two species more than 1 day after treatment.

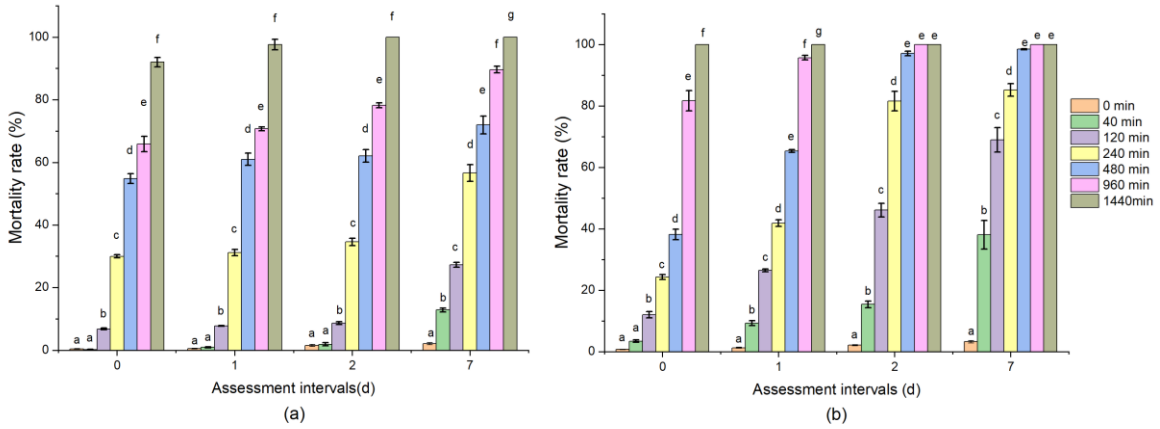

Figure 2.2. The mortality rate of (a) *Rhyzopertha dominica* and (b) *Tribolium castaneum* of different O_3 exposure times 0, 1, 2 and 7 days after O_3 treatments. Bars with different letters are significantly different (Tukey's HSD test, $p<0.05$).

Table 2.1. Regression analysis for mortality rate of *Rhyzopertha dominica* and *Tribolium castaneum* at different assessment intervals. 700ppm ozone of each treatment time (0, 40, 120, 240, 480, 960 and 1440 min) for two species were conducted.

Insects	DAT †	Slope ‡ ± SE	Intercept ± SE	Relative Median Potency *	LT_{50} (95% CI §) (min)	LT_{95}(95% CI) (min)	χ^2 ¶	df
R. dominica	0	2.32 ± 0.05	−6.21 ± 0.15	1.00	472.91 (343.04–653.55)	2419.93 (1465.95–6336.68)	118.02	6
	1	2.48 ± 0.06	−6.48 ± 0.15	1.15	410.12 (278.81–587.64)	1887.41 (1130.49–5594.71)	162.50	6
	2	2.74 ± 0.06	−7.01 ± 0.17	1.25	378.37 (265.87–515.29)	1510.96 (976.73–3617.63)	138.20	6
	7	2.06 ± 0.05	−4.80 ± 0.12	2.22	213.50 (146.17–291.40)	1342.06 (843.67–3033.99)	95.58	6
T. castaneum	0	2.72 ± 0.07	−7.23 ± 0.20	1.00	453.07 (228.14–769.72)	1823.12 (972.31–27304.4)	322.13	6
	1	2.31 ± 0.06	−5.54 ± 0.14	1.80	252.04 (143.69–378.75)	1300.80 (747.48–4884.78)	198.01	6
	2	2.85 ± 0.07	−5.88 ± 0.16	3.91	115.79 (92.92–139.38)	437.12 (341.45–620.62)	40.70	6
	7	2.30 ± 0.07	−4.15± 0.14	7.12	63.60 (49.06–78.09)	329.40 (256.77–463.99)	27.22	6

† Day after O_3 treatment

‡ Mean ± Standard Error

* Relative potency = LC_{50} of 0 DAT/LC_{50} of another DAT (1, 2, 7);

§ Confidence Interval

¶ Chi-square value for goodness-of-fit of Probit model to data

2.3.2. Toxicity of ozone duration on all life stages of *R. dominica* and *T. castaneum*

The mortality rate of all life stages for *R. dominica* and *T. castaneum* exposed to different O_3 durations is summarised in Table 2.2. Based on our preliminary study, 10 and 20 min O_3 exposure did not kill the insects, so O_3 duration longer than 40 min was investigated. 1440 min O_3 treatment could kill all stages of both species. The mortality rate of *R. dominica* adults significantly increased with extended O_3 durations and reached 89.62% at 960-min exposure ($F_{5, 12}$=29.334, p<0.001), while *T. castaneum* reached 91.39% at 240-min exposure and 100% at 960-min treatment ($F_{5, 12}$=209.197, p<0.001). The mortality rate of both *R. dominica* and *T. castaneum* eggs, larvae and pupae also considerably increased with extended O_3 durations and reached 83.33%, 91.67%, and 71.30% at 960-min exposure, while those of *T. castaneum* reached 81.11%, 100%, and 93.33% at 960-min treatment, respectively. In Table 2.2, the different letters in each life stage indicate significant mortality differences of the ozone treatment times between the two species. The results indicate that except for the egg stage, *T. castaneum* was more sensitive to the O_3 treatment than *R. dominica*. Considerable variation was observed in susceptibility among all the life stages of *R. dominica* and *T. castaneum*. In terms of *R. dominica*, the adults and larvae were more susceptible to the O_3 treatment than the pu-pae and eggs. The adults and larvae achieved 86.92% and 91.67% mortality with 960 min O_3 treatment, however the pupae and eggs reached 74.30% and 83.33% mortality. Regarding *T. castaneum*, the observed mortality was 100% for the larvae and adults and 93.33% for the pupae, compared to 81.11% for the eggs with 960 min exposure.

Table 2.2. Efficacy of O_3 treatment duration at 700ppm on all life stages of *Rhyzopertha dominica* and *Tribolium castaneum.*

Life stage	Duration of O_3 treatment (min)	*Rhyzopertha dominica*	*Tribolium castaneum*
		Mortality rate ± SE (%)[†]	Mortality rate ± SE
Eggs	0	0.00 ± 0.00 g	1.67 ± 1.67 g
	40	18.33 ± 3.33 fg	17.92 ± 1.5 fg
	120	38.33 ± 3.33 ef	33.33 ± 3.85 ef
	240	68.33 ± 4.41 bcd	49.01 ± 5.21 de
	480	70.63 ± 5.23 bc	61.52 ± 9.34 cd
	960	83.33 ± 6.01 ab	81.11 ± 2.22 abc
	1440	100.00 ± 0.00 a	100.00 ± 0.00 a
Larvae	0	3.33 ± 1.92 g	3.70 ± 0.93 g
	40	26.67 ± 3.33 f	25.56 ± 2.94 f
	120	53.33 ± 6.67 e	51.38 ± 2.01 e
	240	60.42 ± 5.51 de	82.88 ± 1.93 bc
	480	70.83 ± 2.08 cd	99.67 ± 0.33 a
	960	91.67 ± 2.08 ab	100.00 ± 0.00 a
	1440	100.00 ± 0.00 a	100.00 ± 0.00 a
Pupae	0	0.00± 0.00 g	2.78 ± 1.60 g
	40	12.95 ± 2.50 fg	24.39 ± 3.43 ef
	120	28.62 ± 3.10 de	51.82 ± 4.30 cd
	240	44.21 ± 4.89 c	64.55 ± 2.92 b
	480	63.82 ± 4.20 b	74.24 ± 2.98 b
	960	74.30 ± 1.86 b	93.33 ± 3.33 a
	1440	100.00 ± 0.00 a	100.00 ± 0.00 a
Adults	0	2.06 ± 0.32 h	3.21 ± 0.61 gh
	40	12.85 ± 0.58 g	38.00 ± 8.09 e
	120	27.29 ± 0.77 f	68.97 ± 6.90 c
	240	56.57 ± 2.74 d	85.24 ± 3.51 b
	480	71.95 ± 2.81 c	98.45 ± 0.29 a
	960	89.62 ± 1.06 ab	100.00 ± 0.00 a
	1440	100 ± 0.00 a	100 ± 0.00 a

[†] Mean ± Standard Error. Means in the same column with different letters are significantly different at $p < 0.05$ following Tukey's HSD test.

2.3.3. Effect of ozone treatment duration on germination of barley

Germination rate is one of the most critical parameters that contributes to predicting final crop yield and commercial value, which was measured 7 days after sowing. The effect of O_3 on germination of barley seeds are presented in Table 2.3. Seeds harvested in 2013/2014 and 2019/2020 seasons are refer to as old and new barley, respectively. The germination rate of old harvest seeds increased from 94.34% to 97.17% at 40 min ozonation, while that of new barley seeds increased from 98.35% to 99.06% at 10 and 20 min ozonation. Longer duration of O_3 treatment, such as 480, 960 and 1440 min have been observed to have a considerably negative effect on germination rate of old harvest seeds. Regarding new seeds, the germination rate of new harvest seeds significantly decreased under 960 and 1440-minute O_3 exposure compared with untreated seeds ($p<0.05$). In both old and new barley seeds, O_3 application period of 1440 min were resulted dramatically decreasing in germination rates to 14.39 % and 20.28%, respectively.

Table 2.3. Effect of O_3 duration at 700ppm on barley germination rate.

O_3 treatment duration (min)	Germination rate ± SE (%)	
	Old (2013/2014) †	New (2019/2020)
0	94.34 ± 1.67 ab	98.35 ± 0.56 a
10	94.11 ± 0.97 ab	99.06 ± 0.36 a
20	95.28 ± 0.87 ab	99.06 ± 0.36 a
40	97.17 ± 1.01 a	98.59 ± 0.59 a
120	95.28 ± 1.01 ab	98.82 ± 0.79 a
240	84.21 ± 1.12 bc	96.23 ± 0.71 a
480	86.32 ± 0.93 c	94.34 ± 0.94 a
960	68.16 ± 1.61 d	78.54 ± 2.71 b
1440	14.39 ± 4.55 e	20.28 ± 1.84 c

† Mean ± Standard Error. Means in the same column with different letters are significantly different at $p < 0.05$ following Tukey's HSD test.

2.3.4. Effect of ozone treatment duration on seedling growth

The effect of O_3 on morphological parameters (seedling length and vigour index) of barley seeds are presented in Figure 2.3. In terms of total root length of new harvest barley, there was no significant difference between 10, 20, 40, 120, 240, 480 and 960 min ozonation and untreated samples. However, after 1440 min treatment, root length decreased (6.65 cm) compared to the untreated sample (8.56 cm). For old harvest barley, 480 and 960 min O_3 treatment significantly increased root length to 11.28 and 10.95 cm from 8.34 cm, whereas root length was significantly decreased to 4.79 cm at exposure of 1440 min compared with that of the control (8.34 cm) (Figure 2.3a). New barley shoot length was not significantly inhibited by 10, 20, 40, 120, 240, 480 or 960-min O_3 treatment, but was significantly inhibited at 1440 min ozonation. For old barley seeds, there was a positive effect of O_3 on shoot length at 480- and 960-min treatment, which increased shoot length to 12.61 cm and 13.05 cm compared with the untreated sample (11.11 cm). Shoot length was inhibited (8.49 cm) with extension of O_3 treatment to 1440 min (Figure 2.3b).

Seedling vigour index (SVI) is an important parameter to reflect the potential field performance of different seeds. SVI data is presented in Figure 2.3c. Regarding new barley seeds, in comparison to control, SVI showed no significant difference under short duration ozonation, including 10, 20, 40, 120, 240, 480 min. However, it dramatically decreased as duration time increased to 960 and 1440 min ($p<0.05$ value). In terms of old seeds, only 1440 min O_3 treatment significantly decreased SVI compared with the untreated sample ($p<0.05$). The ratio of root to shoot length (R/S ratio) in both new and old barley seeds significantly decreased at 1440 min O_3 treatment ($p<0.05$). In addition, the R/S ratio in new barley seeds increased subjected to 480- and 960-min exposure, however, in old barley seeds there was no significant difference between 10, 20, 40, 120, 240, 480 or 960 min of O_3 treatment and control in old barley seeds (Figure 2.3d).

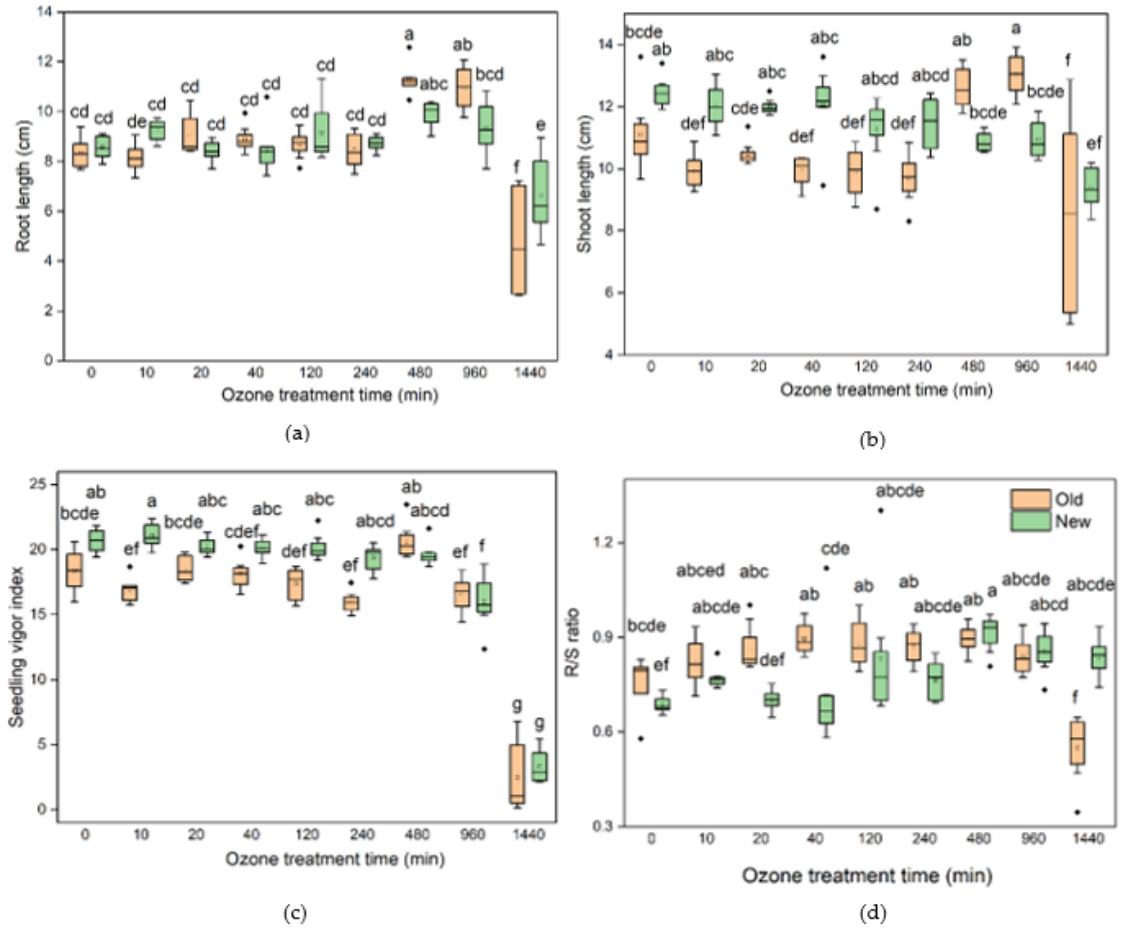

Figure 2.3. Changes in morphological characteristics with O_3 treatment duration on (a) barley root length; (b) barley shoot length; (c) seedling vigour index of barley seeds; (d) the ratio of root to shoot length. Significant differences ($p<0.05$) between each treatment are indicated by letters. Groups do not have significant differences if they have identical marked letters. Statistical reliability of the differences was determined based on Tukey's HSD test.

2.3.5. Effect of ozone on barley grain quality

The soluble protein content of old and new barley seeds was 15.2% and 9.9%, respectively (Figure 2.4a). There was no significant effect of O_3 treatment on the soluble protein content in both samples, irrespective of age. The moisture content of old and new barley varied from 12.2% to 12.6% and 10.7% to 11%, respectively, and showed no change with increasing O_3 duration in both sample groups (Figure 2.4b). The old barley had a higher lightness value than the new barley (From Figure 2.4c). The colour of both new and old barley was not affected by O_3 exposure duration. As can be seen from Figure 2.4d, the hardness values for old and new barley were 55.3 and 45.3, respectively. There was a less apparent trend towards increasing hardness of both old and new seeds, characterised by small fluctuations up to 58 and 47.6, respectively. Generally, TW of both old and new barley was not considerable impacted by extended periods of O_3 treatment. In terms of old barley, TW was decreased from 33.2 to 32.9 g, while new barley was reduced from 35.1 to 34.2 g (Figure 2.4e). Overall, the value of the soluble protein content, moisture content, colour and hardness of old barley were higher, and only thousand weight (TW) was lower than that of the new barley seeds (Figure 2.4).

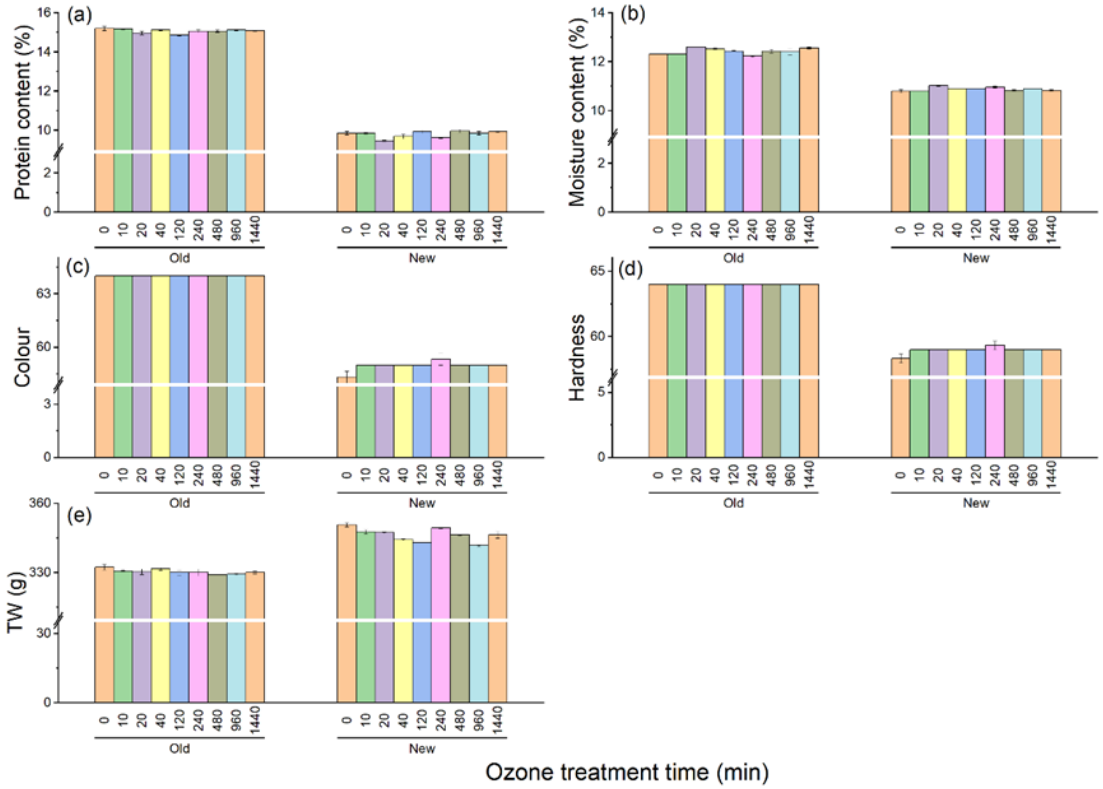

Figure 2.4. Effect of O_3 duration on the quality of barley seeds: (a) soluble protein content; (b) moisture content; (c) colour; (d) hardness; (e) thousand seeds weight. Statistical reliability of the differences was determined based on Tukey's HSD test.

2.4. Discussion

The results suggest that O_3 has efficacy against both *R. dominica* and *T. castaneum*, with the toxicity data indicating that mortality occurs in a relatively short interval and increased with prolonged O_3 treatment. Sufficient exposure time is required to kill insects since disinfestation action of O_3 takes place mainly at the surface of the grain, unless saturation of the grain is reached (Jian et al., 2013). It was observed that 960-min O_3 treatment could kill 65% and 81% of *R. dominica* and *T. castaneum*, even if assessed immediately after O_3 treatment. Lower exposure time led to a higher mortality rate of insects using longer assessment intervals. Thus, both exposure times and mortality assessment intervals should be considered to evaluate pesticide efficacy. Assessment intervals for insect mortality to pesticides should be varied based on insecticide mode of actions, namely 1-2 days for fast-acting insecticides, and 7-14 days for slow-acting insecticides. Our results indicated the delayed toxicity of O_3 since the mortality of both species increased up to 7 days, which is in accordance with Subramanyam

and Li (2017) and Holmstrup et al. (2011). The mechanism by which ozone kills insects is still elusive, with two main hypothesized mechanisms. The first is related to respiratory rate. The respiratory system of insects is considered to be the first site of action for O_3, as it enters insects through spiracles causing disrupted respiration in insects. Discountinuous gas change could lower insect metabolism causing death (Boopathy et al., 2021). The second possible mechanism is based on the strong oxidative properties of O_3, which could cause oxidative damage of essential components resulting in DNA strand breaks and cell membrane oxidation (Ballinger et al., 2005). Gene expression change and damage to DNA exposures does not occur immediately after O_3 exposure, but in the post-exposure period (Holmstrup et al., 2011).

Toxicity data indicated a noticeable difference in susceptibility between *R. dominica* and *T. castaneum*, especially at different life stages. In general, all life stages except for eggs of *R. dominica* have a higher tolerance to O_3 than *T. castaneum*. In addition, longer O_3 exposure times at 700ppm were required for eggs and pupae of both species to reach higher mortality compared with larvae, which agrees with previous research (McDonough et al., 2011; Xinyi et al., 2019). Distinct responses of life stages to O_3 could be attributed to their respiration rates. The respiratory system of insects is considered as the first site of action for O_3. On the basis of phosphine studies (Pimentel et al., 2007), we suggest that the low respiration rate of pupae and eggs might reduce O_3 uptake resulting in greater tolerance (Emekci et al., 2004). Furthermore, the majority of the outer layer of eggs and pupae are made of lipid, sometimes covered with a waxy coat which could provide an additional barrier against O_3.

Current results indicate that to obtain 100% mortality of all stages of both species the required exposure time of O_3 at 700ppm was 1440 min. Short duration O_3 treatment did not significantly influence germination ability in either old or new barley. However, with increasing duration, seed germination was negatively impacted. These observations are in agreement with Wu et al. (2006), who found that the germination ability of stored wheat was damaged with prolonged O_3 treatment. Peroxide or superoxide accumulation, which is elicited by O_3, can damage antioxidant mechanisms, reduce the activity of related enzymes and disturb macromolecule biosynthesis, resulting in suppression of the germination process. This is problematic, as quality of barley is crucial in determining its end utilisation. For example, the germination rate of barley seed must exceed 95% for malting (Shrestha and Lindsey, 2019). Therefore, a high seed germination ability plays a pivotal role in improving crop production and shortening manufacturing costs of the malting process. Some studies have indicated that moderate duration ozonation can accelerate germination of tomato seeds because ROS produced by O_3 promotes

cell signalling (Monroy Vazquez et al., 2017). ROS break seed dormancy and promote germination by regulating hormone levels and reducing ABA (Wang et al., 1998a). In this study, seed germination of two samples of different ages was enhanced under moderate ozonation time, especially for old seeds, which increased from 94.34% to 97.17% at 40 min O_3 treatment, improving the germination ability of aging barley to meet malting barley grade. Further research needs to be conducted, specifically utilising seeds with lower germination ability to explore if moderate O_3 processing time can significantly increase germination ability.

This study also suggested that prolonged O_3 exposure (1440 min) was unfavourable for seedling growth. Both root and shoot length were decreased with extended O_3 duration to 1440 min, which is consistent with reports that maize seedling length decreased with increasing O_3 processing time (Normov et al., 2019). Additionally, root length of both old and new barley significantly increased under moderate O_3 processing time. Seedling growth is a cell division and expansion process, and endogenous ROS, such as hydrogen peroxide (H_2O_2) induced by O_3, play essential roles in cell growth (Gapper and Dolan, 2006; Sudhakar et al., 2007). Root to shoot ratio is a good indicator of the overall health and physiology of plants. It is generally genetically fixed for each cultivar, but can be modified by environmental factors (Bláha, 2019). The ratio of root to shoot length increased when the seeds were subjected to longer O_3 exposure time, signifying that plants reduce shoot growth and enhance root growth in response to O_3 stress. Alexander et al. (2018) suggested that the root to shoot ratio of spring wheat increased with increasing O_3 concentration. Plants apportioning more biomass to roots and less to stems when they encounter unfavourable conditions has been previously established (Bonifas et al., 2005; Nahar and Gretzmacher, 2011).

Quality characteristics including the soluble protein content, colour and TW may affect the final market price of grain. In this research, different O_3 treatment time did not influence soluble protein content, moisture content, TW and colour of both old and new seeds, which closely correlated with previous studies (Sui et al., 2016; Wang et al., 2016). Seed storage is one of the most important factors that affect seed quality. Seed reserves are lost during storage, as manifested by the lower TW of old seeds. Total soluble protein was higher in old seeds compared with the new ones, consistent with Lozano et al. (2018), who reported that the total soluble protein and amino acid content of *Jatropha curcas L.* seeds increased by 160% and 67% during storage, respectively. A possible explanation could be the consequence of soluble protein or amino acid converted from storage proteins to maintain respiration and respond to stress during storage (Azevedo Neto et al., 2009). As the energy sources are used by the embryo

during germination, organic compounds in seeds continually reduce due to respiration processes after harvest, resulting in an energy deficiency during germination and early seedling development. This explains why old seeds in this study appeared to have a lower germination ability and seedling growth capacity compared to new seeds. Additionally, old seeds tend to be more susceptible to O_3 stress. The germination rate of the old seeds significantly reduced at 480 min ozonation, whereas that of new barley did not significantly decrease until 960 min exposure.

Morphology and germination were adversely influenced by ozone overexposure, but can be regulated to achieve desired outcomes if proper measures are taken. For instance, if the barley seeds are utilized for livestock feed and human consumption, it is necessary to supply sufficient ozone treatment to eliminate pests rather than achieve high germination ability. As most farmers grow barley for sale as malting barley, germination ability and other malting quality parameters could also be maintained and even improved based on proper application of ozone. Outcomes of this research were that short ozone exposure enhanced germination ability of aging seed to meet malting barley requirement, demonstrating that ozone has great potential to improve the functionalities of grain products while ensuring food safety.

2.5. Conclusion

More than 2 days after ozone treatment should be considered as the endpoint to evaluate insect mortality rate due to delayed toxicity of O_3. The C×t product of 36 mg h/L (700ppm × 24 h) completely killed all stages of two important stored-grain pests without impacting quality parameters including soluble protein content, moisture content, colour, hardness and TW. Pupae and eggs were more tolerant to O_3 than larvae and adults. Moderate ozone treatment time such as 20 min and 40 min increased germination ability to greater than 95%, however, the germination ability and seedling growth of both old and new barley seeds were impaired due to long exposure to O_3 (1440 min), especially for the longer stored seeds. Overall, the data demonstrated that the multiple effects of ozone treatment on barley seeds could be utilized to control quality attributes of final food products, thus it is imperative to select an optimal O_3 exposure time to achieve the desired outcome for malting, animal feeding or human consumption. Future investigations are needed to evaluate O_3 efficacy against insects in large-sized silos, as well as to elaborate on how metabolites in barley seeds shift in response to O_3 treatment.

Attribution Statements

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The following authors contributed to this manuscript as outlined below.

Authorship order	**Contribution (%)**	**Concept Development**	**Data Collection**	**Data Analyses**	**Drafting of manuscript**
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Name 2 Litao Sun	10	X	X	X	
Name 3 Xiangyang Yu	5	X		X	
Name 4 Yonglin Ren	5	X		X	
Name 5 Garth Maker	5	X		X	

Contribution indicates the total involvement the author has had in this project. Placing an 'X' in the remaining boxes indicates what aspect(s) of the project each author engaged in.

By signing this document, the Candidate and Principal Supervisor acknowledge that the above information is accurate and has been agreed to by all other authors.

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Candidate **Principal Supervisor**

Chapter Three

Effects of ozone treatment on VOCs released from barley grain

Abstract

Ozone is widely used to control pests in grain and has an impact on seed germination. The germination process involves multiple secondary metabolites, such as VOCs, which are altered under ozone treatment. Here, optimised solid-phase microextraction coupled with gas chromatography-mass spectrometry was implemented to explore changes in VOCs from barley seeds under ozone treatment. The data demonstrated that barley released both a greater variety and quantity of VOCs under oxidative stress. The number of alcohols and hydrocarbons gradually decreased, whereas aldehydes and organic acids markedly increased with increasing ozone treatment time. Acetic acid was identified as a potential ozone stress-specific marker. Furthermore, the dosage-dependent function of acetic acid on the germination of barley was verified, namely, a low dosage of acetic acid increased germination and vice versa. This study provided new insights into how barley responds to ozone treatment and highlighted the role of acetic acid in seed germination.

3.1. Introduction

Barley (*Hordeum vulgare*), a widely cultivated and highly adaptable crop, is used predominantly for stockfeed, food products and the brewing industry via the malting process. Germination is a crucial stage for plant growth and can be regarded as a determinant of plant growth and yield productivity. It is also the most vulnerable stage in plant development, being more susceptible to intrinsic and extrinsic factors than other stages. Germination ability is therefore one of the most important parameters to evaluate the value of barley, seeds with >95% germination rate being acceptable for malting.

There has been growing emphasis on the application of ozone (O_3) in grain processing for disinfection (Allen et al., 2003; Bonjour et al., 2011; Savi et al., 2014). Additionally, ozone also has an impact on seed germination. According to Marique et al. (2012), ozone was employed for wheat seed disinfection without altering the seed germination capacity and had no adverse effect on the physical and biochemical properties of the seeds. Some work reported that the germination and seedling growth ability of corn and wheat seeds had been facilitated under ozone treatment (Violleau et al., 2008; Avdeeva et al., 2018a). Conversely, a study investigated the effect of gaseous ozone as an anti-fungal fumigant for stored wheat, suggesting that the germination of wheat seeds had been significantly reduced after too high or too long exposure to ozone gas (Wu et al., 2006). According to our study in chapter two, short ozone exposure seems to have a positive effect, but prolonged ozone treatment may negatively affect

seed germination. Germination involves multiple physiological and biochemical mechanisms that may be altered in response to ozone treatment. Therefore, exploring changes in metabolite levels may be helpful for understanding the biochemical responses of barley seeds to ozone. The majority of research to date has been conducted on the effects of ozone on the composition of the seed, such as protein, starch and lipid content (Wang et al., 2016; Gozé et al., 2017; Vanier et al., 2017), whereas the effect of ozone on emissions from grain, especially barley, has received little attention.

Volatile organic compounds (VOCs) are molecules with low molecular weight (<300 g/mol) and high vapour pressure. Plants release a large variety of VOCs into the surrounding atmosphere, including simple gases, such as oxygen and carbon dioxide, as well as alcohols, ketones, aldehydes, acids, esters, hydrocarbons and terpenes (Qualley and Dudareva, 2009). Certain VOCs have functional properties, while others appear to be by-products of metabolism (Oikawa and Lerdau, 2013). Functional VOCs not only contribute to the odour of food, but have pivotal roles in plant communication, increasingly recognised as an important factor in the ecology of natural and agricultural systems (Ninkovic et al., 2016). For example, some VOCs inhibit seed germination and seedling growth of neighbouring weedy plants to reduce competition (Romagni et al., 2000). Plant VOCs are also important in relieving both biotic and abiotic stresses which can result in the production of reactive oxygen species ROS and cause cell damage. According to the literature, isoprene, monoterpenes, sesquiterpenes and green leaf volatiles were demonstrated to mitigate oxidative stress (Vickers et al., 2009; Stolterfoht et al., 2019). They have been reported to induce plant defences to stress and allow plants to respond faster to subsequent stresses (Cofer et al., 2018). Certain VOCs can also modulate plant growth. Some ketones, such as 2-nonanone and 2-undecanone were reported to stimulate germination of *Lactuca sativa* seeds (Fincheira et al., 2017). Acetic acid, mainly produced through the catabolism of precursor compounds, promotes the germination ability of stored wheat seeds and facilitates seed development in maize (Sholberg and Gaunce, 1996; Dylan and Michael, 2020).

Despite numerous studies that have analysed VOCs of barley seeds to elucidate aroma components (Cramer et al., 2005; Féchir et al., 2021), the volatile metabolite profile of barley under ozone treatment is still unknown. This study aims to characterise the VOCs of barley under different ozone treatments based on optimised solid-phase microextraction (SPME) combined with the gas chromatography-mass spectrometry (GC–MS). VOCs that play a

possible role in ozone-induced resistance will be highlighted. Furthermore, the influence of the VOC acetic acid on barley germination will also be assessed.

3.2. Materials and methods

3.2.1. Grain sample preparation

Barley samples belonging to Spartacus CL were harvested in 2019 from Lake Grace, Western Australia, within 10.9% moisture content. A commercial ozone generator (Model FH-CYJ1520A-20 g/h, maximum concentration =700ppm, Shanghai Fenghua Optoelectronics Technology Co., Ltd., China) was utilised to produce ozone from atmospheric air. Ozonation of barley seeds was carried out in a 2-L and 16.6-cm diameter Hysil semi-batch reactor with a desiccation glass chamber fitted with a lid containing a 2-cm central hole for ozonation. Gas was introduced from the ozone generator directly to the bottom of the reactor and passed through the seeds using a 1-cm diameter hose. One kilogram of barley seeds was placed on a supporting iron wire gauze 5 cm above the reactor bottom to enable gas circulation. The ozone concentration to and from the reactor was continuously measured by an ozone monitor (Shenzhen Yuan Technology Co., Ltd., China). Nine treatments were designed for this study, comprising control, 700ppm ozone-exposure for 10, 20, 40,120, 240, 480, 960 and 1440 min, with three replicates. These samples were stored in a refrigerator at -20°C until further analysis.

3.2.2. SPME procedure

A three-phase SPME fibre (50/30 μm) with a 2-cm combination coating of divinylbenzene/ carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm, Agilent, USA) was utilised in this study. Prior to initial use, all new fibres were conditioned for 60 min at 270℃ as indicated by the manufacturer, using a desk-type thermal oscillator (Model THZ-92AS, Jtone, China).

Five grams of barley seeds were added to 20 mL SPME vials with magnetic screw caps and white PTFE/blue silicone septa (thickness 1.3 mm) (Agilent, USA) (Figure 3.1). Four pre-incubation times (0, 5,10 and 20 min), three extraction temperatures (25, 40 and 50℃), four extraction times (20, 30, 40 and 60 min), three desorption temperature (240, 250 and 260℃) and three desorption times (3, 5 and 7 min) were tested to establish the optimal method. A desk-type thermal oscillator (Model THZ-92AS, Jtone, China) was employed for maintaining the extraction temperature.

3.2.3. GC-MS analysis

An Agilent 7890B GC system coupled with 5977B MSD was employed for compound analysis with an Agilent HP-5MS capillary column (30 m length, 0.25 mm internal diameter, 0.25 µm film thickness, 5% phenyl and 95% dimethylpolysiloxane stationary phase) (Figure 3.1). After extraction, samples were injected in splitless mode with ultra-high purity helium (Air Liquide, Australia) as the carrier gas at a constant flow rate of 0.8 mL/min. The temperature program followed that of Buśko et al. (2010), starting at 40℃ for 1 min, ramped at 10℃/min to 180℃, then at 40℃/min to 260℃ and then held at 260℃ for 6 min. The detector was operated in electron impact (EI) ionisation mode at 70 eV and the spectra were acquired at 3.1 scan/s from 35 to 350 atomic mass units (amu). The transfer line temperature of the MSD was 280℃, and the ion source temperature was 230℃. Volatile compounds were identified using experimentally obtained Kovats retention indices (RI) with the combination of C_7-C_{40} alkane standards and mass spectra in the National Institutes of Standards and Technology Mass Spectrometry (NIST MS) library. All experiments were performed in triplicate.

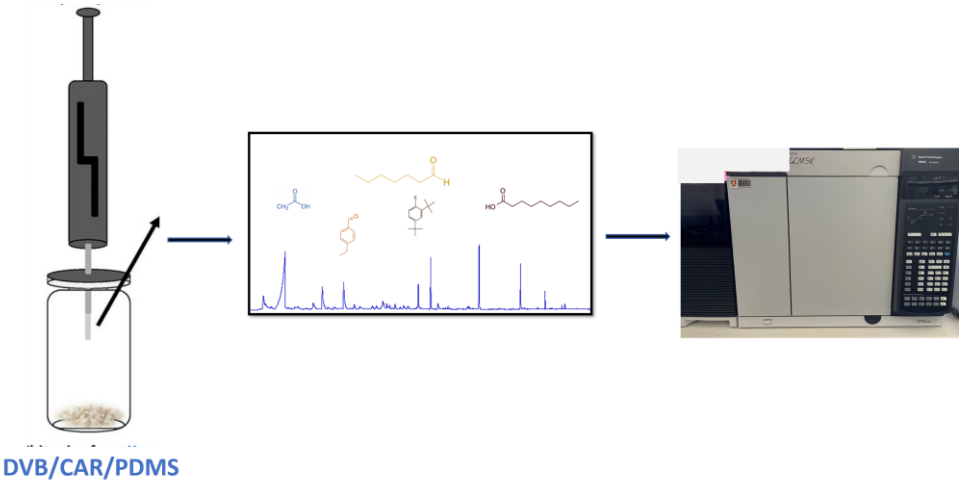

Figure 3.1 Analysis of volatile organic compounds (VOCs) in barley seeds based on headspace solid-phase microextraction coupled with gas chromatography mass spectrometry (HS-SPME-GC-MS).

3.2.4. Acetic acid vapour treatment

Twenty grams of seeds were placed in 250 mL flasks sealed with a PTFE-silicon septum. One hundred mL of acetic acid (analytical grade, 99.7%, Ajax Finechem Pty Ltd, AU) solution at different concentrations was added onto filter paper (Whatman, AU) in the flask to avoid acetic acid unevenly contacting with seeds. The effects on barley germination of six concentrations of acetic acid (0, 0.05, 0.5, 1, 2 and 4 mg/g) with 3 different treatment times (2, 5 and 24 h) were studied. Each treatment was performed with three replicates. The germination test was

carried out based on the between-paper (BP) method of the International Seed Testing Association (ISTA, 2006).

3.2.5. Data analysis

Data acquisition was performed using Mass Hunter Acquisition software (vB.06.00; Agilent Technologies, Santa Clara, USA), and data were expressed as the mean ± standard deviation of three replicates. Multivariate analyses, including principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) with one way-analysis of variance (one way-ANOVA) were performed by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). Tukey's post-hoc test (HSD) based on the *P*-value (α=0.05) was used for identifying statistical differences among different treatments. The cluster heatmap was performed to visualise a hierarchically clustered data matrix. Figures were generated using Origin software 2019b.

3.3. Results and Discussion

3.3.1. HS-SPME-GC-MS optimisation

Eight compounds across the chromatogram with different polarities and volatilities were selected for method optimisation, starting with fibre extraction parameters (Table 3.1). The selected compounds are main aroma contributors in barley or important signal compounds in plant-plant interactions (Hoff et al., 2013; Dong et al., 2015b; Kegge et al., 2015). Three extraction temperatures (25, 40 and 50℃) were selected for optimisation, and extraction efficiency was significantly enhanced with increasing extraction temperature ($p > 0.05$) (Figure 3.2A). The total peak area of the selected compounds was utilized in the following method optimization. The peak area at an extraction temperature of 50℃ was 34-fold higher than at 25℃ and double that at 40℃. The optimal temperature of 50℃ was utilised for the extraction time profile experiment, summarised in Figure 3.2B. The maximum peak area was achieved after 60 min. However, there was no significant difference between 30, 40 and 60 min based on ANOVA ($p > 0.05$). Therefore, 30 min was chosen for further analysis to reduce sample preparation time. The results of pre-incubation time (0, 5, 10 and 20 min) profile experiments indicated that volatile components of barley seeds needed at least 10 min incubation to reach phase equilibrium in vials (Figure 3.2C). Hence, pre-incubation of samples at 50℃ for 10 min was used in this procedure to encourage the accumulation of VOCs in the headspace. Desorption temperature of 250℃ was shown to be the most effective (Figure 3.2D). A desorption time of 7 min was selected (Figure 3.2E), as there was a significant increase ($p >0.05$) by extending the desorption time from 3 or 5 to 7 min. A range of different oven programs

were used in literatures, but appeared to have minimal impact on the resulting data. (Perkowski et al., 2012). Therefore, the GC parameters proposed by Buśko et al. (2010), who worked on volatile metabolites in various cereal grains, were employed. All the parameters used for further analysis are listed in Table 3.2.

3.3.2. The effect of ozone on barley VOC profiles

In this study, volatile compounds of barley seeds subjected to 9 different ozone treatments were analysed. For GC-MS data, the peaks were integrated in the total ion chromatogram (TIC). Any peak with more than 5000 absolute peak area was considered as detected and subsequently integrated. A total of 88 peaks were obtained, and 68 volatile compounds in barley seeds were identified based on retention time and mass spectral data from the NIST Mass Spectral Library (Table 3.3). Siloxane derivatives were not reported as they originate from the fibre or column bleeding. Metabolite profiling data was normalised by generalised logarithm transformation (Table 3.4).

Table 3.1. Target compounds used for HS-SPME method optimization.

Compound	RT (s)	RI (lit)[a]	RI (cal)[b]	M (%)[c]	CAS number
1-Pentanol	3.789	765	760	85.4	71-41-0
Hexanal	4.159	800	801	88.9	66-25-1
1-Hexanol	5.228	868	873	91.2	111-27-3
Heptanal	5.65	901	901	87.9	111-71-7
1-Octen-3-ol	6.977	980	985	70.8	3391-86-4
Hexanoic acid	7.368	990	1010	80.4	142-62-1
1-Hexanol, 2-ethyl	7.764	1030	1035	93.2	104-76-7
6,10,14-trimethyl-2-Pentadecanone	16.995	1844	1843	84.3	502-69-2

[a] Retention indices reported in the NIST library based on literature.

[b] Retention indices calculated from C_7-C_{40} *n*-alkanes.

[c] Match (%) obtained from NIST data base.

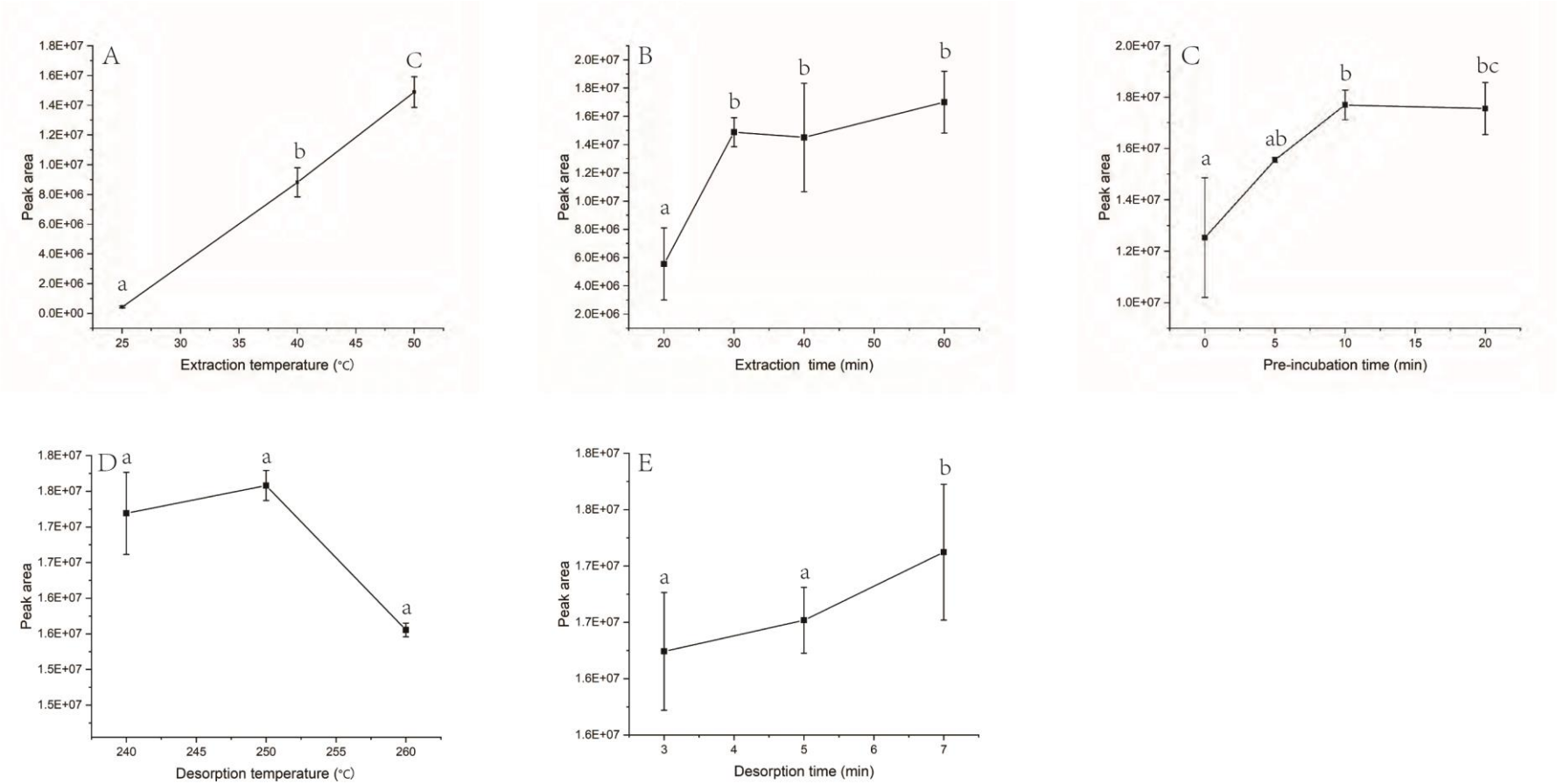

Figure 3.2. Influence of extraction procedure on GC-MS peak area response using (A) extraction temperature; (B) extraction time; (C) pre-incubation time; (D) desorption temperature and (E) desorption time. Data points represent the mean ±SE of three replicates

Table 3.2. Optimised conditions for the determination of volatile and semi-volatile components of barley seeds.

HS-SPME-GC-MS	
HS vial	20 mL amber headspace vial
Sample volume	5 g whole kernel of barley seeds
SPME fibre	DVB/CAR/PDMS 50/30 m, 2 cm, 23 Ga
Incubation conditions (equilibrate)	50℃ for 10 min
Extraction conditions	50℃ for 30 min
Desorption conditions	250℃ for 7 min
GC	
Injector mode	Splitless
GC column	HP-5MS (30 m×0.25 mmID×0.25 mm)
Carrier gas	UHP helium (99.999%)
Gas flow	0.8 mL/min (constant)
GC oven program	40℃ (1 min)/5℃/min to 110℃/
	10℃/min to 250℃/250℃ (6 min)
MS	
Ion source	Electron Ionization 70eV
Data acquisition rate	3.1 scans/Sec
Mass scan range	35-500 amu
Ion source temperature	230°C
Transfer line temperature	280°C
MS quad temperature	250°C

Table 3.3. Volatile compounds detected in all barley samples by SPME-GC-MS.

Chemical groups	Compounds[a]	RT[b]	RI (lib)[c]	RI (cal)[d]	M (%)[e]	Ozone treatment time (min)								
						0	10	20	40	120	240	480	960	1440
Alcohols	3-Methyl-1-butanol	3.39	736	738	74.9	+	+	+	+	-	-	-	-	-
	1-Pentanol	3.79	765	771	85.4	+	+	+	+	+	+	+	-	-
	1-Hexanol	5.23	868	873	91.2	+	+	+	+	+	+	+	+	-
	1-Heptanol	6.83	970	975	71.1	+	+	+	+	+	+	+	+	-
	1-Octen-3-ol	6.98	980	985	70.8	+	+	+	+	+	+	+	+	-
	2-Ethyl-1-Hexanol	7.76	1030	1035	93.2	+	+	+	+	+	+	+	+	+
	1- Octanol	8.42	1071	1077	77.0	+	+	+	+	+	+	+	+	-
	trans-Linalool oxide (furanoid)	8.69	1086	1094	81.5	-	-	-	-	-	-	+	+	+
	1-Nonanol	9.95	1173	1178	86.0	+	+	+	+	+	+	+	-	-
	Terpinen-4-ol	10.08	1177	1186	75.4	+	+	+	-	-	-	-	-	-
	4,8-Dimethyl-1-nonanol	10.72	1230	1231	76.0	-	-	-	-	-	+	+	+	-
	2-Tridecanol	14.37	1510	1507	91.0	+	+	+	+	+	+	+	+	-
	2,4-Di-tert-butylphenol	14.56	1519	1524	84.1	+	+	+	+	+	+	-	-	-
	1-Tetradecanol	15.71	1676	1622	71.3	-	-	-	-	-	+	+	+	-
Aldehydes	Pentanal	2.96	699	703	79.5	+	+	+	+	+	+	+	+	+
	Hexanal	4.16	800	801	88.9	+	+	+	+	+	+	+	+	+
	Heptanal	5.65	901	901	87.9	+	+	+	+	+	+	+	+	+

	2-ethyl-hexanal	6.53	955	956	77.3	-	+	+	+	+	+	-	-	-
	Benzaldehyde	6.70	962	967	76.5	-	-	-	-	+	+	+	+	+
	Octanal	7.29	1003	1004	91.1	+	+	+	+	+	+	+	+	+
	Nonanal	8.88	1104	1107	92.2	+	+	+	+	+	+	+	+	+
	4-Ethyl-benzaldehyde	9.87	1180	1173	80.7	-	-	-	-	-	-	+	+	+
	Decanal	10.40	1206	1208	92.1	+	+	+	+	+	+	+	+	+
	Undecanal	11.83	1307	1310	83.6	-	+	+	+	+	+	+	+	-
	Tridecanal	14.45	1512	1515	90.4	-	+	+	+	+	+	+	+	-
	Tetradecanal	15.56	1613	1619	71.1	-	-	-	-	-	+	+	+	-
Benzene derivatives	p-Xylene	5.08	865	863	70.1	+	-	-	-	-	-	-	-	-
	Styrene	5.35	891	881	73.7	+	-	-	-	-	-	-	-	-
	4-Ethyl-1,2-dimethylbenzene	8.77	1100	1099	68.1	-	-	-	-	-	-	-	-	+
Carboxylic esters	Hexanoic acid, methyl ester	6.04	925	925	81.3	-	-	-	-	-	-	+	-	-
	Octanoic acid, methyl ester	9.18	1126	1126	82.3	-	-	-	-	-	-	+	+	-
	Nonanoic acid, methyl ester	10.65	1225	1226	75.7	-	-	-	-	-	+	+	+	-
	2-Methyl-propanoic acid 3-hydroxy-2,2,4-trimethylpentyl ester	12.80	1380	1383	75.7	+	+	+	+	+	-	-	-	-
Heterocyclics	2(3H)-Furanone, dihydro-5-methyl-	6.57	958	959	70.1	-	-	-	-	-	-	-	+	-
	2(3H)-Furanone, 5-ethenyldihydro-5-methyl-	7.97	1043	1048	73.9	+	+	+	+	+	+	+	+	+
	2(3H)-Furanone, 5-ethyldihydro-	8.20	1057	1063	73.1	+	+	+	+	+	+	+	+	+
	2(3H)-Furanone, 5-butyldihydro-	11.26	1261	1269	61.3	-	-	-	-	-	-	+	+	+

	2(3H)-Furanone, dihydro-5-pentyl-	12.68	1363	1373	74.5	-	-	-	-	-	-	-	+	-
	1-Phenyl-1-butene	8.64	1080	1091	80.2	-	-	-	-	-	-	+	+	+
	Undecane, 3-methyl-	9.84	1170	1170	70.5	+	+	+	-	-	-	-	-	-
	1-Dodecene	10.15	1190	1191	86.8	+	+	+	+	+	+	+	+	-
	Dodecane	10.27	1200	1199	85.9	+	+	+	+	+	+	+	+	+
Hydrocarbons	Undecane, 2,6-dimethyl-	10.48	1210	1214	77.8	+	+	+	-	-	-	-	-	-
	1-Tridecene	11.58	1292	1292	89.2	+	+	+	+	+	+	+	+	+
	Heptylcyclohexane	12.41	1346	1353	70.1	+	+	+	+	+	-	-	-	-
	Tridecane, 3-methyl-	12.63	1371	1370	73.2	+	+	+	-	-	-	-	-	-
	1-Pentadecane	14.18	1492	1492	71.5	-	-	-	-	-	+	+	+	-
	2-Hexanone, 3-methyl	4.67	844	841	76.2	-	+	+	+	+	+	+	+	-
	2-Heptanone	5.51	891	892	82.5	-	-	-	-	-	-	+	+	-
	2-Heptanone, 6-methyl	6.52	956	956	73.8	-	-	-	-	-	-	+	+	+
	2-Heptanone, 4,6-dimethyl-	8.01	1045	1051	71.6	-	-	-	-	-	-	+	+	+
	2-Nonanone	8.70	1092	1095	87.6	+	+	+	+	+	+	+	+	-
Ketones	4-Decanone	9.30	1137	1135	70.0	-	-	-	-	-	+	+	+	-
	2-Decanone	10.21	1193	1195	73.7	-	+	+	+	+	+	+	-	-
	2-Undecanone	11.65	1294	1297	86.5	-	+	+	+	+	+	+	+	+
	2-Pentadecanone	16.20	1698	1704	80.0	+	+	+	+	+	+	+	+	-
	2-Pentadecanone, 6,10,14-trimethyl-	17.00	1844	1843	84.3	+	+	+	+	+	+	+	+	-
Organic acids	Acetic acid	2.58	610	*	90.3	+	+	+	+	+	+	+	+	+

	Propanoic acid, 2-methyl	3.96	772	785	89.1	-	-	-	-	-	-	+	-	-
	Pentanoic acid	5.89	903	916	78.9	-	-	-	-	+	+	+	+	+
	Benzoic acid, 2-amino-4-methyl-	5.93	949	919	81.8	+	-	-	-	-	-	-	-	-
	Pentanoic acid, 2-methyl-	6.38	959	947	63.1	-	-	-	-	-	-	-	-	+
	Hexanoic acid	7.37	990	1010	80.4	+	+	+	+	+	+	+	+	+
	Formic acid, 2-ethyhexyl	8.50	1092	1082	73.8	-	+	+	+	+	+	+	+	+
	Octanoic acid	10.20	1180	1195	74.1	-	-	-	-	-	-	-	+	+
	Nonanoic acid	11.53	1273	1288	70.3	-	-	-	-	-	-	-	-	+
Others	Hexanenitrile	5.31	877	881	80.5	-	-	-	-	-	-	+	+	+
	Octanenitrile	8.55	1082	1085	80.5	-	-	-	-	-	-	-	-	+

[a] Only identified compounds are shown: +, detected; - not detected.

[b] Retention time.

[c] Retention index by searching NIST library.

[d] Retention index calculated by C_7-C_{40} alkanes external standards.

[e] Match (%) obtained from NIST data base.

* Based on standard.

Table 3.4. Analysis of volatile compounds by SPME-GC-MS analysis of barley under different ozone treatment times.

Compounds	Peak area (log_{10}) at different ozone treatment times (min)								
	0	10	20	40	120	240	480	960	1440
Alcohols									
1-Butanol, 3-methyl-	6.28 ± 0.14	5.40 ± 0.20	5.62 ± 0.20	6.26 ± 0.41	n.d.	n.d.	n.d.	n.d.	n.d.
1-Pentanol	6.08 ± 0.05	6.50 ± 0.12	6.65 ± 0.10	6.52 ± 0.19	6.10 ± 0.03	6.02 ± 0.05	6.19 ± 0.17	n.d.	n.d.
1-Hexanol	6.85 ± 0.07	7.59 ± 0.13	7.74 ± 0.04	7.70 ± 0.08	7.11 ± 0.09	7.09 ± 0.06	6.34 ± 0.21	6.34 ± 0.11	n.d.
1-Heptanol	5.47 ± 0.14	6.04 ± 0.07	6.23 ± 0.03	6.00 ± 0.13	5.58 ± 0.12	5.36 ± 0.10	5.22 ± 0.07	5.51 ± 0.03	n.d.
1-Octen-3-ol	5.01 ± 0.22	5.14 ± 0.13	5.83 ± 0.32	5.66 ± 0.10	5.85 ± 0.03	5.26 ± 0.13	6.34 ± 0.06	5.94 ± 0.06	n.d.
1-Hexanol, 2-ethyl	6.65 ± 0.05	7.13 ± 0.05	7.32 ± 0.04	7.32 ± 0.07	7.41 ± 0.06	7.18 ± 0.01	7.25 ± 0.02	6.97 ± 0.02	6.83 ± 0.14
1- Octanol	5.49 ± 0.08	6.44 ± 0.08	6.53 ± 0.06	6.26 ± 0.06	6.11 ± 0.04	6.16 ± 0.07	6.93 ± 0.01	6.63 ± 0.05	n.d.
trans-Linalool oxide	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.00 ± 0.04	5.37 ± 0.14	5.24 ± 0.12
1-Nonanol	5.78 ± 0.17	6.56 ± 0.17	6.64 ± 0.02	6.45 ± 0.15	5.64 ± 0.08	5.71 ± 0.06	6.16 ± 0.07	n.d.	n.d.
Terpinen-4-ol	5.35 ± 0.05	5.48 ± 0.16	5.30 ± 0.07	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-Nonanol, 4,8-dimethyl-	n.d.	n.d.	n.d.	n.d.	n.d.	5.07 ± 0.11	6.01 ± 0.01	5.77 ± 0.08	n.d.
2-Tridecanol	6.26 ± 0.03	6.21 ± 0.29	6.59 ± 0.02	5.81 ± 0.09	5.24 ± 0.06	5.09 ± 0.25	5.86 ± 0.05	5.12 ± 0.05	n.d.
2,4-Di-tert-butylphenol	5.84 ± 0.06	5.26 ± 0.15	5.27 ± 0.02	5.12 ± 0.09	4.27 ± 0.38	4.37 ± 0.17	n.d.	n.d.	n.d.
1-Tetradecanol	n.d.	n.d.	n.d.	n.d.	n.d.	5.16 ± 0.09	5.21 ± 0.12	5.21 ± 0.18	n.d.
Aldehydes									

Pentanal	5.31 ± 0.22	5.99 ± 0.16	6.12 ± 0.05	5.89 ± 0.10	6.56 ± 0.04	6.02 ± 0.06	6.27 ± 0.08	6.47 ± 0.12	6.14 ± 0.11
Hexanal	6.55 ± 0.10	7.39 ± 0.14	7.60 ± 0.08	7.57 ± 0.12	7.96 ± 0.11	7.44 ± 0.06	7.74 ± 0.13	7.83 ± 0.06	6.98 ± 0.14
Heptanal	6.02 ± 0.12	6.43 ± 0.06	6.55 ± 0.09	6.65 ± 0.06	7.03 ± 0.08	6.66 ± 0.09	6.60 ± 0.10	6.99 ± 0.10	5.09 ± 0.48
Hexanal, 2-ethyl	n.d.	5.93 ± 0.07	6.07 ± 0.02	5.86 ± 0.08	6.16 ± 0.03	5.76 ± 0.07	n.d.	n.d.	n.d.
Benzaldehyde	n.d.	n.d.	n.d.	n.d.	6.10 ± 0.04	5.69 ± 0.05	6.38 ± 0.03	6.53 ± 0.07	6.87 ± 0.18
Octanal	6.41 ± 0.12	6.63 ± 0.08	6.74 ± 0.09	6.78 ± 0.08	7.29 ± 0.05	6.94 ± 0.04	6.88 ± 0.04	7.12 ± 0.05	6.43 ± 0.19
Nonanal	5.98 ± 0.12	7.38 ± 0.13	7.50 ± 0.04	7.53 ± 0.05	7.67 ± 0.06	7.55 ± 0.02	7.42 ± 0.03	7.57 ± 0.05	6.42 ± 0.42
Benzaldehyde, 4-ethyl-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.29 ± 0.07	5.52 ± 0.05	5.68 ± 0.15
Decanal	5.59 ± 0.15	7.08 ± 0.22	7.25 ± 0.04	7.05 ± 0.07	7.10 ± 0.08	6.91 ± 0.04	6.68 ± 0.04	6.86 ± 0.04	5.71 ± 0.17
Undecanal	n.d.	5.78 ± 0.21	5.93 ± 0.05	5.57 ± 0.04	6.05 ± 0.10	5.69 ± 0.04	5.76 ± 0.02	6.07 ± 0.05	n.d.
Tridecanal	4.91 ± 0.01	5.82 ± 0.19	6.03 ± 0.02	5.74 ± 0.08	6.44 ± 0.14	6.17 ± 0.09	6.39 ± 0.05	6.21 ± 0.06	n.d.
Tetradecanal	n.d.	n.d.	n.d.	n.d.	n.d.	5.22 ± 0.09	5.15 ± 0.18	5.19 ± 0.03	n.d.
Aromatic derivatives									
p-Xylene	5.46 ± 0.09	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Styrene	4.68 ± 0.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Benzene,4-ethenyl-1,2-dimethyl-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.08 ± 0.09
Carboxylic esters									
Hexanoic acid, methyl ester	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.45 ± 0.09	n.d.	n.d.
Octanoic acid, methyl ester	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.55 ± 0.05	4.94 ± 0.06	n.d.

Nonanoic acid, methyl ester	n.d.	n.d.	n.d.	n.d.	n.d.	4.54 ± 0.13	5.90 ± 0.02	5.31 ± 0.09	n.d.
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	5.30 ± 0.03	5.18 ± 0.10	5.22 ± 0.15	4.86 ± 0.07	4.82 ± 0.09	n.d.	n.d.	n.d.	n.d.
Heterocyclics									
2(3H)-Furanone, dihydro-5-methyl-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.02 ± 0.04	n.d.
2(3H)-Furanone, 5-ethenyldihydro-5-methyl-	5.36 ± 0.09	5.51 ± 0.04	5.49 ± 0.03	5.26 ± 0.11	5.41 ± 0.09	5.07 ± 0.06	5.83 ± 0.01	5.62 ± 0.07	5.45 ± 0.23
2(3H)-Furanone, 5-ethyldihydro-	5.46 ± 0.04	5.93 ± 0.04	5.84 ± 0.10	5.96 ± 0.02	6.22 ± 0.07	6.26 ± 0.05	6.84 ± 0.03	6.61 ± 0.04	6.53 ± 0.06
2(3H)-Furanone, 5-butyldihydro-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.35 ± 0.08	5.47 ± 0.06	5.49 ± 0.05
2(3H)-Furanone, dihydro-5-pentyl-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.86 ± 0.09	n.d.
Hydrocarbons									
1-Phenyl-1-butene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.64 ± 0.13	5.78 ± 0.04	5.80 ± 0.20
Undecane, 3-methyl-	5.30 ± 0.09	5.61 ± 0.11	5.53 ± 0.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-Dodecene	6.21 ± 0.04	6.04 ± 0.14	6.10 ± 0.10	5.94 ± 0.06	5.96 ± 0.08	5.80 ± 0.06	6.30 ± 0.06	5.96 ± 0.03	n.d.
Dodecane	6.13 ± 0.02	6.10 ± 0.06	6.04 ± 0.01	6.13 ± 0.08	6.28 ± 0.03	6.07 ± 0.06	6.36 ± 0.02	6.31 ± 0.01	5.66 ± 0.13
Undecane, 2,6-dimethyl-	5.30 ± 0.20	5.45 ± 0.07	5.42 ± 0.12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-Tridecene	5.20 ± 0.03	5.12 ± 0.10	5.13 ± 0.02	5.18 ± 0.14	6.19 ± 0.14	6.18 ± 0.05	6.89 ± 0.07	6.83 ± 0.15	5.76 ± 0.10
Heptylcyclohexane	4.87 ± 0.09	4.76 ± 0.18	4.92 ± 0.10	4.68 ± 0.16	4.41 ± 0.08	n.d.	n.d.	n.d.	n.d.
Tridecane, 3-methyl-	5.39 ± 0.02	4.80 ± 0.07	4.79 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

1-Pentadecane	n.d.	n.d.	n.d.	n.d.	n.d.	4.84 ± 0.15	5.84 ± 0.02	4.98 ± 0.11	n.d.
Ketones									
2-Hexanone, 3-methyl	n.d.	6.60 ± 0.17	6.63 ± 0.13	6.55 ± 0.14	6.87 ± 0.08	6.00 ± 0.10	5.32 ± 0.15	6.29 ± 0.27	n.d.
2-Heptanone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.39 ± 0.08	5.56 ± 0.04	n.d.
2-Heptanone, 6-methyl	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.03 ± 0.05	6.05 ± 0.13	5.46 ± 0.13
2-Heptanone, 4,6-dimethyl-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.62 ± 0.06	5.82 ± 0.04	4.84 ± 0.20
2-Nonanone	5.89 ± 0.07	5.74 ± 0.07	5.74 ± 0.03	5.60 ± 0.14	5.67 ± 0.16	5.30 ± 0.03	6.04 ± 0.04	5.79 ± 0.10	n.d.
4-Decanone	n.d.	n.d.	n.d.	n.d.	n.d.	5.84 ± 0.11	6.43 ± 0.03	6.32 ± 0.03	n.d.
2-Decanone	n.d.	5.44 ± 0.13	5.76 ± 0.01	5.47 ± 0.10	5.59 ± 0.14	5.08 ± 0.03	5.15 ± 0.15	n.d.	n.d.
2-Undecanone	n.d.	5.85 ± 0.19	6.05 ± 0.05	5.29 ± 0.14	5.43 ± 0.22	6.52 ± 0.03	5.98 ± 0.06	5.64 ± 0.02	5.49 ± 0.12
2-Pentadecanone	4.74 ± 0.13	5.25 ± 0.12	5.43 ± 0.08	5.08 ± 0.05	4.97 ± 0.14	4.89 ± 0.28	5.17 ± 0.13	5.23 ± 0.06	n.d.
2-Pentadecanone, 6,10,14-trimethyl-	4.96 ± 0.05	5.38 ± 0.25	5.69 ± 0.02	5.46 ± 0.30	5.44 ± 0.22	5.27 ± 0.24	5.56 ± 0.13	5.28 ± 0.13	n.d.
Organic acids									
Acetic acid	7.03 ± 0.09	7.09 ± 0.28	7.22 ± 0.06	7.45 ± 0.07	8.12 ± 0.13	7.91 ± 0.11	8.51 ± 0.07	8.43 ± 0.05	8.33 ± 0.10
Propanoic acid, 2-methyl	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.38 ± 0.31	n.d.	n.d.
Pentanoic acid	n.d.	n.d.	n.d.	n.d.	5.91 ± 0.13	5.35 ± 0.1	6.24 ± 0.04	6.47 ± 0.10	6.28 ± 0.03
Benzoic acid, 2-amino-4-methyl-	6.64 ± 0.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pentanoic acid, 2-methyl-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.73 ± 0.03
Hexanoic acid	4.61 ± 0.08	5.63 ± 0.12	5.76 ± 0.03	5.59 ± 0.03	6.77 ± 0.07	6.83 ± 0.01	7.01 ± 0.05	7.18 ± 0.05	7.12 ± 0.13

Formic acid, 2-ethyhexyl	n.d.	5.43 ± 0.11	5.66 ± 0.04	5.65 ± 0.03	6.41 ± 0.03	6.21 ± 0.02	5.64 ± 0.04	5.54 ± 0.11	5.29 ± 0.12
Octanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.97 ± 0.12	5.72 ± 0.09
Nonanoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.79 ± 0.21
Nitriles									
Hexanenitrile	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.42 ± 0.05	6.39 ± 0.08	6.64 ± 0.12
Octanenitrile	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.34 ± 0.10

n.d., not detected

As shown in Figure 3.3A and B, the ozone treatment noticeably increased both the number and abundance of VOCs in comparison to untreated samples. Barley seeds released more VOCs as ozone treatment time increased from 0 to 480 min (49 VOCs) (Figure 3.3B). The total peak area of VOCs also showed the same trend, increasing 10-fold when subjected to 480 min ozonation (Figure 3.3A).

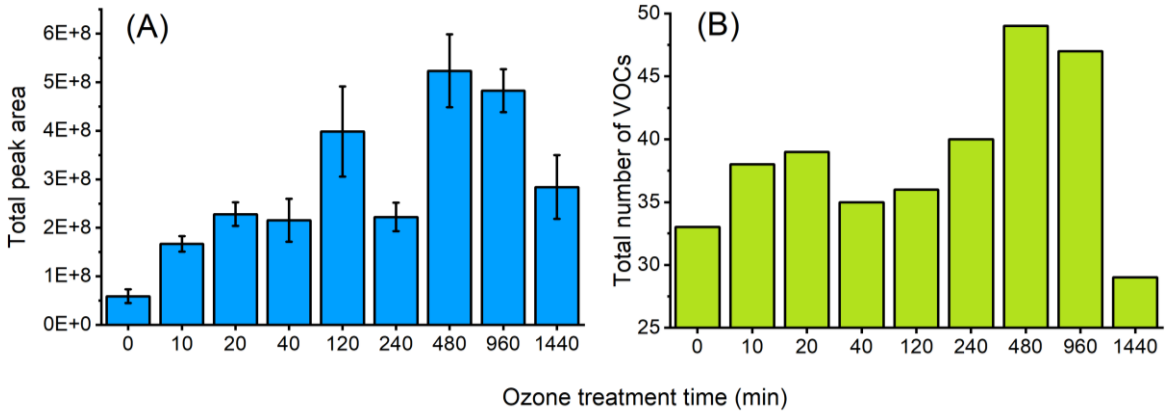

Figure 3.3. The total amount (A) and variety (B) of VOCs in barley seeds under different ozone treatment times, as measured by SPME-GC-MS. Data points represent the mean ± SE of three replicates.

Ozone treatment has previously been reported to increase VOC emissions from plants. Total VOC emissions from *Ceratonia siliqua* in summer and *Olea europaea* and *Quercus ilex rotundifolia* leaves in spring increased when subjected to ozone fumigation compared with filtered air and non-filtered air (Llusia et al., 2002). Certain VOCs triggered by ozone can rapidly react with reactive oxygen species (ROS) to reduce cell damage in response to oxidative stresses (Loreto et al., 2001). These findings align with the literature, where the composition and the amount of released VOCs have been associated with abiotic and biotic stress factors, such as temperature, drought, oxidative stress via ozone treatment and herbivore attack (Ebel et al., 1995; Vuorinen et al., 2004; Vallat et al., 2005). The multiple biotic and abiotic stress factors can also interact to trigger VOC emissions from plants (Holopainen and Gershenzon, 2010). The functions of the VOCs could be a part of programmed plant response to mitigate the negative consequences of stress. Intriguingly, the 1440-min ozone treatment reduced the variety and total amount of VOCs. This may be due to the fact that many VOCs break down by ozonolysis after prolonged oxidative treatment, which accords with the duality of ozone proposed by Pinto et al. (2010) , namely that ozone both increased and decreased VOC levels.

Identified compounds can predominantly be classified into 8 chemical groups, including 14 alcohols, 12 aldehydes, 3 benzene derivatives, 4 carboxylic esters, 5 heterocyclics, 9 hydrocarbons, 10 ketones and 9 organic acids. Alcohols, aldehydes, esters, ketones, and organic acids are products mainly related to α- and β-oxidation, the lipoxygenase pathway and aldol condensation (Schwab and Schreier, 2002; Zunino et al., 2015). Volatile alcohols are regarded as potential aroma-active compounds generated from common lipid oxidation. For example, 3-methyl-1-butanol has a whiskey/malt/burnt odour, and 1-hexanol has a herbal odour (Dong et al., 2015b). Aldehydes originate from enzymatic oxidation of the double carbon-carbon bond of unsaturated fatty acids in cereals. For instance, nonanal and hexanal are regarded as lipid oxidation volatiles, and influence the aroma of cereal products. Volatile aldehydes and some ketones can also originate from Strecker degradation, which can convert α-amino acids into aldehydes and ketones with one fewer carbon atom by oxidative decarboxylation. The representative VOCs generated from Strecker degradation are 3-methyl-butanal with a malty flavour and benzaldehyde with an almond-like aroma (Waldvogel, 2010). Heterocyclic compounds including furans and furanones are produced by the peroxidation of lipids (Dong et al., 2013). Organic acids are derived directly from pyruvate or the branched tricarboxylic acid cycle. Hydrocarbons, comprising alkanes and alkenes (C_{10-16}), derived from decarboxylation of long-chain fatty acids, are mainly used to form epidermal wax and energy storage components in seeds (von Wettstein - Knowles, 2007). There is little published literature on benzene derivatives.

Based on hierarchical clustering, VOCs in barley at different ozone treatment times were scattered into four main clusters (Figure 3.4). Barley samples subjected to 0, 20, 240 and 1440 min were selected for further analysis, in accordance with findings of our germination result showing percent germination increased at 20 min ozone treatment, started to decrease at 240 min and dramatically decreased at 1440 min.

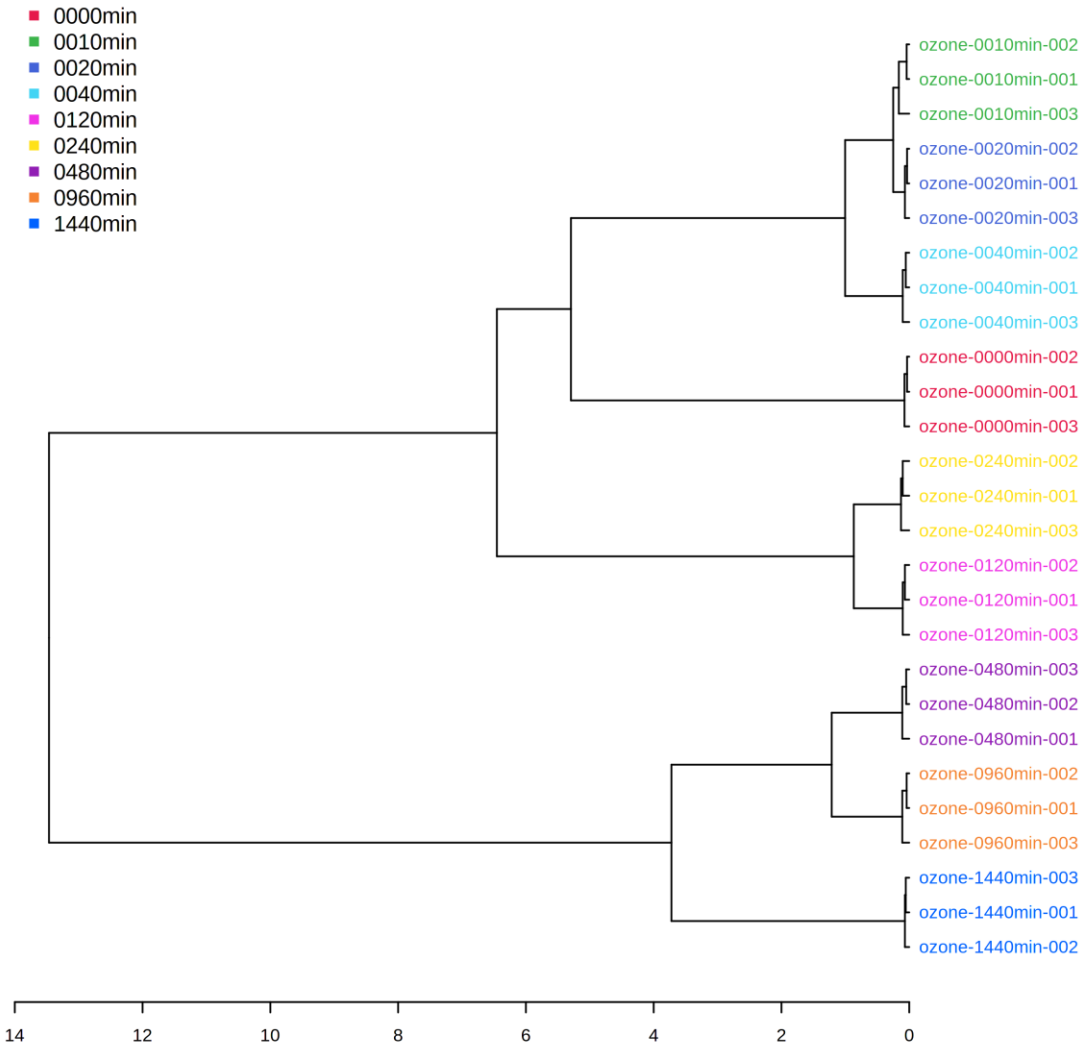

Figure 3.4. Clustering pattern shown as a dendrogram (distance measure using Spearman, and clustering algorithm using ward.D) of barley VOCs at different ozone treatment times.

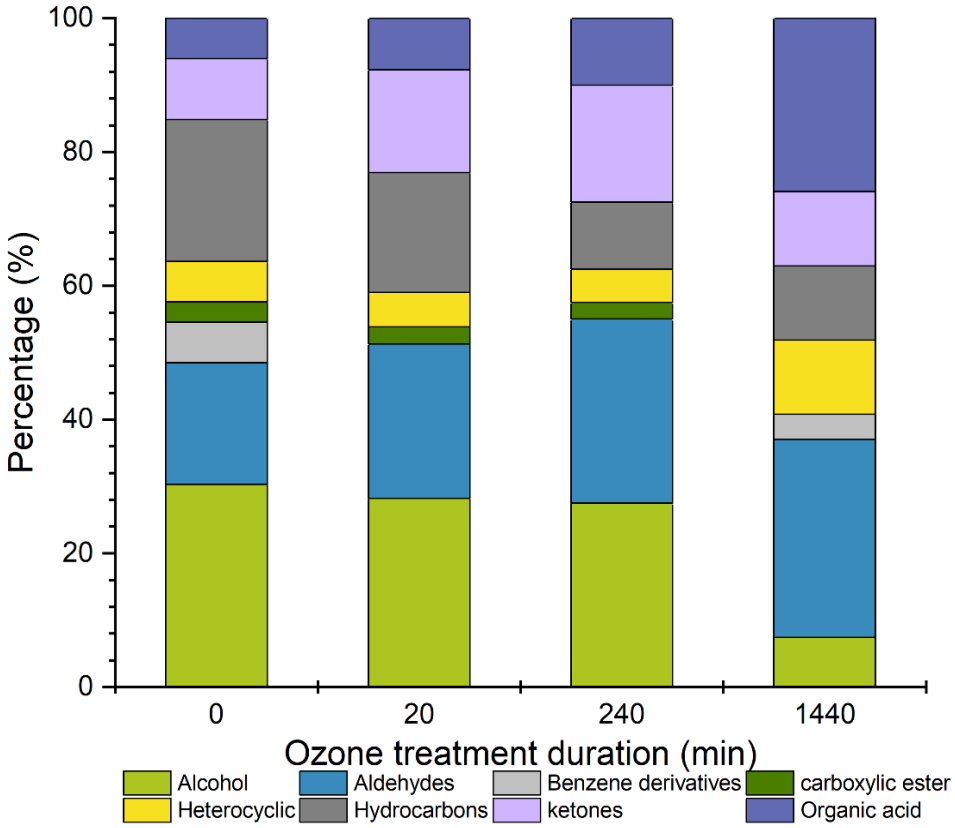

Figure 3.5. Relative abundances of metabolite classes of VOCs. Percentage is the ratio of the number of compounds in each group to the total number of compounds.

Chemical groups in barley seeds showed various patterns when subjected to different ozonation times. As shown in Figure 3.5, alcohols, hydrocarbons and aldehydes were the dominant groups in untreated barley seeds, representing about 30.03%, 21.2% and 18.2% of the total number of VOCs, respectively, followed by ketones, organic acids, heterocyclics, carboxylic esters, accounting for 9.1%, 6.1%, 6.1% and 3.0%, respectively. Generally, the number of alcohols and hydrocarbons decreased with extending ozone duration, declining to ~7.0% and ~10.0% of total VOCs after 1440 min ozone treatment, respectively. After 1440 min ozone treatment, aldehydes and organic acids were the main groups in barley seeds, accounting for 27.6% and 24.1% in the total number of VOCs, respectively, followed by heterocyclics (10.3%), hydrocarbons (10.3%), ketones (10.3%) and alcohols (6.9%). The reduction in alcohols and hydrocarbons, and increase in aldehydes and organic acids, could be connected to the oxidation of those compounds by ozone. Ozone can react with hydrocarbons, and all C–H bonds of alkanes are reactive (Fokin and Schreiner, 2002). The carbon-carbon double bond of alkenes is broken down by ozone and replaced by a carbon-oxygen double bond. Hydrocarbons,

including alkane and alkenes, can be oxidised by ozone to form alcohols, aldehydes, ketones and organic acids. The total number of ketones generally increased when the seeds were subjected to a relative short ozone treatment time (within 240 min), but rapidly declined at 1440 min. Carboxylic esters exhibited a similar trend to ketones but disappeared after 1440 min ozone treatment. Heterocyclics showed a stable tendency within 240 min ozone treatment, and increased at 1440 min. A possible explanation is that, initially, alcohols and aldehydes partially oxidised from hydrocarbons, can undergo further oxidation to ketones and organic acids, which are relatively stable to ozone. Subsequently, these two groups may decompose into carbon dioxide and water as the ketones and carboxylic acids are subjected to attack by radical hydroxyls (•OH) produced by ozonation.

3.3.3. Comparison of VOCs in barley at different ozone treatment times using multivariate analysis

Partial least squares-discriminant analysis (PLS-DA) was performed to evaluate variations among barley under nine ozone treatments. The metabolite profiling data was normalised by generalised logarithm transformation. The first two principal components (PCs) explained 90.6% of the total variance with 56.6% of PC1 and 34.0% of PC2, and the scores plot emphasised the separation between barley samples among four groups (Figure 3.6A). Ozone treatment times that increased or decreased germination ability were separated along PC1, accounting for the majority of explained variance. Cross-validation procedure was used to assess the quality of the PLS-DA models, summarizing by the value of R2 and Q2. R2 measures the goodness of fit, while Q2 measures the predictive ability of the model. The R2 and Q2 were 0.9978 and 0.9942 in this study, which indicated the perfect fitness and predictability of this model. Variable importance in projection (VIP) reflects the importance of variables in PLS-DA classification (Akarachantachote et al., 2014). Integrating the results of the statistical analyses and the VIP, nine VOCs with FDR<0.05 and VIP> 1.2 were selected, including hexanoic acid, 2,4-di-tert-butylphenol, 2-tridecanol, benzaldehyde, pentanoic acid, 3-methyl-1-butanol, 3-methyl-tridecane, acetic acid and 2-nonanone (Figure 3.6B). 2,4-di-tert-butylphenol and 3-methyl-1-butanol presented a negative correlation with ozone treatment time, mainly attributed to the hydroxyl free radicals quickly react with ozone. As a naturally occurring compound in at least 169 species, 2,4-di-tert-butylphenol possesses herbicidal and antioxidant properties, as well as toxicity on the root and leaf tissues of grassy weeds (Chuah et al., 2016), however there is a lack of information on 2,4-di-tert-butylphenol in barley. 3-methyl-1-butanol is regarded as a potential aroma-active compound in barley, which is also an immediate precursor of more flavour-active esters. Benzaldehyde is responsible for the fresh and slightly green notes of

barley, based on sensory attributes (Dong et al., 2015b). 2-nonanone and 3-methyl- tridecane were reported in specific wheat cultivars, but there are no reports for barley. Hexanoic acid, pentanoic acid and acetic acid are perceived as the odour compounds in barley (Takemitsu et al., 2019), which accumulated with extending ozone treatment time. The accumulation of organic acids is in agreement with other studies that demonstrated that ozone oxidises starch and introduces carbonyl and carboxyl groups, and lowers the pH of wheat flour (Sui et al., 2016; Lee et al., 2017). The reduction in pH appeared to be related to ozone duration and intensity (Lee et al., 2017). According to a previous study, phytotoxicity of organic acids to plant growth increased with longer acid carbon chains, thus acetic acid was used for further analysis.

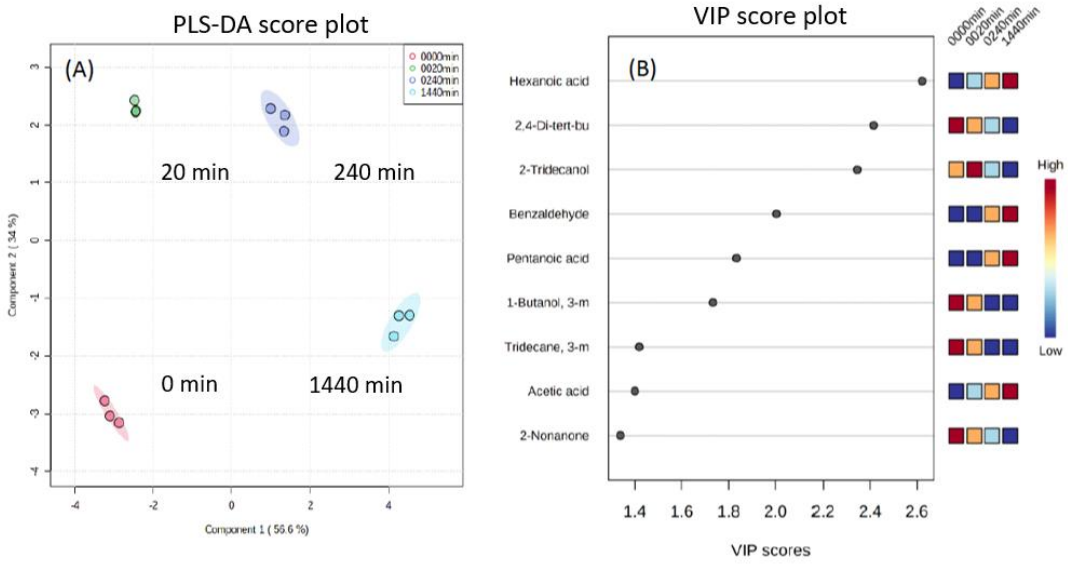

Figure 3.6. (A) Partial least squares-discriminant analysis (PLS-DA) scores plot for separation of barley samples at different ozone treatment times; (B) Variable importance in the projection (VIP) scores for different ozone treatment times. Coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each ozone treatment.

3.3.4. Validate the effect of acetic acid on barley seeds germination

In this study, barley seeds were subjected to 0, 0.05, 0.5, 1, 2 and 4 mg/g of acetic acid at 2, 5 and 24 h. According to Figure 3.7, both concentration and duration of acetic acid affected the germination ability of barley. In terms of concentration, an increasing tendency in barley germination was observed at 0.05 and 0.5 mg/g acetic acid, increasing approximately 3% compared with the control. The germination rate started to decrease at 1 mg/g acetic acid treatment for the 24-h treatment and dramatically declined at all treatments when acetic acid

concentration increased to 2 and 4 mg/g. Regarding acetic acid processing time, the 24-h treatment decreased germination rates at each concentration level.

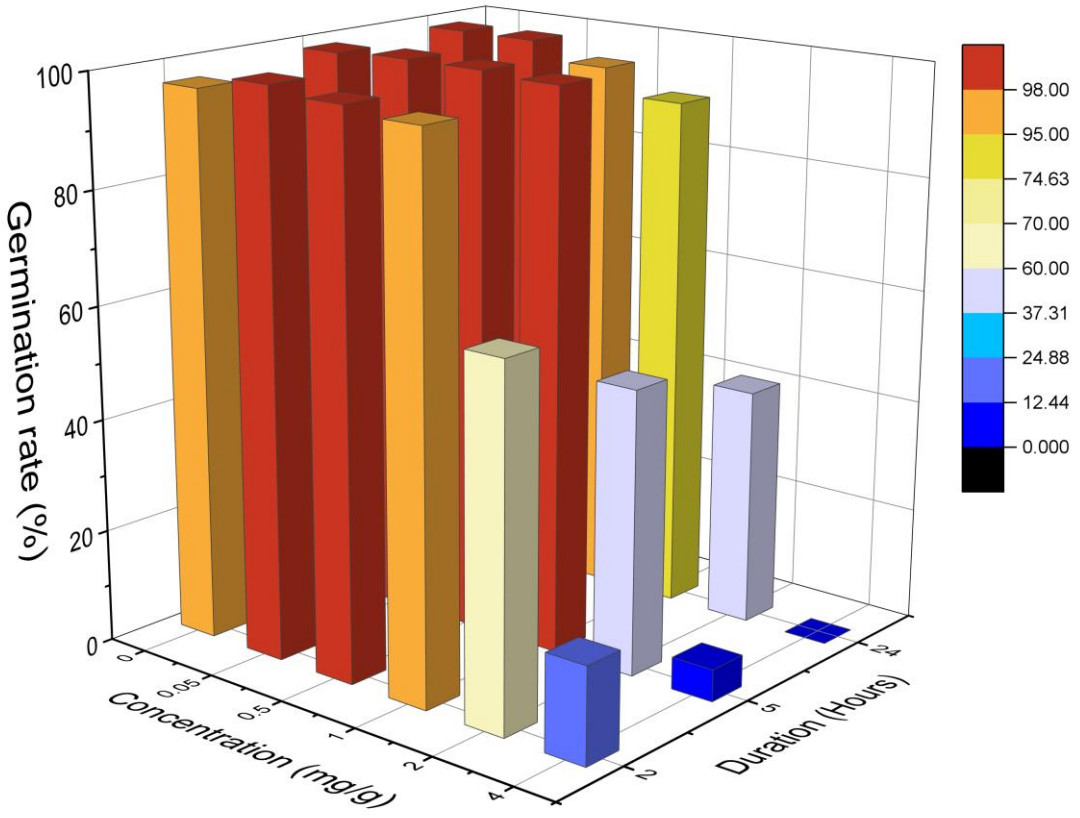

Figure 3.7. Three-dimensional bar chart showed the effect of different concentration and duration of acetic acid treatments on the germination of barley.

The slight stimulation of acetic acid at the lower concentrations at a short duration was discovered in this research. The acetyl group, derived from acetic acid, is fundamental to all forms of life, and plays a pivotal role in carbohydrate and fat metabolism when bound to coenzyme A. Acetyl coenzyme A (Acetyl-CoA) acts via the tricarboxylic acid (TCA) cycle to facilitate the synthesis of soluble carbohydrates necessary for germination (Muscolo et al., 2007). Longer processing times and higher concentrations of acetic acid inhibited the germination ability of barley seeds, which agrees with a study showing that 10 and 20% acetic acid inhibited maize seed germination (Pujisiswanto et al., 2013). Acetic acid interferes with the integrity of the cell membrane by enhancing electrolyte leakage of seeds which results in

decreasing germination ability (Pujisiswanto et al., 2013). Spancer and Ksander (1997) suggested that acetic acid may cause degradation of membrane proteins, causing loss of germination ability. Our research demonstrated that acetic acid, as the main product from ozone oxidation of VOCs, slightly facilitated barley germination ability at lower concentrations and short duration but strongly inhibited germination ability at long processing time and high concentration. Thus, exogenous application (fumigation) with acetic acid at a safe dose was able to stimulate the germination of barley seeds. However, metabolic profiles of additional VOCs such as terpenes, combined with enzyme assays, should be taken into consideration in further research to give a better depiction of how ozone influences the germination of barley.

3.4. Conclusions

In this study, an optimized analytical procedure for the analysis of VOCs was implemented to explore the alteration of VOCs in barley seeds in response to ozone treatments. With prolonging ozone treatment time, barley produced greater diversity and quantity of VOCs under oxidative stresses, suggesting some VOCs play important roles in adaptation to unfavourable growth conditions. Chemical groups in barley seeds showed various patterns when they were subjected to different ozonation times. Generally, the number of alcohols and hydrocarbons decreased with extending ozone duration, whereas aldehydes and organic acids markedly increased as the dominant groups with increasing ozone treatment time. Meanwhile, the PLS-DA results demonstrated that the VOC characteristics of barley under different ozone treatment were quite different. Hexanoic acid, 2,4-di-tert-butylphenol, 2-tridecanol, benzaldehyde, pentanoic acid, 3-methyl-1-butanol, 3-methyl-tridecane, acetic acid and 2-nonanone could be considered as the potential ozone stress specific markers. Moreover, this research demonstrated that acetic acid was a main product from ozone oxidation of VOCs, and that it slightly facilitated germination ability of barley at lower concentrations and short duration and strongly inhibited germination ability at long processing time and high concentration. Metabolic profiles of VOCs such as terpenes combined with enzyme assay should be taken into consideration in further research to give a better depiction of how ozone influence the germination of barley.

Attribution Statements

The following chapter has been drafted in accordance with the "*Foods*".

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The following authors contributed to this manuscript as outlined below.

Authorship order	**Contribution (%)**	**Concept Development**	**Data Collection**	**Data Analyses**	**Drafting of manuscript**
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Name 6 Litao Sun	2.5	X		X	
Name 7 Yitao Han	2.5			X	

Contribution indicates the total involvement the author has had in this project. Placing an 'X' in the remaining boxes indicates what aspect(s) of the project each author engaged in.

By signing this document, the Candidate and Principal Supervisor acknowledge that the above information is accurate and has been agreed to by all other authors.

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Candidate **Principal Supervisor**

Chapter Four

Effect of ozone treatment on metabolite profile of germinating barley

Abstract

Ozone is widely used to control pests in grain and impacts seed germination, a crucial stage in crop establishment which involves metabolic alterations. In this study, dormancy was overcome through after-ripening (AR) dry barley seed storage more than 4 weeks, alternatively, 15 min ozone treatment could break the dormancy of barley immediately after harvest, with accelerated germination efficiency remaining around 96% until four weeks. HS-SPME and liquid absorption coupled with GC-MS were utilized for metabolite profiling of 2-, 4- and 7-day germinating seeds. Metabolic changes during barley germination reflected by time-dependent characteristics. Alcohols, fatty acids, and ketones were major contributors to time-driven changes during germination. In addition, greater fatty acids were released at the early germination stage when subjected to ozone treatment.

4.1. Introduction

Barley *(Hordeum vulgare)* is a widely cultivated and highly adaptable crop predominantly used for stockfeed, food products and the brewing industry through the malting process. Besides a small amount of them used as seed for establish the next season's crop, harvested barley seeds is used for different purposes. The quality of barley significantly influences its end utilization since the germination rate of barley seed must be >95% for the malting process (Shrestha and Lindsey, 2019). There are many reasons causing failure of germination. A failure of an intact viable seed to complete germination under favorable conditions is defined as seed dormancy (Baskin and Baskin, 2004). Seed dormancy is a complex trait that is determined by genetic factors and substantial environmental conditions. Environmental factors such as moisture, temperatures, and light affect seed dormancy and regulate the timing of germination under natural conditions (Biddulph et al., 2007; Khan and Weber, 2007).

The majority of barley varieties have been bred with dormancy traits to prevent preharvest sprouting (Nakamura, 2018); however, dormancy that persists after harvest delays germination resulting in yield and malting losses. There are different methods for eliminating seed dormancy, including chemical, mechanical, and biological scarification; stratification; phytohormone application; exposure to photoperiod and thermoperiod; and combinations of them (Finch-Savage and Leubner-Metzger, 2006; Olszewski et al., 2010; Smirnova and Sadanov, 2019). Additionally, the potential for embryo dormancy can be overcome through after-ripening (AR) in dry seed storage (de Casas et al., 2012), which has been demonstrated in many species, including Arabidopsis thaliana, wheat, and barley (Liu et al., 2013; Gao et al.,

2012; Romagosa et al., 2001). However, induction of secondary dormancy and deterioration can occur in seeds during dry storage (Basbouss-Serhal et al., 2016).

Embryo dormancy is generally regulated by the content of phytohormones abscisic acid (ABA) and gibberellic acid (GA) (Bewley et al., 2012; Tuttle et al., 2015). It has been reported that ozone (O_3) may trigger antioxidants that regulate hormone levels, particularly abscisic acid (ABA), to break tomato seed dormancy and enhance germination (Sudhakar et al., 2011; Monroy Vazquez et al., 2017). Our previous study demonstrated that both a greater variety and quantity of VOCs were released under oxidative stress and that acetic acid can regulate barley germination depending on the dosage. However, metabolite changes during seed germination in response to ozone treatment remain elusive. Seed germination can be affected by intrinsic and extrinsic factors. The germination process is characterized by various metabolic processes resulting in distinct metabolic and time-dependent alterations. Seeds store food reserves such as proteins, carbohydrates, and lipids which are the source of energy, carbon, and nitrogen during germination and seedling establishment. Starch degradation into sugars and protein degradation into amino acids during seed germination have been extensively studied (Fincher, 1989; Sreenivasulu et al., 2008). Lipids are a minor component of seeds, playing a vital role in the structure and function of cell membranes and acting as an energy store to allow metabolism to continue during abiotic stress. During germination, fatty acids are released through lipid mobilization and then degraded through the β-oxidation and glyoxylate cycles, and subsequently converted into sugars (Graham, 2008). In addition, fatty acids are oxidized to hydroperoxide by lipoxygenase and then degraded to volatiles alkanes, aldehydes, ketones, and alcohols (Frankel, 1983; Wanasundara et al., 1999).

Numerous works focused on proteins (Qin et al., 2021), transcripts (Leymarie et al., 2007) and primary metabolites (Frank et al., 2011; Gupta et al., 2019) during barley germination, but the volatile metabolic profile of barley during germination is still unknown. In addition, metabolite changes in germinating seeds in response to ozone treatment remain elusive. This study aims to characterise VOCs and metabolites of germinating barley based on solid-phase microextraction (SPME) and liquid absorption combined with the gas chromatography-mass spectrometry (GC–MS). Furthermore, the impact of ozone on metabolite profiles in the germinating seeds will also be assessed.

4.2. Materials and methods

4.2.1. Barley seed material

Barley samples of varieties Scope CL and Flinders were harvested in 2020 from the site in Narrogin, Western Australia (32.94 S, 117.24 E), within 10.8% and 10.6% moisture content, respectively. The essential characteristics are shown in Table 4.1.

4.2.2. Ozone generation

A commercial ozone generator (Model FH-CYJ1520A-20 g/h, maximum concentration = 700ppm, Shanghai Fenghua electronics Technology Co., Ltd., Shanghai, China) was utilized to produce ozone from atmospheric air. Ozonation of barley seeds was carried out in a 2-L and 16.6-cm diameter Hysil semi-batch reactor with a desiccation glass chamber fitted with a lid containing a 2-cm central hole for ozonation. Gas was introduced from the ozone generator directly to the bottom of the reactor and passed through the seeds using a 1-cm diameter hose. One kilogram of barley seeds was placed on a supporting iron wire gauze 5 cm above the reactor bottom to enable gas circulation. The ozone concentration to and from the reactor was continuously measured by an ozone monitor (Shenzhen Yuan Technology Co., Ltd., Shenzhen, China).

Table 4.1. Basic information of the two varieties of barley seeds used in this study.

Properties	Scope CL	Flinders
Harvest year	2020	2020
Moisture content (%)	10.80	10.60
Thousand grain weight (g)	50.00	40.90
Germination rate (%)	92.90	91.90

Seeds were introduced into the reactor and treated with different O_3 treatment times at 700ppm, T1= 15 min, T2= 120 min, while the air was passed for the control (C) barley seeds. After treatment, seeds were removed from the reactor, dried at room temperature, and stored in a closed plastic container for further study.

4.2.3. Germination test

The germination tests were performed using treated seeds by giving different periods of rest time before the germination test, 1) germination test performed immediately after treatment

(T1, T2, C); 2) one week after O_3 treatment (1T1, 1T2, 1C); 3) two weeks after O_3 treatment (2T1, 2T2, 2C); 4) four weeks after O_3 treatment (4T1, 4T2, 4C); 5) thirteen weeks after O_3 treatment (13T1, 13T2, 13C). These experimental conditions followed a complete factorial design with three independent factors: variety, treatment duration, and rest time before germination. After ozone treatment, the sample was stored at 25±1℃, 11% RH. The germination test was carried out based on the between-paper (BP) method of the International Seed Testing Association methods (ISTA, 2006). As shown in Figure 4.1, 424 (eight replicates of 53 seeds) of each treatment were randomly selected. A large filter paper was saturated with 60 mL distilled water and folded in half, creating a double thickness. A steel template (290 × 580 mm) was placed over this wet paper, with holes in this stencil allowing barley seeds to be placed 30 mm apart. Once the seeds were positioned correctly, the template was removed, and the upper half of the filter paper was folded over the seed area. The folded paper containing the seeds was then loosely rolled from one side perpendicular to the base and tied with elastic bands. All treatments were stored individually in polythene zip-lock bags and incubated for 7 days at 25±1°C in the dark. Visible radicle protrusion was used as the criterion for germination. The germination rate was detected 7 days after the germination test. Meanwhile, germinated seeds were collected for further metabolic profiling 2, 4 and 7 days after the start of the germination test.

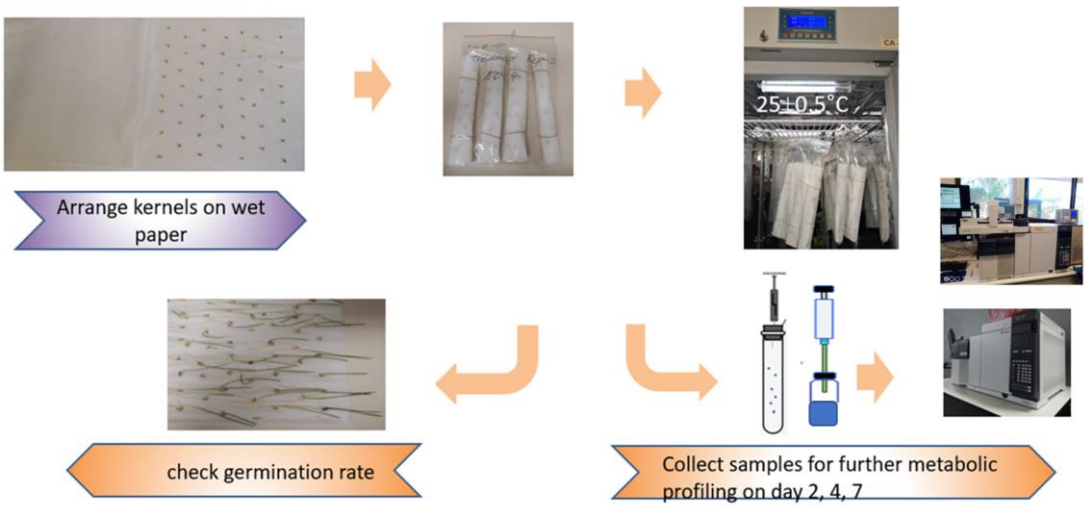


Figure 4.1. Diagram of germination test and sample collection.

4.2.4. Liquid absorption VOC collection

Grain samples collected at 2, 4 and 7 d during germination were used for metabolic analysis. 0.35 g germinated seeds were placed in 2 mL Eppendorf microcentrifuge tubes. 500 ul acetonitrile and 3 silicon beads were added to the vial and ground at 4000 rpm for 120 s. 500 ul acetonitrile was added and vortexed at 4000 rpm for 60 s, then ground at 4000 rpm for a further 120s. The solution was stood at room temperature for 1 h. After completion of the liquid extraction, 200 ul supernatant was transferred to a glass auto-sampler vial, with 1 μL of the liquid adsorbent being injected into the GC-MS by liquid auto-sampler.

4.2.5. HS-SPME VOC collection

One gram samples of germinating barley seeds were transferred into 25 mL SPME vials with screw caps. A three-phase SPME fibre (50/30 μm) with a 2-cm combination coating of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm, Agilent Technologies, Santa Clara, California, USA) was utilized for VOC absorption. After exposure to the headspace of the vial at 25 °C for 60 min, the fiber was removed from the vial and desorbed for 7 min onto the GC injection port operated in splitless mode. A vial containing moistened filter paper (deionized water) was used for control.

4.2.6. GC-MS analysis of seed volatiles

For the HS-SPME sample, an Agilent 7890B GC system coupled with 5977B MSD was employed for compound analysis with an Agilent HP-5MS capillary column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness, 5% phenyl and 95% dimethylpolysiloxane stationary phase). After extraction, samples were injected in splitless mode with ultra-high purity helium (Air Liquide, Perth, Australia) as the carrier gas at a constant flow rate of 1.0 mL/min. The temperature program started at 40℃ for 2 min, ramped at 5℃/ min to 180℃, then at 20℃/min to 260℃ and then held at 260℃ for 4 min. The detector was operated in electron impact (EI) ionisation mode at 70 eV and the spectra were acquired in a range from 40 to 400 atomic mass units (amu). The ion source and quadruple temperatures were maintained at 230°C and 150°C, respectively. The transfer line temperature of the MSD was 280°C.

For liquid samples, the Agilent 7693A Automatic Liquid Sampler (ALS) GC system coupled with 5977E MSD was employed for compound analysis with an Agilent HP-5MS capillary column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness, 5% phenyl and 95% dimethylpolysiloxane stationary phase). After extraction, samples were injected in splitless mode with ultra-high purity helium (Air Liquide, Perth, Australia) as the carrier gas at a

constant flow rate of 1.0 mL/min. The temperature program started at 40℃ for 4 min, ramped at 5℃/ min to 180℃, then at 15℃/min to 300℃ and then held at 300℃ for 4 min. The total analysis time was 44 min and the run was carried out with a solvent delay of 2 min. The detector was operated in electron ionisation (EI) mode at 70 eV and the spectra were acquired in a range from 40 to 600 atomic mass units (amu). The ion source and quadrupole temperatures were maintained at 230°C and 150°C, respectively. The transfer line temperature of the MSD was 280°C. The individual peaks were categorised by comparison with NIST 05- NIST Mass Spectral. Volatile compounds were identified using an experimentally obtained Kovats retention index (RI) with the combination of C7-40 alkane standards and mass spectra in the National Institutes of Standards and Technology Mass Spectrometry (NIST MS) library.

4.2.7. Data analysis

Data acquisition was performed using Mass Hunter Acquisition software (vB.06.00; Agilent Technologies, Santa Clara, California USA), and data were expressed as the mean ± standard deviation of three replicates. Multivariate analyses, including partial least squares discriminant analysis (PLS-DA) with one way-analysis of variance (one way-ANOVA), were performed by MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/). Tukey's post-hoc test (HSD) based on the *P*-value (α=0.05) was used for identifying statistical differences among different treatments. The cluster heatmap was performed to visualise a hierarchically clustered data matrix. Figures were generated using Origin software (Version 2021b, OriginLab, Northampton, Massachusetts, USA).

4.3. Result and discussion

4.3.1. The effect of storage time on barley germination

In this study, two different malting barley varieties were utilized for the germination test. To best used for malting, barley requires a germination greater than 95%. In this study, after the harvest, the germination rate of Scope CL and Flinder was 92.93% and 91.98%, respectively (Figure 4.2), Viable grain may not germinate under favourable conditions if the seed is dormant, which prevents preharvest sprouting in the field. However, dormancy that persists after harvest is undesirable because it prevents malting barley germination resulting in malting loss (Jacobsen et al., 2002). The germination data demonstrated that the general trend of two varieties' germination ability gradually increased during storage. The germination rate of Scope CL increased from 92.93% to 95.23 % after 4 weeks of storage, while that of Flinder increased from 91.58% to 94.81% after 2 weeks of storage, which is in accordance with previous research

suggesting that during dry storage at room temperature, germination percentage of seeds increased (Favier, 1995; Soltani et al., 2017). Primary dormancy is often lost during after-ripening in many species, including wheat and barley (Romagosa et al., 2001; Liu et al., 2013). Peanut seeds increased ethylene production during after-ripening to reverse dormancy (Ketring and Pattee, 1985). Dormancy loss during after-ripening in sunflower seeds was correlated with a reduction in sensitivity to abscisic acid (ABA), an inhibitor of germination (Rodríguez et al., 2018). During after-ripening, wheat seeds became sensitive to treatment with gibberellin (GA), which promoted germination, and then became insensitive to ABA (Tuttle et al., 2015). Although much progress has been made in understanding after-ripening, the specific mechanisms of dormancy alleviation by after-ripening remain poorly understood.

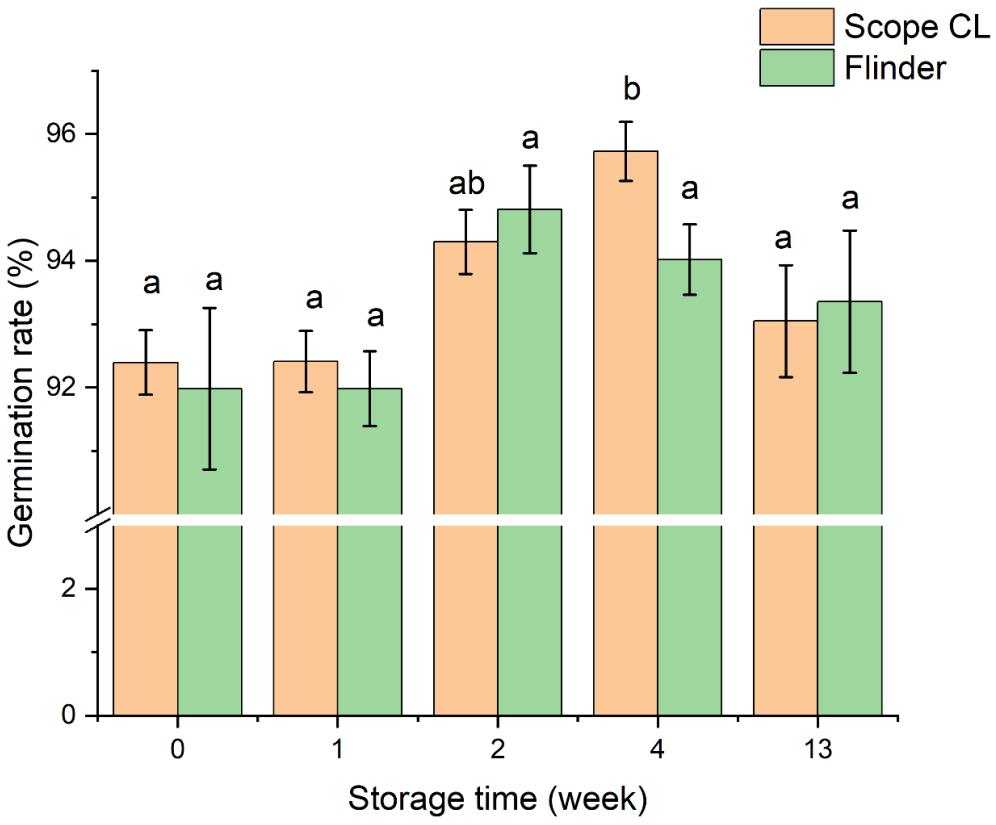


Figure 4.2. Effect of storage time on barley germination in Scope CL and Flinder varieties.

4.3.2. Influence of ozone in barley seed dormancy alleviation

The effect of O_3 on seed dormancy alleviation is presented in Figure 4.3. The data demonstrated that 15 min O_3 treatment at 700ppm promoted germination ability of Scope CL and Flinder to 95.52% and 93.40% (germination test performed immediately after treatment). Samples treated with O_3 for 15 min remained at the accelerated germination efficiency during storage up to four weeks. In terms of Scope CL, the germination rate of 1T1, 2T1 and 4T1 was enhanced to 95.52%, 95.99% and 96.46%, but 13T1 did not show sustained germination acceleration efficiency. Flinder showed the same tendency. The germination rate of 1T1, 2T1 and 4T1 was enhanced to 94.10%, 94.88% and 95.52%; however, 13T1 had decreased to 92.92%.

Some studies have indicated that O_3 can accelerate the germination of tomato seeds because reactive oxygen species (ROS) produced by O_3 promote cell signalling (Sudhakar et al., 2011). Ozone could also induce gene expression for GA synthesis (*GA20ox1*) and ABA catabolism (*ABA80 OH*) (Ma et al., 2020). Some studies suggested that O_3 promotes VOC release, such as acetic acid acetaldehyde, ethanol and ethyl acetate, which were highly correlated with seed germination (Michalak et al., 2021). Seeds treated for 120 min were found to be injured, with the seeds recovering from injury as time progressed. Initially, the germination rate of Scope CL and Flinder was 85.38% and 86.09% when subjected to 120 min ozone treatment. However, it gradually increased to 93.08% and 93.63% after 4 weeks of storage, indicating that, as time progressed, O_3 treatment loses its effect.

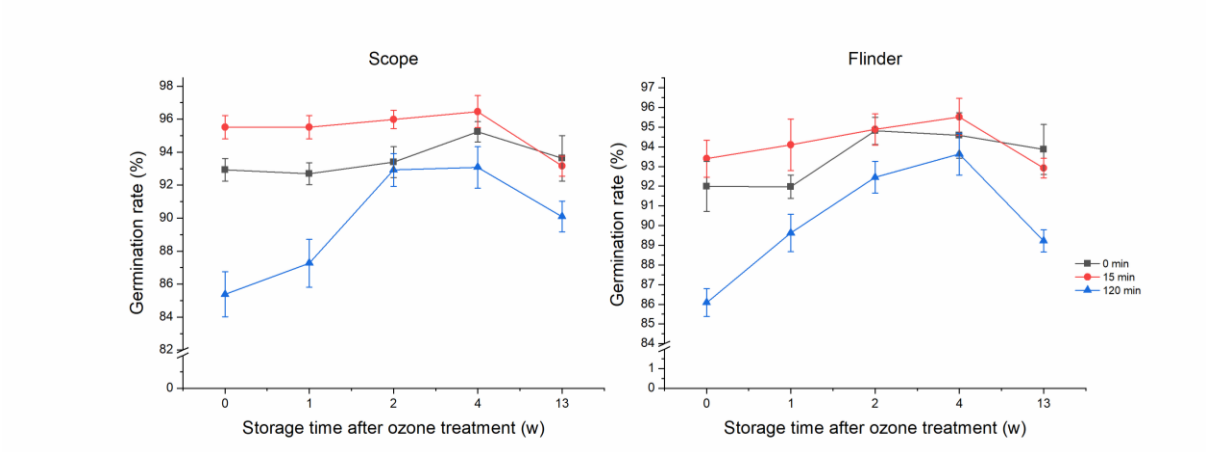


Figure 4.3. The effect of ozone treatments on seed dormancy alleviation based on 3 independent factors, i.e., variety, ozone treatment duration, and storage time after ozone treatment.

4.3.3. The changes in metabolites profiles during germination and their response to ozone treatment

In this study, the dormancy of both Scope CL and Flinder was reversed with 15 min ozone treatment and showed the largest difference after one week storage compared with control. Therefore, the Scope CL sample after one week of storage was utilized for VOC profiling. For GC-MS data, peaks were integrated into the total ion chromatogram (TIC). Any peak with more than 5000 total peak area was considered as detected and subsequently integrated. A total of 24 and 27 volatile compounds in barley seeds were identified by SPME and liquid adsorption methods based on retention time and mass spectral data from the NIST Mass Spectral Library. Metabolite profiling data were normalised by generalised logarithm transformation (Tables 4.2 and 4.3). As shown in Figure 4.4A, identified compounds based on HS-SPME can be classified into six chemical groups. Alcohols, hydrocarbons, and aldehydes were the dominant groups, representing about 28.6%, 23.8%, and 19% of the total number of VOCs, respectively, followed by ketones and phenols heterocyclics, accounting for 14.3%, 9.5% and 4.8%, respectively. However, compounds obtained by liquid absorption showed different chemical patterns. Ketones, heterocyclics and phenols were the main groups, occupying 35.7%, 28.6 and 14.3%, followed by fatty acids, aldehydes and alkaloids, representing about 10.7%, 7.1% and 3.6% (Figure 4.4B). In this study, alcohols and hydrocarbons were obtained only by HS-SPME. Volatile alcohols regarded as potential aroma-active compounds generated from common lipid oxidation are easily extracted by HS-SPME due to their high volatility. Fatty acids were only extracted by liquid absorption, which meant that the HS-SPME method with 25℃ extraction temperature might not allow trapping of sufficient fatty acids. Hordenine was also detected only by the liquid absorption method. As an active compound during barley germination, hordenine has numerous health benefits, including inhibiting melanin content, promoting weight loss and potential anti-cancer activity (Kim et al., 2013; Anwar et al., 2020). Compounds obtained from both methods were combined for further analysis.

Table 4.2. Analysis of volatile compounds measured by HS-SPME-GC-MS of germinating barley seeds under different ozone treatment times.

Chemical groups	Compounds	RT[a]	RI (lib)[b]	RI (cal)[c]	Peak area (log_{10})					
					Control			Ozone (15 min)		
					D2	D4	D7	D2	D4	D7
Alcohol	2-Propanol, 1-methoxy-	4.11	661		n.d.[d]	n.d.	n.d.	5.44 ± 0.40	n.d.	n.d.
	1-Butanol, 3-methyl-	5.45	736	732	n.d.	4.32 ± 0.21	4.74 ± 0.04	n.d.	n.d.	4.53 ± 0.06
	1-Butanol, 2-methyl-	5.54	739	736	n.d.	4.18 ± 0.24	4.19 ± 0.03	n.d.	4.01 ± 0.19	4.37 ± 0.07
	2-Pentanol,4-methyl-	6.67	758	778	n.d.	n.d.	4.67 ± 0.31	n.d.	4.63 ± 0.24	4.90 ± 0.86
	2,3-Butanediol	7.01	788	791	n.d.	n.d.	4.80 ± 0.09	4.55 ± 0.44	5.12 ± 0.36	4.94 ± 0.09
	3-Hexen-1-ol, (Z)	9.02	839	856	n.d.	4.93 ± 0.39	5.21 ± 0.20	n.d.	n.d.	4.91 ± 0.01
	1-Hexanol	9.42	868	868	n.d.	n.d.	4.51 ± 0.04	n.d.	n.d.	n.d.
	1-Hexanol, 2-ethyl-	14.67	1030	1029	n.d.	4.68 ± 0.15	n.d.	n.d.	n.d.	4.36 ± 0.17
	2-Nonen-1-ol, (E)-	19.09	1169	1171	4.23 ± 0.14	4.58 ± 0.34	4.80 ± 0.14	4.63 ± 0.28	4.82 ± 0.06	n.d.
Aldehyde	Acetaldehyde	2.75			n.d.	5.20 ± 0.09	n.d.	n.d.	5.07 ± 0.26	n.d.
	Hexanal	7.26	801	801	4.24 ± 0.16	4.96 ± 0.06	4.60 ± 0.22	4.65 ± 0.24	4.37 ± 0.04	4.61 ± 0.23
	Octanal	13.85	1003	1003	n.d.	n.d.	4.53 ± 0.18	n.d.	n.d.	n.d.
	2-Nonenal, (E)-	18.82	1162	1162	3.70 ± 0.14	4.90 ± 0.15	5.46 ± 0.17	n.d.	4.58 ± 0.48	n.d.
Heterocyclic	Furan, 2-pentyl-	13.48	993	995	3.90 ± 0.16	4.53 ± 0.20	4.28 ± 0.08	4.09 ± 0.25	3.81 ± 0.21	4.34 ± 0.10
Hydrocarbon	7-Tetradecene	24.33	1369	1356	n.d.	4.49 ± 0.14	4.59 ± 0.21	n.d.	3.71 ± 0.05	4.55 ± 0.09
	(-)-Aristolene	26.39	1453	1435	4.30 ± 0.14	4.62 ± 0.27	4.35 ± 0.08	4.35 ± 0.09	4.05 ± 0.07	4.66 ± 0.02
	Aromadendrene	26.58	1461	1443	4.41 ± 0.38	4.84 ± 0.30	n.d.	4.13 ± 0.19	3.65 ± 0.10	n.d.

	β-Guaiene	26.87	1490	1454	4.70 ± 0.12	4.58 ± 0.04	4.28 ± 0.07	4.53 ± 0.12	4.26 ± 0.14	4.57 ± 0.11
Ketone	Acetone	2.02			n.d.	5.62 ± 0.07	6.31 ± 0.12	n.d.	5.48 ± 0.10	6.30 ± 0.13
	Acetoin	4.91	713	712	n.d.	5.82 ± 0.24	6.12 ± 0.14	n.d.	5.99 ± 0.39	5.72 ± 0.09
	5-Hepten-2-one, 6-methyl-	13.34	986	991	n.d.	4.42 ± 0.25	n.d.	4.34 ± 0.07	n.d.	n.d.
	4-Acetyl-1-methylcyclohexene	17.43	1110	1116	4.36 ± 0.14	4.76 ± 0.06	n.d.	n.d.	n.d.	n.d.
Phenol	Phenol	13.07	981	982	4.08 ± 0.28	n.d.	n.d.	4.39 ± 0.19	n.d.	n.d.
	2,4-Di-tert-butylphenol	28.39	1519	1515	n.d.	4.51 ± 0.26	n.d.	n.d.	n.d.	n.d.

[a] Retention time.

[b] Retention index by searching NIST library.

[c] Retention index calculated by C_7-C_{40} alkanes external standards.

[d] Not detected.

Table 4.3. Analysis of volatile compounds of germinating barley seeds under different ozone treatment times by liquid absorption.

Chemical groups	Compounds	RT[a]	RI (lib)[b]	RI (cal)[c]	ck			Ozone (15 min)		
					D2	D4	D7	D2	D4	D7
Aldehyde	Benzeneacetaldehyde	17.06	1045	1047	n.d.[d]	6.09 ± 0.04	5.47 ± 0.02	5.45 ± 0.29	5.57 ± 0.17	5.30 ± 0.26
	2-Decenal, (Z)-	23.70	1252	1263	5.25 ± 0.06	5.65 ± 0.10	5.43 ± 0.01	5.11 ± 0.01	5.18 ± 0.07	5.16 ± 0.03
	2-Undecenal	26.52	1367	1365	5.20 ± 0.05	n.d.	n.d.	4.95 ± 0.04	4.74 ± 0.01	5.01 ± 0.13
Alkaloid	Hordenine	29.21	1495	1479	6.15 ± 0.01	6.02 ± 0.03	6.63 ± 0.16	6.13 ± 0.06	6.38 ± 0.12	6.55 ± 0.08
Fatty acid	Acetic acid	5.18	610		4.94 ± 0.09	7.19 ± 0.03	7.04 ± 0.05	n.d.	6.74 ± 0.05	7.01 ± 0.05
	Palmitic acid	36.87	1968	1962	5.43 ± 0.01	6.85 ± 0.02	6.91 ± 0.06	6.12 ± 0.21	6.62 ± 0.08	n.d.
	Oleic acid	38.23	2133	2141	5.20 ± 0.07	6.55 ± 0.08	6.73 ± 0.01	5.71 ± 0.12	6.44 ± 0.13	6.88 ± 0.01
	stearic acid	38.37	2172	2161	4.36 ± 0.17	5.55 ± 0.02	5.53 ± 0.05	n.d.	n.d.	6.73 ± 0.09
Heterocyclic	2-Furanmethanol	10.88	860	869	n.d.	6.22 ± 0.08	5.67 ± 0.18	n.d.	5.66 ± 0.13	5.52 ± 0.44
	2-furancarboxaldehyde, 5-methyl	14.30	964	965	n.d.	5.82 ± 0.12	5.33 ± 0.04	n.d.	n.d.	5.44 ± 0.12
	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	14.98	989	984	4.61 ± 0.23	6.29 ± 0.13	5.76 ± 0.19	n.d.	5.18 ± 0.25	5.73 ± 0.40
	Furaneol	17.61	1070	1063	n.d.	6.37 ± 0.01	5.90 ± 0.02	n.d.	5.48 ± 0.05	5.79 ± 0.23
	Maltol	19.30	1110	1116	5.19 ± 0.02	6.24 ± 0.03	5.12 ± 0.03	n.d.	n.d.	5.50 ± 0.06

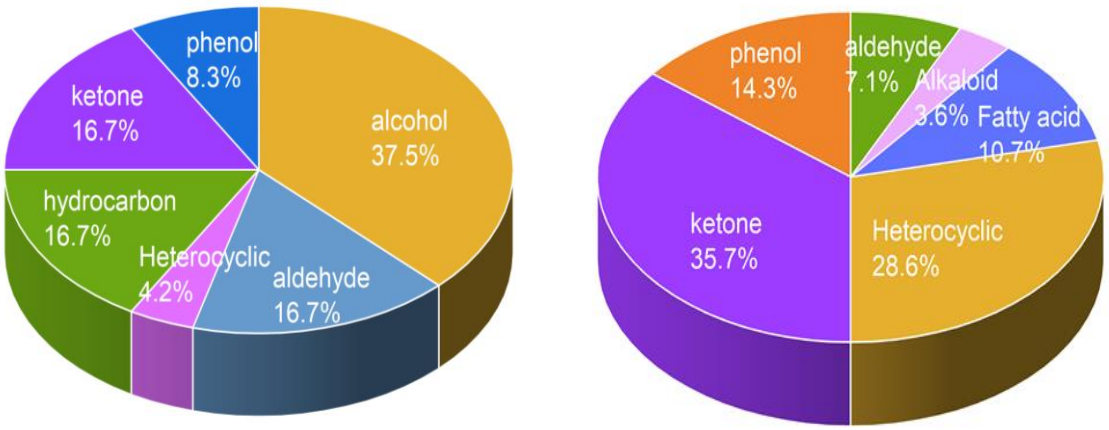
	2(3H)-Furanone, dihydro-4-hydroxy-	20.68	1185	1161	4.34 ± 0.10	6.49 ± 0.18	5.62 ± 0.07	n.d.	n.d.	5.68 ± 0.19
	5-Hydroxymethylfurfural	22.72	1232	1229	4.55 ± 0.09	6.94 ± 0.08	5.76 ± 0.17	n.d.	n.d.	5.61 ± 0.19
Ketone	2-Propanone,1-hydroxy	5.87	666		4.66 ± 0.02	6.76 ± 0.01	6.74 ± 0.09	n.d.	6.51 ± 0.06	6.71 ± 0.02
	4-Cyclopentene-1,3-dione	11.51	883	886	n.d.	6.16 ± 0.07	5.67 ± 0.01	n.d.	5.49 ± 0.15	5.68 ± 0.20
	2-Cylopenten-1-one, 2-hydroxy-	13.13	926	932	5.06 ± 0.15	6.44 ± 0.10	5.99 ± 0.05	5.38 ± 0.17	5.72 ± 0.11	5.93 ± 0.21
	2-Hydroxy-gamma-butyrolactone	15.36	1011	995	6.30 ± 0.13	6.42 ± 0.01	6.18 ± 0.02	5.16 ± 0.35	5.78 ± 0.05	6.16 ± 0.07
	1,2-Cyclopentanedione,3-methyl-	16.54	1028	1031	5.72 ± 0.09	n.d.	n.d.	n.d.	n.d.	5.25 ± 0.14
	2-Pentanone, 5-(acetyloxy)-	17.32	1053	1055	n.d.	n.d.	n.d.	n.d.	4.94 ± 0.19	5.53 ± 0.05
	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-	20.27	1150	1147	n.d.	7.00 ± 0.07	6.49 ± 0.13	n.d.	5.79 ± 0.06	6.58 ± 0.20
	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	21.53	1196	1188	n.d.	5.73 ± 0.10	n.d.	n.d.	n.d.	5.36 ± 0.08
	2-Heptadecanone	36.36	1904	1903	5.18 ± 0.01	5.18 ± 0.01	n.d.	4.97 ± 0.09	4.70 ± 0.02	n.d.
Phenol	4-Vinylphenol	22.38	1223	1217	n.d.	5.54 ± 0.01	5.27 ± 0.12	n.d.	n.d.	5.32 ± 0.20
	2-Methoxy-4-vinylphenol	25.25	1316	1318	n.d.	5.44 ± 0.07	5.39 ± 0.18	4.76 ± 0.13	5.01 ± 0.08	5.58 ± 0.17
	Phenol, 4-ethenyl-2,6-dimethoxy-	31.68	1567	1584	n.d.	5.52 ± 0.05	5.78 ± 0.13	n.d.	5.23 ± 0.04	5.92 ± 0.02

[a] Retention time.

[b] Retention index by searching NIST library.

[c] Retention index calculated by C_7-C_{40} alkanes external standards.

[d] Not detected.



(A) **(B)**

Figure 4.4. Chemical patterns of germinating barley seeds based on (A) HS-SPME and (B) liquid absorption coupled with GC-MS.

Since oxidative conditions resulted in high variability in barley VOC profiles based on our previous research, partial least squares-discriminant analysis (PLS-DA) was carried out to evaluate variation among germinating barley with ozone treatment. In the original data, metabolites with more than 50% missing values were removed from subsequent analysis; the expression of metabolites was normalized by log-transformation and mean-centered scaling. Compounds presented variation in different germinating times, as shown by the complete separation among metabolite samples in the PLS-DA, indicating that considerable metabolic changes happened during germination. The first two principal components (PCs) explained 73.5% of the total variance with 33.9% of PC1 and 39.6% of PC2, and the scores plot emphasised the separation between barley samples among different germinating days (2, 4 and 7 days after imbibition) (Figure 4.5). Barley samples at different germination stages were separated along PC1, and ozone-treated and control samples were separated along PC2. The difference in metabolites profiles between ozone-treated and control samples started at 2 days and it reached the highest 4 days during germination, suggesting ozone played a critical role in metabolite changes during germination, especially at 4 days. Clear separation among the treatments (CK and ozone treatment) was also detected at 7 days during germination, indicating that ozone treatments substantially affected germination patterns.

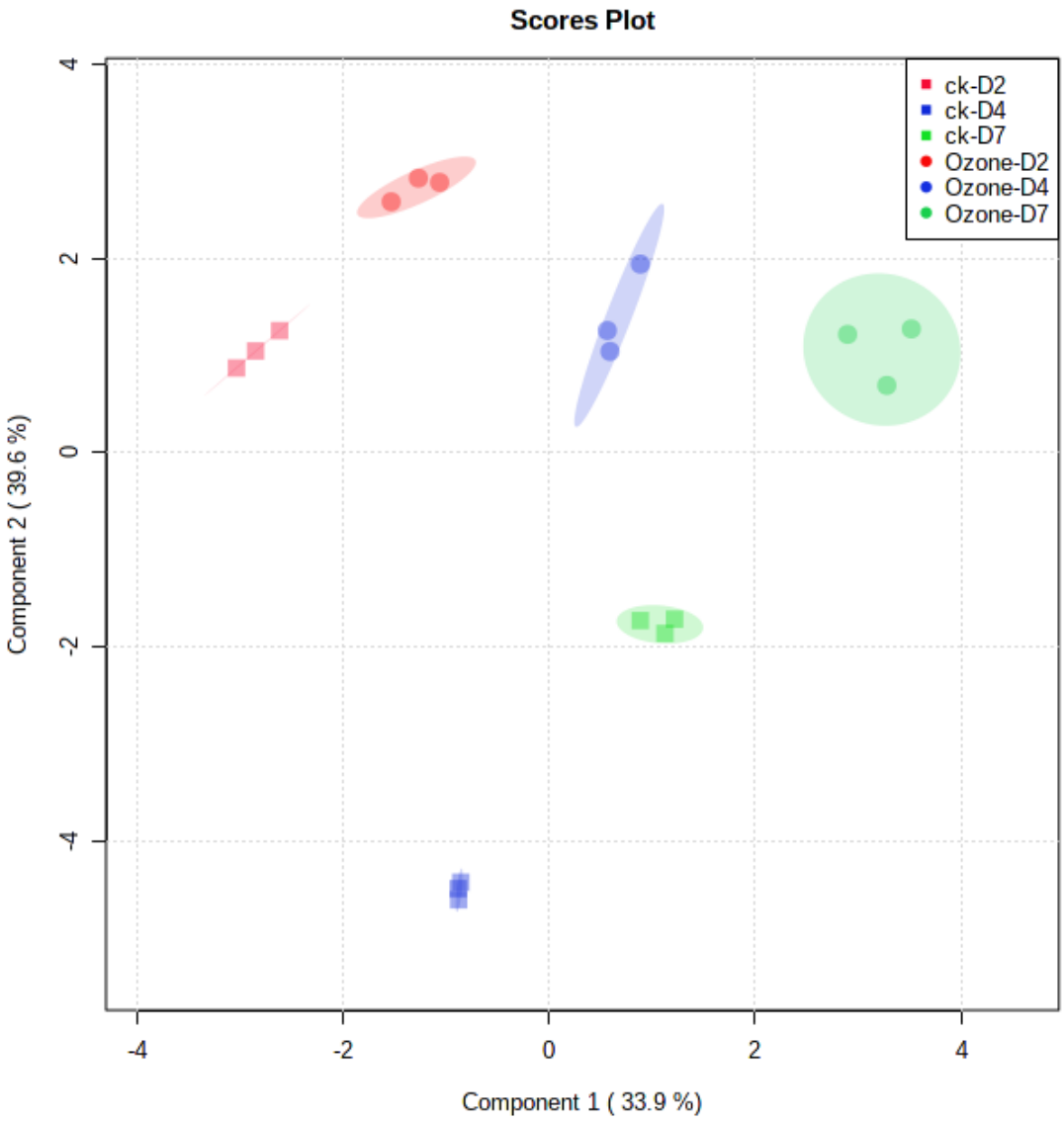


Figure 4.5. Partial least squares-discriminant analysis (PLS-DA) scores plot for metabolite profiles of germinating barley with ozone treatment.

Chemical groups in barley seeds showed various patterns during the germination process. Fatty acids, alcohols, and ketones were major contributors to time-driven changes during germination (Figure 4.6). The data demonstrated that more fatty acids were released from days 2 to 7 in both ozone-treated and control samples. Seeds contain lipids for energy storage, and the mobilization of storage lipid during seed germination begins with the hydrolysis of the glyceride into free fatty acids and glycerol (Borek et al., 2015). Here, the ozone-treated sample produced a greater quantity of fatty acid 2 days after imbibition, but fewer fatty acids 4 days after imbibition compared with the control. As an oxidizing agent, ozone could promote lipid oxidation to release more fatty acids and alcohols at an early stage. Subsequently, fatty acid

could be broken down into acetyl coenzyme A (acetyl-CoA) through β-oxidation and enter the citric acid cycle (TCA cycle) to supply carbon skeletons and energy for germination (Jin et al., 2013).

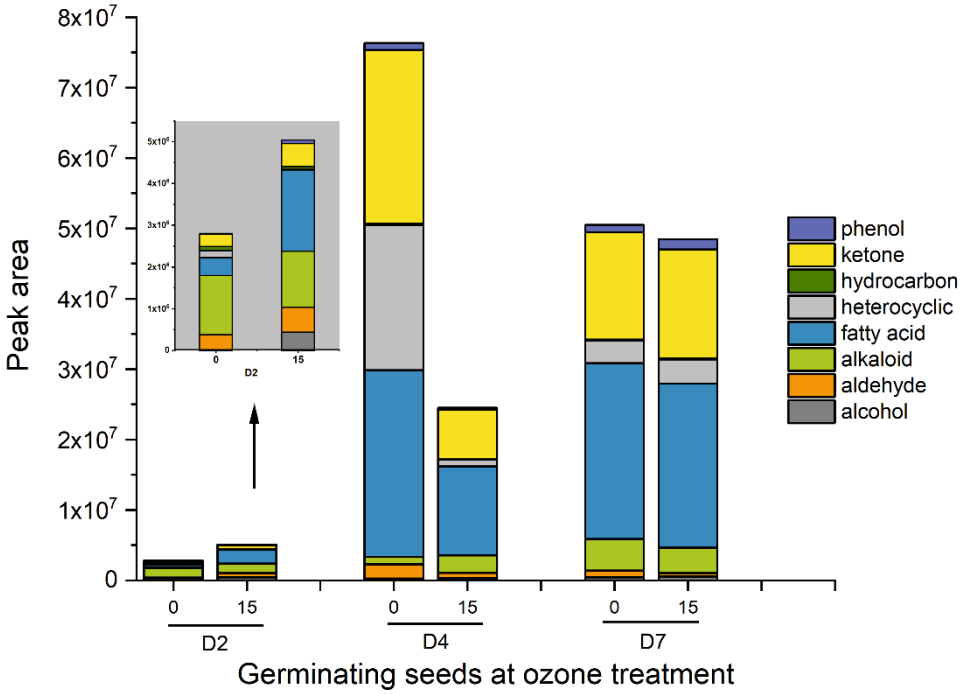


Figure 4.6. Volatile compound composition of germinating seeds (2, 4 and 7 days during germination) at ozone (0 and 15 min) treatment. The compounds were analysed using solid-phase microextraction (SPME) and liquid absorption.

4.3.4. Dynamic changes of metabolites in barley during germination

Metabolic responses during germination are presented in a heatmap (Figure 4.7). Variable importance in projection (VIP) reflects the importance of variables in PLS-DA classification. Integrating the results of the statistical analyses ANOVA and VIP score, 12 VOCs with FDR<0.05 and VIP>1.2 were selected, including 2 alcohols, 3 acetic acids, 4 ketones, 1 aldehyde, 1 phenol, and 1 hydrocarbon (Figure 4.8). 2,3-butanediol increased during germination process in both ozone-treated and control samples. 2,3-butanediol is a crucial volatile component detected in barley, which has been reported to enhance seed germination and seedling growth (Ryu et al., 2003). Ozone treatment induced more 2,3-butanediol (Figure 4.7), which could facilitate barley germination. In addition, oleic acid and stearic acid are the main components of germinating barley, which increased in both ozone-treated and control

samples during germination. The increase in fatty acids may have been due to glyceride hydrolysis. The same variation was also observed in peanut and foxtail millet germination (Chiou et al., 1997; Li et al., 2020). Palmitic acid increased and reached the maximum at 7 days germination in control samples, but 4 days germination in ozone treated samples. Palmitic acid is a long chain fatty acid, playing a vital role in regulating energy metabolism. Ozone induced greater palmitic acid levels at an earlier stage compared to control, which might be attributed to enhanced germination ability by supplying more energy. Acetone was not detected at day 2 germination, appeared at day 4, and then increased at day 7 in barley germination, in accordance with trends observed in lima beans (Murphy, 1985). In addition, acetone production in seeds was closely correlated with lipid metabolism during germination (Murphy, 1985). As an aldehyde in barley seeds contributing to rancidity in beer (Filipowska et al., 2021), (E)-2-nonenal is derived from enzymatic or nonenzymatic oxidation of lipids and fatty acids (Hambraeus and Nyberg, 2005). Here, (E)-2-nonenal increased during germination, however, ozone treated sample showed a reduction of (E)-2-nonenal at day 7 germination, suggesting ozone could be applied for eliminating off-flavours of grains. 2-methoxy-4-vinylphenol gradually increased during germination. It is an aromatic substance responsible for the natural aroma (Janeš et al., 2009), which could induce exogenous dormancy in wheat seeds (Darabi et al., 2007). Aromadendrene is not sufficiently studied in the literature, so will not be further discussed.

Correlation analysis was used to identify metabolites that were functionally related or co-regulated in germinating barley seeds (Figure 4.9). Ten differential metabolites obtained above were included, and 100 metabolite pairs were analysed. The results indicated oleic acid and stearic acid were highly positive correlated with each other. In addition, oleic acid and stearic acid were positive correlated to ketones including 5-(acetyloxy)-2-pentanone, 2-hydroxy-gamma-butyrolactone and acetone, while palmitic acid showed significant negative correlations with the above ketones and positive correlation with (E)-2-nonenal. 5-(acetyloxy)-2-pentanone, 2-hydroxy-gamma-butyrolactone and acetone were also highly positive correlated with each other. This result suggests that ozone induced increase in fatty acid and ketone during germination, while ozone treatment appeared to induce conversion of metabolic products from palmitic acid to ketones.

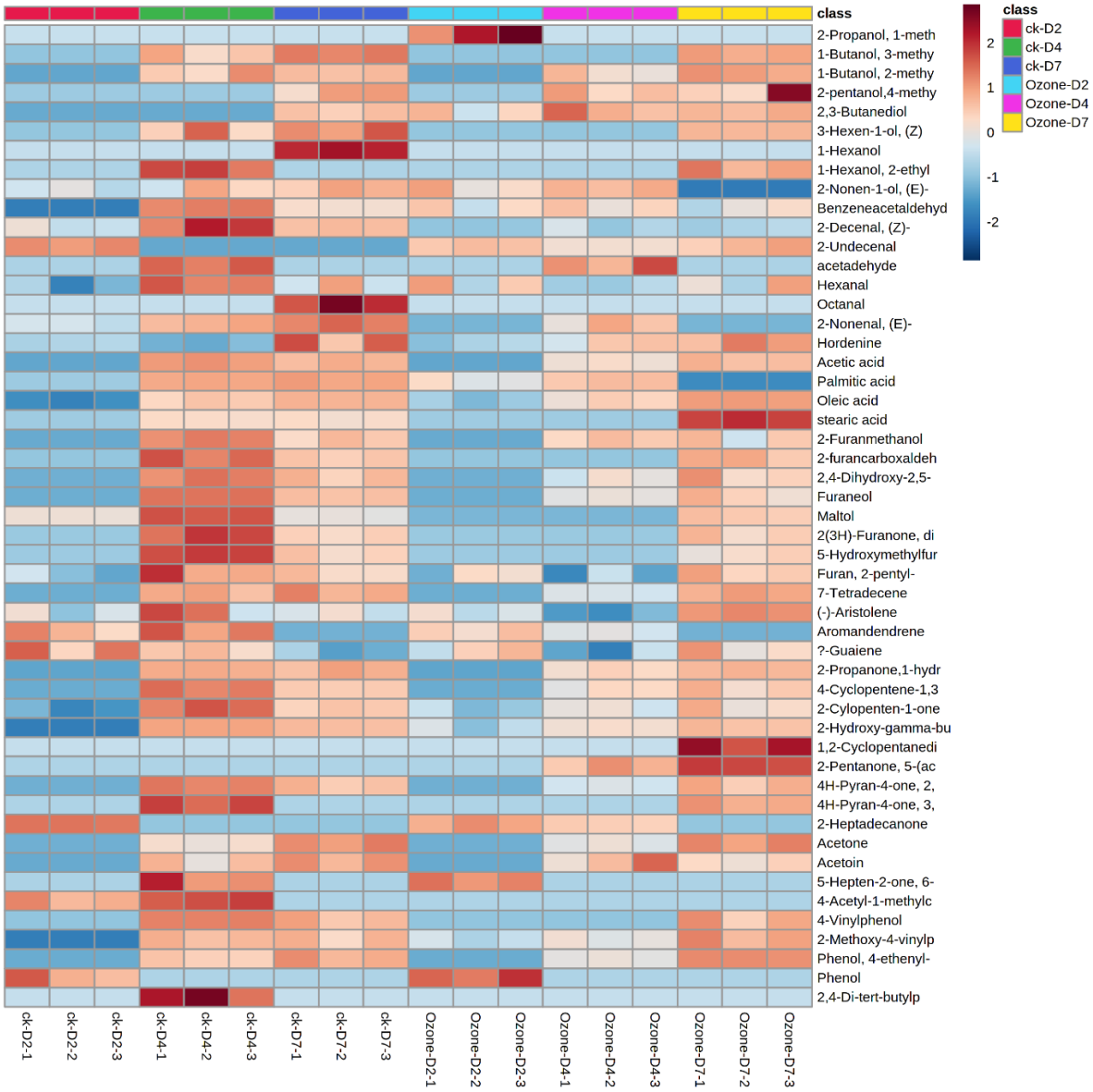


Figure 4.7. Heatmap of metabolites at different stages of germination. Red means a higher abundance of metabolites, blue means a lower abundance.

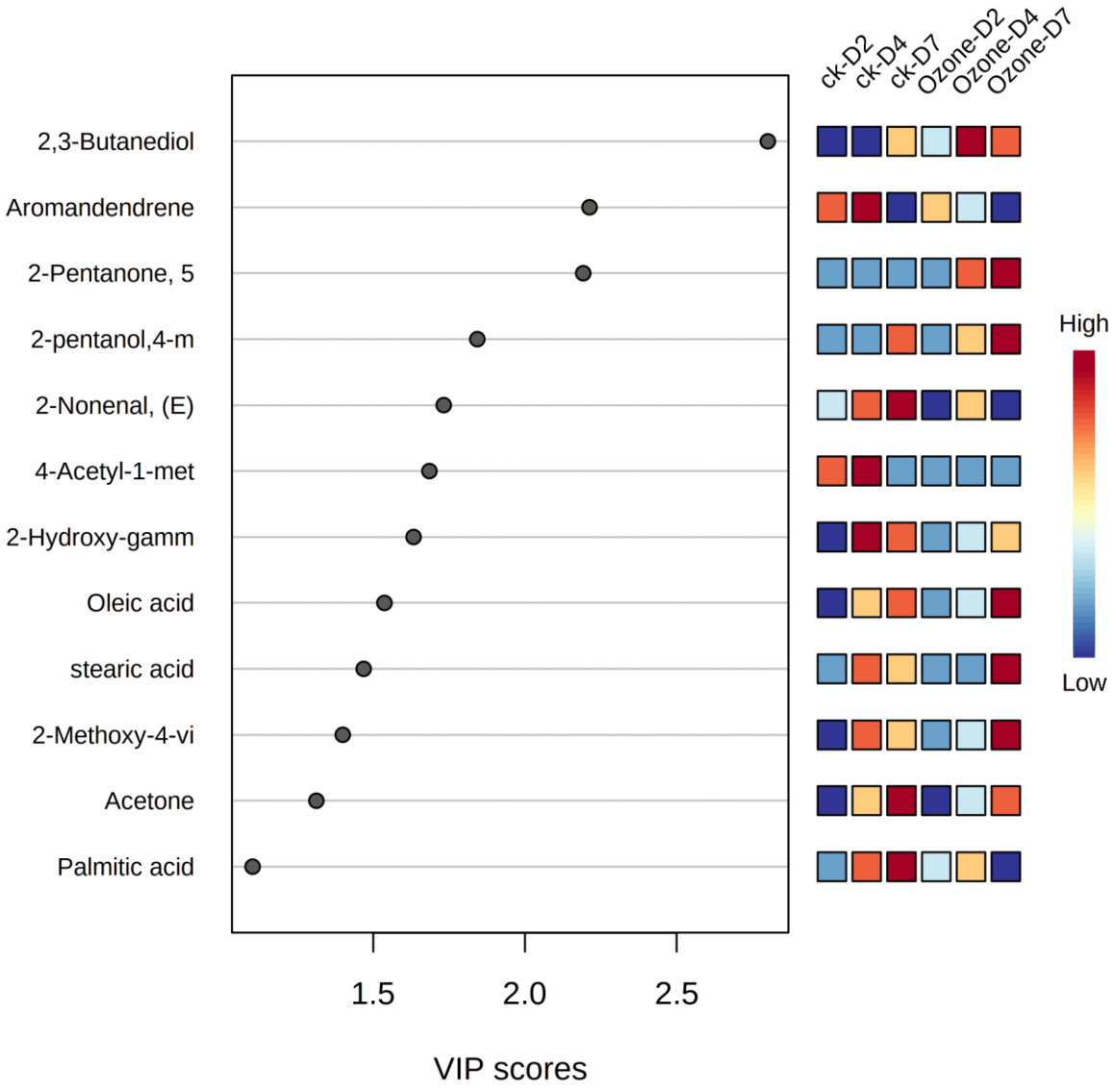


Figure 4.8. Variable importance in the projection (VIP) scores for metabolites in germinating barley at zone treatment.

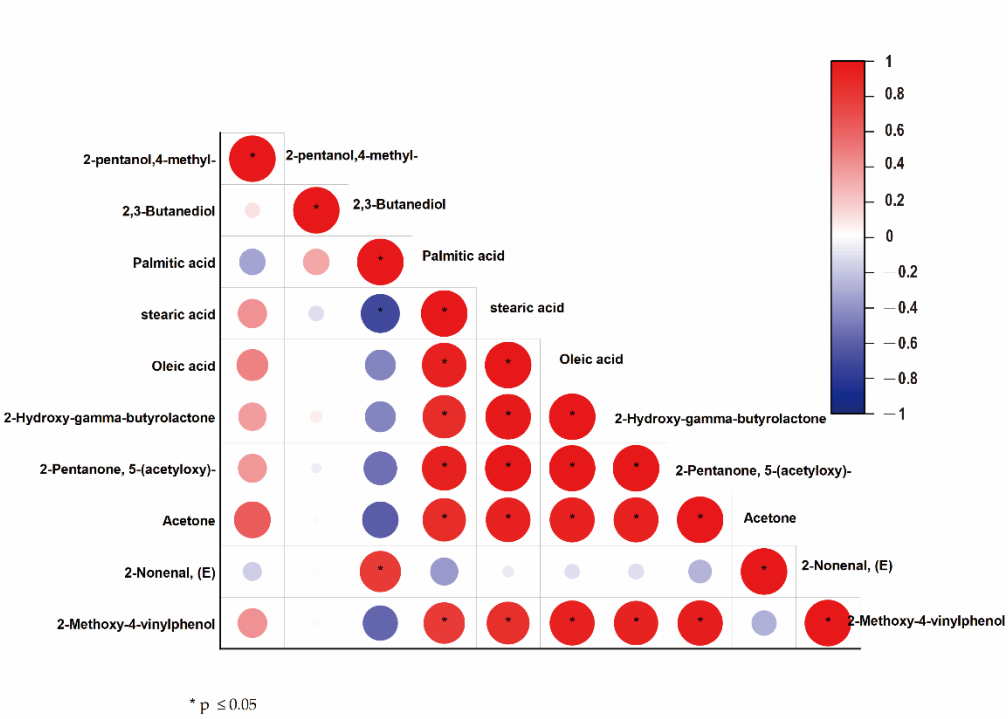


Figure 4.9. Correlation analysis of differential compounds in ozone-treated germinating barley. Correlation coefficient (positive or negative) between a pair of metabolites is represented by colour based on Pearson analysis. Red means positive correlation, while blue represents negative.

4.4. Conclusion

In this study, dormancy in two varieties of malting barley persisted after harvest and was lost during after-ripening up to four weeks, however, 15 min ozone treatment could break dormancy of barley immediately and it remained at the accelerated germination efficiency ~96% until four weeks. HS-SPME and liquid absorption coupled with GC–MS were utilized for metabolite profiling. Ketones, alcohols and fatty acids contributed to variation in different germinating time of barley. Ozone also played an important role in metabolite changes during germination, especially at day 4 of germination. In addition, ozone induced an increase of fatty acid levels to enhance germination by supplying carbon skeletons and energy for germination via TCA cycle.

Chapter Five

General Discussion

5.1. Findings of the thesis

Ozone (O_3) is a naturally occurring substance. As a disinfestation agent, it has distinct advantages, such as being highly toxic to a broad range of microorganisms and rapid auto-decomposition to oxygen without leaving residues. Despite numerous studies that have assessed the efficacy of O_3 against pests in maize and wheat, relatively few studies have focused on the toxicity of O_3 on stored grain pests with barley. VOCs play essential roles in plant communication, stress release, and plant growth, but the volatile metabolite profile of barley under ozone treatment remains unknown. This thesis focused on understanding stored grain insects and barley response to ozone, which has been achieved by a series of experiments including bioassay of insect mortality in the laboratory, HS-SPME-GC/MS and liquid absorption-GC/MS. The major findings are as follows:

1) Ozone was effective against all life stages of *Rhyzopertha dominica* and *Tribolium castaneum* in barley without negatively affecting barley commercial values.
2) Germination ability and seedling growth of old and new barley seeds were impaired due to extended exposure to O_3.
3) Barley released both a greater variety and quantity of VOCs under oxidative stress.
4) Acetic acid was identified as a potential ozone stress-specific marker, which has a dosage-dependent function on the germination of barley.
5) Ozone treatment could break dormancy of barley immediately and remained at the accelerated germination efficiency up to four weeks.
6) Alcohols, fatty acids, and ketones were major contributors to time-driven changes during germination.
7) Ozone induced a significant increase of fatty acid levels in the early germination stage to enhance germination.

5.2. Efficacy of ozone to control storage grain insects in barley

As the second largest crop in Australia, a large amount of barley is exported to the global market. Ideally, it is better to export newly harvested gain to the global market, because it can be sold at a higher price and reduce storage costs. However, grain production in any country varies from year to year. Thus, it should be stored strategically from over-production years to compensate under-production years. Insect pests cause post-harvest losses of barley. There are major insect species in barley belonging to the orders Coleoptera (beetles), Lepidoptera (moths), and Psocoptera (psocids). Coleoptera: *Sitophilus Granarius (L.)* are primary pests of grain,

which develop on wheat, corn and barley. *Sitophilus Granarius* infestations can result in the reduction in the weight and quality of grain (Schwartz and Burkholder, 1991). *Rhyzopertha dominica* has been reported from India, China, Australia, Argentina and other countries (Song et al., 2011; Opit et al., 2012; Huang et al., 2019), which is feed primarily on stored cereal seed including wheat, maize, rice, oats, barley, sorghum and millet (Kavallieratos et al., 2005). possesses powerful jaws which used in causing serious damage to the grain. As the secondary insect, *T. castaneum* does not cause damage to whole rice, wheat, barley oats and other cereal products but mainly feeds on processed stored products, broken or infested grain by other insects (Gerken and Campbell, 2020). *Trogoderma granarium* is a major pest of stored grain in India and has been transported and established in Africa, Asia, Australia, and Europe and widely distributed throughout the world (Athanassiou et al., 2019). Both adult and destroy stored grain including wheat, barley, oat, millet and maize (Kavallieratos et al., 2017). *Sitotroga cerealella* and *L. bostrychophila Badonnel* belonging to Lepidoptera and Psocoptera cause barley, wheat, oat and rice loss during storage (Athanassiou et al., 2010; Bushra et al., 2013).

Ozone is a promising fumigant with distinct advantages, which could overcome deficiencies of other fumigants. Ozone can quickly decompose into oxygen in relative short time, however, continuous application can overcome this drawback. Bonjour reported that 50ppm of O_3 for at least 4 days continually was required to control *T. castaneum* in stored wheat (Bonjour et al., 2011), since O_3 concentration inside the grain would decrease to less than 30ppm in less than 7 min if stop the O_3 supply (Dos Santos et al., 2007). This O_3 fumigation time was similar to the fumigation time using phosphine (Jian et al., 2013). Ozone can be obtained from dioxygen by discharging an electrical current of high voltage. Unlike other fumigant stored in cylinder for transportation, ozone can be generated on site to avoid transportation problems, reducing transport costs. Ozone generator, a device designed to produce the gas ozone in commercial production, have high acceptability and availability for markets and farmers. However, ozone has some limitations restricting its wide usage scale. Commercial fumigants like phosphine can penetrate the grain kernel and cause death of internal pests, whereas ozone has low penetration ability. When applied to control pests in grains, O_3 decomposes since it interacts with active sites present in or on the kernel surface. Once active sites are saturated, O_3 moves freely through grain layers, with O_3 concentration gradually increasing to an effective dosage for target pests (Sousa et al., 2008). Our results demonstrated that the successful application of O_3 against stored grain insects in barley requires sufficient concentration and exposure time. For ozone application in silos, as a highly reactive gas, it is difficult to move through the entire grain

causing the uneven distribution, and discrepancy efficacy to pests which in different positions (Pandiselvam et al., 2017). The O_3 could be supplied from the headspace of bins and the fan could move air downward because O_3 is heavier than air. Three important parameters for evaluating the effect of ozone: saturation time, decomposition kinetics, and the half-life of the ozone (Boopathy et al., 2021), which mainly depend on of the grain moisture content and bed thickness (Pandiselvam and Thirupathi, 2015).

This research also suggested that O_3 displays delayed toxicity. Unlike some pesticides, which disrupting the insect central nervous system to kill insects immediately (Brautbar and Howard, 2002), ozone displays a different mode of action. According to the literature, the first site of action for O_3 is the respiratory system. When O_3 enters through spiracles, insects trigger discontinuous gas exchange to reduce oxidative tissue damage (Liu et al., 2007). In addition, as a strong oxidizing agent, O_3 could cause cell membrane damage and DNA strand breakages (Tiwari et al., 2010), which do not occur immediately after O_3 exposure, but in the post-exposure period. Toxicity data indicated a noticeable difference in susceptibility between two species, especially at different life stages. Distinct responses of life stages to O_3 could be attributed to their body structures and respiration rates.

5.3. Effect of ozone on grain quality and germination

This research suggested that short duration O_3 treatment did not negatively influence germination ability, seedling growth and quality parameters in either old or new barley, which is consistent with reports that ozone does not alter critical chemical elements, intrinsic properties of grain and density of the kernel which are contributors for milling, processing quality and germination (Mendez et al., 2003; Pandiselvam et al., 2019), However, with increasing duration of ozone treatment, inhibition of germination was observed in this study. The O_3 exposure time required to obtain 100% mortality of all stages of both species adversely affected germination ability. Oxidative stress can damage antioxidant mechanisms, reduce the activity of related enzymes and disturb macromolecule biosynthesis, resulting in suppression of the germination process. Although some morphological and spectral changes were reported to be influenced by ozone exposure, this can be regulated to achieve desired outcomes if proper measures are taken (Srivastava et al., 2021). For instance, if barley seeds are utilized for livestock feeding and human consumption, it is necessary to supply sufficient ozone treatment to eliminate pests and reduce deoxynivalenol rather than achieve high germination ability.

Moreover, ozone treatment could also be modified to meet baking and flour-making requirements. Zhu (2018) reported that ozone treatment tended to decrease starch's retrogradation while increasing paste clarity. In addition, it decreased the swelling power and viscosity of the starch. Ozone can oxidise the hydroxyl group of starch to carbonyl and carboxyl groups (Klein et al., 2014), which may cause cross-linking or degradation of starch molecules, depending on the reaction condition. Farmers aim to maximise the price they can sell their grains for. Most farmers grow barley for sale as malting barley, and germination ability and other malting quality parameters could be maintained and even improved based on the proper application of ozone. This research found that short ozone exposure could break barley seed dormancy and enhance germination. Moderate ozonation treatment enhanced the germination ability of aging seeds to meet the malting barley requirement. Ozone treatment was reported to improve the malting quality by improving barley germination and enhancing hydrolase activity of malts, including α-amylase, β-amylase, β-glucanase and endo-protease (Ma et al., 2020). High β-glucan levels contributes to poor malt quality resulting in poor quality beer and reduced brewing process efficiency (Jin et al., 2004). Turbidity, an important indicator for brewing process efficiency, is affected by macromolecule hydrolysis, such as proteins, polyphenols, β-glucan, and dextrin (Cai et al., 2016). Diastatic power also plays an important role in malting quality. Brewers want to seek malts with high diastatic power to enable the degradation of more starch into yeast fermentable sugars (Gibson et al., 1995). Wort free amino nitrogen content and diastatic power increased while β-glucan content, turbidity decreased under ozone treatment, which is beneficial for improving wort quality and the efficiency beer brewing (Ma et al., 2020). All these studies demonstrate that ozone has great potential to improve the functionalities of grain products while ensuring food safety. Thus, it is imperative to select an optimal O_3 exposure time to maximise contributors of final product quality and achieve the desired functional outcomes.

The majority of barley varieties have been bred with dormancy traits to prevent preharvest sprouting (Nakamura, 2018); however, dormancy that persists after harvest delayed germination resulting in yield and malting losses. The potential for embryo dormancy could be overcome through after-ripening (AR) in the dry seed storage (de Casas et al., 2012). In our research, dormancy was overcome through after-ripening (AR) in dry barley seed storage of more than 4 weeks, however dry storage increases the risk of grain deterioration, such as attacking by pathogen and pests resulting quality loss. In addition, long-term dry storage induce secondary dormancy (Basbouss-Serhal et al., 2016). Ozone treatment could shorten dormancy time. In our study, 15 min ozone treatment could break the dormancy of barley immediately

after harvest, with accelerated germination efficiency remaining around 96% until four weeks. Our finding suggested that grower and beer brewers can shorten dormancy period by apply short time ozone treatment to break dormancy and induce germination.

5.4. Ozone influences VOCs release in barley germination

Germination is a crucial stage of plant development, involving multiple physiological and biochemical mechanisms that are altered in response to ozone treatment. The data demonstrated that barley released greater variety and quantity of VOCs under oxidative stress. VOCs not only contribute to the odour of food, but also have pivotal roles in plant communication, stress response and growth (Brilli et al., 2019). Acetic acid was identified as a potential ozone stress-specific marker mainly produced through the catabolism of precursor compounds and showed dosage-dependent function on the germination of barley. This research found that lower concentrations at a shorter duration could increase barley germination, whereas longer processing times and higher concentrations of acetic acid inhibited the germination ability of barley seeds. The acetyl group, derived from acetic acid, is fundamental to all forms of life and plays a pivotal role in carbohydrate and fat metabolism when bound to coenzyme A to facilitate the synthesis of soluble carbohydrates necessary for germination via the TCA cycle. However, as an organic acid, excessive application of acetic acid is toxic to seeds, as it may cause degradation of membrane proteins, causing loss of germination ability (Pujisiswanto et al., 2013). The germination process can be classified into three phases: imbibition, metabolic activation, and radical protrusion, each characterised by various metabolic processes resulting in distinct and time-dependent metabolic alterations. Ketones, alcohols and fatty acids contributed to variations in different germinating times of barley. Increased fatty acid levels were observed in the early germination stage when subjected to 15-min ozone exposure. Seeds contain lipids for energy storage, and mobilization of storage lipid during seed germination begins with the hydrolysis of the glyceride into free fatty acids and glycerol (Borek et al., 2015). Ozone could have increased lipid metabolism to produce more free fatty acids, which could enhance germination by supplying carbon skeletons and energy for germination via the TCA cycle.

Barley released both a greater variety and quantity of VOCs under oxidative stress (Dong et al., 2022). There is strong relationship between VOCs and flavor of brewing product, which is often judged by consumers. VOCs in barley play an important role in the aroma composition of beer. Trans-2-nonenal is an aldehyde contributing to an unpleasant off-flavor and odor of rancid butter in stored beer (Svoboda et al., 2011). In our study ozone treated sample showed

a reduction of (E)-2-nonenal at day 7 germination, suggesting ozone could be applied for eliminating off-flavours of grains. Benzaldehyde is responsible for the fresh and slightly green notes of barley, based on sensory attributes (Dong et al., 2015a). High abundance of benzaldehyde released in barley under ozone treatment contributing to pleasant flavor of beer.

5.5. Limitations of thesis and future directions

This thesis evaluated the efficacy of O_3 against two coleopteran insect pests: *Rhyzopertha dominica* and *Tribolium castaneum* in barley in chambers and explored how barley metabolites change in response to ozone treatment. Complete control of all life stages of two phosphine susceptible strains was obtained at 700ppm for 1440 mins of ozone exposure. Two species with different phosphine-resistance responding to ozone treatment need to be assessed in the further study. Phosphine is a widely used fumigant, however, continuous use has led to the evolution of resistant populations (Nayak et al., 2020). The mechanism of resistance development was related to the insect respiration rate. It was suggested that the populations with lower carbon dioxide production showed a higher resistance ratio (Pimentel et al., 2007). Lu et al. (2009) reported that ozone could increase insect respiration which may enhance fumigant toxicity. Thus, it is possible to combine ozone with phosphine to increase the efficacy of fumigants against pests. Moreover, it has been reported that different genotypes of rice confer different resistance levels to *R. dominica* infestation (Locatelli et al., 2019), however, there is less research on barley resistance. Thus, selection of barley genotypes that restrain insect pest development could be an alternative to minimize grain damages by postharvest pests. The germination rate of three varieties (Scope CL, Flinders and Spartacus CL) of barley utilized in our research, were promoted under 15-20 min of ozone treatment. Scope CL malt has good extract with moderate enzyme activity which is suitable for export as grain and as malt, but not suitable for the manufacture of shochu in Japan. Spartacus CL is suitable for export as grain and as malt, but limited volumes of grain have been processed in Australia to date. Flinders malt has excellent malt extract and it can be malted without application of gibberellic acid (GRDC, 2019). Due to the phenotype discrepancies of these three varieties, the comparison of three varieties metabolites and even metabolic responses to ozone should be addressed in the further study.

O_3 has a relative short half-life, making it rapidly auto-decompose to oxygen without leaving residues. Thus, O_3 is commonly produced onsite to ensure a continuous supply. The penetration of O_3 gas initially starts from the seed coat. It then goes into the grain, depending on the moisture content, bed thickness and surface morphology of the grain, and finally reaches the

target pests and pathogens (Pandiselvam et al., 2015; Subramanyam et al., 2017a). Therefore, the temperature, moisture content, type of grain and insect species should be considered when applying O_3 to grain processing in the future. The fumigation process is usually carried out in silos depending on the type of grain stored (Bell, 2000). Thus, grain engineering properties are essential to design a storage silo for O_3 fumigation. Currently, a closed-loop fumigation system was employed to maintain the ozone concentration inside the grain bulk to achieve entomologically lethal concentrations (Hardin et al., 2009). Another crucial challenge is that, as a powerful oxidant, O_3 oxidizes with the metal container resulting in corrosion of the storage bin; thus, it is imperative to optimize the concentration, equipment contact and use of ozone resistant materials to inhibit corrosion.

In this study, barley produced a greater diversity and quantity of VOCs under oxidative stresses, suggesting some VOCs play essential roles in adaptation to unfavourable growth conditions. Acetic acid is an important biomarker for O_3 stress response, with the effect of acetic acid on barley seed germination highlighted. The mechanism of how acetic acid affects germination is likely to be complex. Subsequent research to evaluate the mechanism of the effect of acetic acid on germination could be undertaken. We propose different that the effects of acetic acid on germination mainly depend on concentration, and less on the specific grain; this work evaluated and discussed the effect of acetic acid on germination without considering grain type. Thus, the response of different grains to acetic acid should be considered in further research. For seed hydration, stored carbohydrates, proteins and lipid are hydrolysed to sugars, amino acids, and fatty acids as an energy source and carbon skeleton. Thus, metabolic profiles of primary metabolites such as sugars, amino acid, as well as lipids, combined with enzyme assay should be taken into consideration in further research to give a better depiction of how ozone influence the germination of barley.

5.6. Conclusions

This PhD project focused on understanding stored grain insects and barley response to ozone. This is the first time that the susceptibility of all life stages of *R. dominica* and *T. castaneum* to O_3 in barley seeds has been explored, along with investigation of the influence of O_3 on germination ability, seedling growth and quality parameters. This study provides new insight into pest control, selecting optimal O_3 exposure time to transfer ozone into quality contributors of final products and achieve the desired functional outcomes. Additionally, the results of this thesis have advanced our understanding of how VOCs of barley alter in response to O_3

treatment based on HS-SPME-GC-MS analysis. Additionally, these findings indicate that ozone could be utilized to improve the quality of malting barley by enhancing germination ability. VOCs such as acetic acid could also be used as a regulator to control germination. Moreover, the current study was the first investigation of the effect of O_3 on metabolite profiles in germinating barley, helping identify the factors affecting germination or malt quality.

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