ABSTRACT

MASHAHEET, ALSAYED M.S. Effects of Near-Ambient O_3 and CO_2 on Wheat Performance and Interactions with Leaf and Stem Rust Pathogens. (Under the direction of Drs. David S. Marshall and Shijin Hu).

Wheat is a key crop for global food security, and the staple food for 30% of the world population. It provides about 20% of the food proteins and calories to more than 4.5 billion people in 94 developing countries. By 2050, the world will need 70% more food, nearly 4 billion tonnes of cereals, most of which are needed in developing countries, where elevated ozone and resurgent rust diseases are challenges. However, there is limited information on how ozone and rust currently interact with wheat plants, and how these interactions influence the entire wheat phytobiome. Moreover, there is no information on the relative ozone responses of key breeding material and associated genetic markers, which significantly limit breeding wheat for ozone tolerance.

The first objective of this dissertation was to identify the relative ozone responses of key host germplasm, such as a panel of eight rust near-universal susceptible genotypes, and to investigate their suitability for breeding and phytobiome research. Visible injury results showed consistent differential O_3 responses in two gas exposure systems. Thatcher and LMPG-6 were the most sensitive genotype, whereas Chinese Spring and Little Club were the most tolerant. Biomass data showed similar results, accept for Rusty that was ranked moderately susceptible according to visible injury, and was the most sensitive according to biomass production. LMPG-6 could be used as O_3-sensitive, in contrast to Chinese Spring. Similarly, Thatcher is a suitable O_3-sensitive in contrast to Little Club or Morocco for studying O_3 effects on different races of leaf rust.

The second objective of was to identify the genetic control of O_3 tolerance at the chromosome level in the wheat variety Chinese Spring. Tolerance is currently identified only to the subgenome level (subgenome AABB). Visible injury results suggested that the observed O_3 tolerance is correlated with chromosomes 7A, with no evidence for sensitivity factors associated with any of subgenome D chromosome. A mapping population from the cross between Chinese Spring and O_3-sensitive varieties, (e.g. Thatcher) could determine the exact location(s) of O_3 tolerance gene(s) and identify the associated genetic marker(s).

The third objective was to identify the relative O_3 responses of some winter wheat genotypes, and to investigate their suitability for breeding resilient hard red winter wheat varieties, suitable for the eastern United States. O_3 responses of 12 hard and 10 soft red winter wheat genotypes were tested against some varieties representing other cereal species. Injury results indicated that winter wheat were more tolerant than spring wheat, similar in sensitivity to oat, and more sensitive than triticale and barley. Tested hard wheat genotypes were found to be more sensitive to O_3 than soft wheat. This indicated the importance of the O_3 responses of parents used to breed for improved hardness. Tested red wheat genotypes showed decreasing sensitivity as function of release date, with higher rate of declining sensitivity for varieties released after the year 2000. The visible injury difference between Coker 9553 and MD01W28-08-11 was confirmed by yield trials in open-top chambers. These two parents have contrasting adult-plant responses to stem rust (including races in Ug99-lineage), therefore, they are a suitable pair for simultaneous breeding for both stressors.

The fourth objective was to dissect the effects of O_3 and CO_2 concentration, exposure timing and duration on the disease components of stem and leaf rusts on winter wheat. Consistent disease increase of both leaf and stem rust of wheat were evident under near-ambient but not under relatively high O_3 concentrations. The O_3-induced disease increase was more pronounced with increased susceptibility to the pathogen, and was associated with larger pustule size, and accelerated pustule formation, which are characteristics likely to increase rust epidemics.

Effects of Near-Ambient O_3 and CO_2 on Wheat Performance and Interactions with Leaf and Stem Rust Pathogens.

by

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DEDICATION

To my wife and “life” Camellia, my daughters Habeba and Jana, and my son Adam, for always being the best supportive family. To my Mom, who devoted all her days and nights encouraging me to finish this dissertation. To the memory of my Dad and grandparents, who lived and died, fighting for me. To my brothers and sisters, who worked very hard making sure, I am getting the education I had in my dreams. To my nieces and nephews, for continuously motivating me, and I hope this will be just the first PhD dissertation in our small family, with many more to come.

To my committee members, collaborators, technicians and students who contributed to this work. To the memory of Dr. Fitz Booker, for helping me to join such wonderful university and research group. To all my professors, teachers, colleagues, friends and “students”, for giving me such wonderful continuing learning experiences.

BIOGRAPHY

Alsayed Mohamed Saad Mashaheet was borne, and grew up in a small village ‘Sabry’, in a small district ‘Dilingat’, in the largest agricultural governorate in Egypt ‘Beheira’. He loved science and science communication since his early childhood. In 2000, Sayed joined Alexandria University, to pursue Bachelor of Science in Agriculture. Soon he realized that this is where he belongs, and he completed his B.Sc. in 2004, with excellence and honor degree. In 2006, Sayed started his faculty position at Alexandria University, teaching environmental plant diseases.

Sayed received his Master’s degree in 2010, in a joint-supervision program, under Samia Madkour from Alexandria University in Egypt, and Costas Saitanis from the Agricultural University of Athens, Greece. He conducted his master’s research in Athens in 2008, using sensitive plants to biomonitor ambient ozone pollution. In 2012, Sayed was awarded a fully funded PhD scholarship by the mission sector of the Egyptian ministry of higher education, to study elevated ozone interactions with rust diseases on wheat. In the fall of 2012, Sayed joined the Department of Plant Pathology at North Carolina State University, where he had the privilege of being advised by professors known for their global contribution in the fight against elevated ozone and wheat rusts.

For more than four years, Sayed extensively used four advanced gas exposure systems to screen wheat for ozone tolerance, and to study wheat responses to ozone, and their impacts on wheat/rust interactions. The results of those studies are presented in this dissertation. In addition, Sayed screened a doubled haploid population for both rust and ozone stress responses, a continuing work aiming at selecting resilient varieties having high yield, ozone tolerance and rust resistance, and identifying the molecular markers behind these traits. Furthermore, he screened most of the historical and modern wheat varieties for ozone tolerance, as a first step towards breeding ozone tolerant varieties for Egypt. In addition to his research, Sayed received the certificate of accomplishment in teaching from NC State University.

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I would like also to thank Dr. Costas Saitanis for reviewing the first draft of several chapters of this dissertation, and all his advices to improve my writing and research skills. I also thank Dr. Rich Zobel for expanding my understanding of wheat roots, and for his collaboration on several studies covering ozone effects on root architecture. I would like to thank Dr. Christina Cowger and her valuable discussions and providing seeds of the Thatcher. I would to thank all the post docs and visiting scholars including Ahmed Abdallah, Abdelrazk abdelrhim, Rafiullah for their assistance with wheat screening studies. I thank Dr. Yue Jin and Sam Gale for the training on stem rust race identification at the Cereal Disease Lab in Minnesota, and providing seeds of the universal susceptible varieties, and Jim Kolmer for validating leaf rust race used. I also thank Dr. Consuelo Arellano and Dr. Lucky Mehra for their assistance with the statistical analysis. I would like also to thank Tan Tuong for his assistance and training on tissue sectioning and staining. I would like also to thank all the faculty of the plant pathology department for their valuable discussion. I am so thankful to Mike Benson and Mina Mila for the wonderful teaching assistance experience they provided. I would like to acknowledge the PhD scholarship I received from the mission sector of the Egyptian Ministry of Higher Education, and the research funding from USDA-ARS.

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1. General Introduction

Sustainable increase in wheat (*Triticum aestivum* L.) production is needed for attaining global food security. This sustainability requires resilient wheat cultivars that can cope simultaneously with major biotic and abiotic stresses, while maintaining healthy phytobiomes, and high yield. The rapidly evolving rust pathogens and the elevated ambient ozone levels (E-O_3) are two concomitant threats and limiting factors to wheat production worldwide. However, they are not addressed simultaneously, despite of the necessity and the potential, under current limited resources. This is due to the lack of information needed in two main areas: (1) the relative O_3 responses of key wheat breeding materials, and the genetic control and markers associated with these responses, and (2) the effects of O_3 responses of wheat cultivars on wheat interactions with rust pathogen, under current and future carbon dioxide (CO_2) concentrations. This dissertation aims at identifying and addressing some significant knowledge gap in these two critical areas.

1.1. Relative O_3 responses of key wheat breeding materials, and the genetic control and markers associated

1.1.1. Elevated Ozone (E-O_3) and Climate Change

Climate change is defined as the change in the state (i.e. mean or variability) of the climate that persists for an extended period (decades or longer) due to natural internal processes (e.g. the solar cycles and volcanic eruptions) or external forcings (e.g. persistent anthropogenic changes in the composition of the atmosphere or in land use) **(IPCC, 2014a)**. While climate is changing continuously, many of the currently observed changes in global climate are unprecedented over decades to millennia and they are different from previous changes **(IPCC, 2014b)**. This is because the current changes are mainly due to anthropogenic activities rather than natural processes. These human derived external forcings have led to increased global warming gases in the atmosphere, at relatively high rates of increase comparing to previous naturally driven climate changes **(IPCC, 2014b)**. Ground level O_3 is one of these global

warming gases that imposes eminent threat to humans, animals and plants, as well as their biological and physical environments **(De Kok et al., 2016)**.

Ozone is a colorless gas has been associated to electrical storms during all the human history, yet it was first identified and named as a distinct chemical compound by Schönbein in 1840, and its molecular formula as a triatomic Oxygen molecule was determined in 1865 by Soret and confirmed by him in 1867 **(McElroy and Fogal, 2008)**. The name Ozone was derived from the Greek word ozein which means "to smell", due to its slightly sweet, water melon-like odor, that could be recognized at very low concentrations as low as 7.6 - 36 ppb **(Iriti and Faoro, 2009)**.

Ozone in the atmosphere is spatially classified into two pools: stratospheric O_3 (also known as ozone layer) and tropospheric or ground level O_3 (also known as surface O_3 or ambient O_3). Unlike the protective O_3 naturally formed in the stratosphere, O_3 in the ground level layer of the troposphere is a secondary air pollutant and a greenhouse gas, associated with human hospital admission and mortality **(Schwartz, 2016)**, as well as phytotoxicity **(Li et al., 2015)**. The phytotoxic effects of O_3 were recognized as early as 1958, when atypical diseases symptoms were observed on ponderosa pine needles in the near of Los Angeles, and on tobacco leaves in the eastern USA, which were attributed to photochemical smog containing ozone as the major phytotoxic component **(Heggestad and Middleton, 1959)**.

1.1.1.1. Formation, life time, transmission, removal and deposition of Tropospheric O_3

Ground level tropospheric O_3 at a given location could be classified into three origins: (1) stratosphere-originated, (2) long- or short-term transmitted, and locally originated O_3 **(Cooper et al., 2014)**. In the year 2000, the total tropospheric O_3 budget were estimated to be about 5650 Tg, of which, 5100 Tg were estimated to be chemically produced in the troposphere, and 550 Tg were estimated to be originated from the stratosphere **(Stevenson et al., 2006)**. In the same year 4650 Tg were chemically removed, whereas 1000 Tg were removed by dry deposition to dry surfaces and ecosystem fluxes **(Stevenson et al., 2006)**.

The only reliable quantitative ozone measurements from preindustrial non-anthropogenic O_3 levels in the late 19th century were made at Montsouris near Paris where ozone averaged 11 ± 2 ppb from 1876 to 1910 and maximum concentration during the 19th century occurred during spring **(Volz and Kley, 1988)**. This non-anthropogenic O_3 originates from several sources such as stratospheric O_3 transferred to the troposphere, lightning, and photochemical reactions between precursors emitted from natural sources. O_3 concentrations has been increased over the 20th century due to human activities, with highly variable localized peaks that shifted towards summer **(Cooper et al., 2014)**. These local concentrations are determined by precursors' concentrations, baseline O_3 concentrations (O_3 concentrations in free-air before being affected by local O_3 formation), local peak O_3 concentrations, and seasonal cycle **(Colette et al., 2016)**.

Tropospheric O_3 is formed by photochemical reactions between nitrogen oxides (NO_x) and non-methane volatile organic compounds (NMVOC) **(Booker et al., 2009)**. These reactions are dependent on the sun light, concentration of precursors and the ratio between them, and favored by high temperatures and stagnant weather condition **(Khoder, 2009; Madkour and Laurence, 2002)**. Besides the precursors' concentrations, weather and meteorological conditions are major factors controlling stratosphere-troposphere O_3 exchange, long- and short-term O_3 transmission, and local O_3 formation, which eventually determine local O_3 concentrations. Local O_3 formation is also highly dependent on the ratio between the two major precursor groups (NO_x and NMVOCs), as unbalanced ratios result in titration effect of the dominant precursor removing O_3 that is already formed **(Monks et al., 2015)**, especially if NO_x is the dominant precursor **(Archibald et al., 2011)**. Therefore, in some areas, O_3 formation could be NO_x-limited or VOC-limited **(Chang et al., 2016)**.

Ambient O_3 pollution is a diverse problem both spatially and temporally. Spatial variations in O_3 levels result from the relative location of sources of the precursors, and the ratio between them (NO_x/VOCs) **(Khoder, 2009)**. Whereas, the temporal variations result mainly from the changes in precursor emission (e.g. weekend phenomenon), meteorological effects (e.g. stagnant weather conditions and inversion layer formation), temperature and sun

light, all of which are contributing to seasonal and diurnal variations in O_3 levels **(Khoder, 2009)**. Tropospheric O_3 levels usually are low in the morning and increase after sunrise to achieve the peak in the afternoon and evening **(Wang et al., 2008)**. Similarly, O_3 concentrations in the northern hemisphere are usually low in winter and increase in spring to reach the peak in summer **(Fiore et al., 2009)**. These two types of O_3 peaks coincide with high plant activity during the day, and growing season for most of the major crops in spring and summer.

O_3 is not only an urban pollutant, as it is transmitted along with its precursors to sub-urban and rural areas, where humans, animals and plants are experiencing higher O_3 concentrations **(Sicard et al., 2016)**. The lifetime of O_3 molecules are 22±2 days in the troposphere, which allow regional and hemispheric-scale transmission **(Stevenson et al., 2006)**. For example, O_3 transmitted from significant sources in Asia seems to be contributing to increased background O_3 levels in remote areas with low or decreased precursors' emissions in Europe and North America **(Fiore et al., 2009; Sicard et al., 2016)**. This could be clearly observed in the increased base-line O_3 levels in the free air of the troposphere since 1970, despite the reduced precursors' emission in Europe and North America **(Cooper et al., 2014)**.

Ground level O_3 showed varied regional trends from 1990 until 2010 **(Monks et al., 2015)**. In Western Europe, ambient O_3 levels increased in the 1990s then leveled off or decreased after 2000, meanwhile, rural O_3 levels in eastern USA have decreased in summer, leveled off in spring, but increased in winter **(Cooper et al., 2014)**. This decrease is mainly due to the decreased precursors' emission **(Monks et al., 2015)**. Contrarily, in the western USA more than half of the rural sites showed increased O_3 levels in spring **(Cooper et al., 2014)**. Shifts in the high O_3 peaks season has been observed since 1970s, as peaks tends to shift (3-6 days/decade) to take place earlier during spring, becoming more similar to the preindustrial trend **(Cooper et al., 2014; Parrish et al., 2013)**.

The yearly average surface O_3 base line, measured in the marine boundary layer, of rural or remote sites of the northern hemisphere, during the period 2005-2010 ranged from 29-

49 ppb **(Cooper et al., 2014).** O_3 levels increase from sea level with the increase in altitude as values measured at higher altitude from different parts of the world ranged from 37-59 ppb **(Cooper et al., 2014)**. In both of the previous data sets, the highest concentrations were recorded in western Japan, due to its location immediately downwind of precursor emissions from continental East Asia where ozone has increase rapidly since 1980, and is expected to continue increasing until 2050 **(Lee et al., 2014)**. O_3 concentrations in urban polluted areas all over the world are significantly higher than base line, especially in China, where midday summertime (June, July, August) median ozone mixing ratio of 75 ppb is common **(Cooper et al., 2014)**.

1.1.1.2. Deposition, diffusion, and flux of tropospheric O_3 into plant canopy and tissue

After being formed in the atmosphere, O_3 molecule could be scavenged or removed from air by several mechanisms, such as UV interception, rain, deposition to dust particles and soil or and chemical reactions with other gases such as NO_x and VOCs **(Fares et al., 2012)**. Because of its lifetime (about 22 days), O_3 could also be transferred and diffused into plant canopy, where O_3 molecule could be titrated by plant produced VOCs (biogenic VOCs), or could be deposited onto plant cuticle **(Monks et al., 2015)**. Limited dry deposition onto cuticle **(Cape et al., 2009)**, makes O_3 flux through dry cuticle effectively negligible **(Kerstiens and Lendzian, 1989)**. However, cuticle deposition may represent a major sink for O_3 if the receptive canopy is wet **(Altimir et al., 2006)**, because of its relatively high solubility in water (10 times more than CO_2) on leaf surface **(Zuccarini, 2009)**, which enables it to penetrate into the leaf, when this water is absorbed via stomata. This could increase the effective O_3 flux into the leaf causing acute symptoms at O_3 levels expected only to cause chronic effects **(Altimir et al., 2006)**. However, in some cases water films over leaves may reduce deposition **(Monks et al., 2015)**. The O_3 molecule could also be titrated close to soil surface by NO_x emitted **(Monks et al., 2015)**.

O_3 molecules could also penetrate directly through opened stomata and dissolve into reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), hydroxyl radicals ($HO^{\cdot}$), superoxide ($O_2^{\cdot -}$) or hydrogen peroxide (H_2O_2), in the apoplast **(Miller et al., 2010)**. These ROS react directly in the apoplastic fluid with extracellular proteins, the cell wall and/or plasma membrane **(Pellinen et al., 1999; Vahisalu et al., 2010)**. Under moderate external O_3 concentrations, the influx of O_3 and ROS generated during the early phase will be detoxified by the apoplastic antioxidants capacity (such as ascorbate). However, under high external O_3 concentrations, high stomatal conductance, low mesophyll resistance to gas diffusion, late and/or partial stomatal closure response, late and/or insufficient antioxidant response, the influx of O_3 during the early phase exceeds the apoplastic antioxidant capacity to detoxify ROS formed from O_3 degradation **(Vaultier and Jolivet, 2015)**.

1.1.1.3. Perception of O_3 stress in plant tissue

ROS produced by O_3 dissolution in the apoplast probably do not directly enter the cells to activate ROS-generating systems. Current evidence suggests the initial ROS formation activates the heterotrimeric Gβγ-protein directly or indirectly, via oxidation at the cell surface. Changes induced by O_3 or O_3-dervitives in cell wall, or changes induced by cell wall derived H_2O_2 in the apoplast are possible initial steps of O_3-stress signaling **(Vaultier and Jolivet, 2015)**.

Extracellular ROS detrimentally affect the plasma membrane through lipid peroxidation damage of membranes, and formation of gel-phase lipids that increase leakiness and cause higher membrane permeability and rapid loss of K^+ **(Heath and Frederick, 1979)**. Some of the oxygenated lipids do not remain anchored in membranes, but are released (e.g. Oxylipins), and may work as signaling molecules (e.g. jasmonic acid). Cysteine-rich receptor-like kinases (CRKs) are membrane-localized kinase proteins with extracellular domains at the interface between the cell wall and the plasma membrane. CRKs are sensitive to redox modifications and have transmembrane domains, so that, they act as extracellular-ROS sensors

for O_3 signal perception at the cell surface **(Vaultier and Jolivet, 2015; Wrzaczek et al., 2010)**.

The reaction of non-biological ROS (derived from the degradation of O_3 in the leaf apoplast) with the extracellular ascorbate pool causes relative increases in cytosolic calcium $[Ca^{2+}]_{cyt}$. This proportional increase in $[Ca^{2+}]_{cyt}$ induces the Ca^{2+} influx and the activation of an anion channel. In turn, the Ca^{2+} activates anion channels causing plasma membrane depolarization and subsequently, O_3 signal amplification by activating a plasma membrane NADPH-oxidase **(Kadono et al., 2010)**. H_2O_2 resulting from NADPH-oxidase activity increases $[Ca^{2+}]_{cyt}$ by activating the plasma membrane Ca^{2+} channels, acting as a feedback loop on anion channel activation in a biphasic calcium signal **(Kadono et al., 2010)**. This short-lived, spike-like elevation in $[Ca^{2+}]_{cyt}$, followed by the smaller and more prolonged increase is an O_3-induced calcium signature **(Short et al., 2012)**.

1.1.1.4. ROS intracellular perception and Active production

When ROS formation from O_3 exceeds the apoplastic antioxidant capacity, an endogenous, active, self-propagating ROS generation tacks place. This active oxidative burst, which continues after the end of the O_3 exposure is similar to the one observed in hypersensitive response (HR) that takes place in host-pathogen incompatible reactions, and leads to the programmed cell death (PCD) **(Kangasjärvi et al., 2005)**. O_3 intracellular signaling goes through three processes, perception, transduction, and response induction by the network of regulatory mechanisms in the cells affected. It involves at least three separate signaling cascades downstream of the perception of O_3. First, G_β or $G_{\beta\gamma}$ signaling to the chloroplast **(Joo et al., 2005)**. Second, the activation of MAP kinases by O_3 **(Joo et al., 2005)**. Third, G_α subunit required for O_3-lesion formation **(Booker et al., 2004; Joo et al., 2005)**.

O_3-induced ROS production starts first in the chloroplasts of the stomatal guard cells, and spreads later to the adjacent cells **(Joo et al., 2005)**. The early chloroplastic oxidative burst is a result of signaling through the $G_{\beta\gamma}$-protein complex that is perceived in the chloroplast **(Kangasjärvi et al., 2005)**. This triggers ROS production in the chloroplasts of the guard cell.

This intracellular ROS are likely to be sensed through accumulation, which alters the redox balance of the cell **(Van Breusegem et al., 2001)**. This first peak of ROS is perceived by the G_{α} protein that activates NADPH oxidases on the plasma membrane to produce extracellular ROS (second peak), which spread to the adjacent cells and is perceived by $G_{\beta\gamma}$-protein complex, mediating the intercellular signaling, which in turn, repeats the two peaks cycle again in the neighboring cells **(Kangasjärvi et al., 2005)**.

1.1.1.5. Activation of MAP kinase cascades and Ethylene production

The activation of mitogen-activated protein kinase (MAPK) cascades also starts within minutes from the beginning of O_3 exposure in parallel to G-proteins; however, it is independent from the G-protein pathway and does not involve G-proteins. It also does not appear to be directly involved in the activation of ROS production, since it takes place in both O_3-sensitive and -tolerant accessions **(Kangasjärvi et al., 2005)**. Activation of MAP kinases by phosphorylation generally leads to nuclear localization of transcription factors and their activation by the MAPK. Among MAP kinases, AtMPK6 and AtMPK3 are rapidly activated by O_3. The activation of AtMPK6 results in increased ethylene synthesis only in the sensitive accessions. The down-regulation of ethylene synthesis is slower or less efficient in the O_3-sensitive accessions comparing to the tolerant ones **(Kangasjärvi et al., 2005)**. The endogenous, cell death-driving ROS production triggered by O_3 is ethylene-dependent **(Kanna et al., 2003)**.

1.1.1.6. Development and spread of visible O_3-lesions

Ethylene and salicylic acid (SA) are needed for the development of the visible O_3-lesions. The endogenous, cell death-driving ROS production triggered by O_3 is ethylene-dependent **(Kanna et al., 2003)**. Ozone exposure induces SA synthesis within a few hours after the beginning of the exposure. ROS production drives the SA-dependent cell death. SA has a vital role in cell death, and that without SA, active PCD is not initiated in O_3- exposed plants. Lesion propagation is clearly an ethylene-dependent process **(Kangasjärvi et al., 2005)**.

1.1.2. Potential plant tolerance mechanisms to Elevated Ozone (E-O_3)

1.1.2.1. Canopy architecture

Plant canopies with higher leaf area index (LAI) had lower O_3 concentrations in the lower canopy than canopies with lower LAI **(Finkelstein et al., 2004)**. This means that canopy closure could give some protection to the lower canopy with older and more sensitive leaves, which may lead to more tolerance.

1.1.2.2. Cuticle modification

Epicuticular waxes are directly in contact with atmospheric O_3, so that, they are putative sites of primary perception. However, O_3-induced alterations to these epicuticular waxes had no effect on O_3 responses in most of the cases **(Vaultier and Jolivet, 2015)**. In contrast, comparative metabolite profiling among birch genotypes showed that some compounds related to leaf cuticle wax formation could provide growth-related tolerance to O_3 **(Kontunen-Soppela et al., 2007)**.

1.1.2.3. Regulation of O_3 flux to leaves (stomatal control).

The magnitude of ROS from O_3 breakdown in the apoplast of plant leaves is determined by the amount of O_3 entering the leaf through stomatal opening, and the apoplast antioxidant capacity. The early responses guard cells to O_3 stress determine the flux into the leaf. Plants responding to O_3 with adequate stomatal responses are more likely to be more tolerant. However, O_3 is known to interfere with guard cells functions, and therefore stomatal conductance. O_3-induced ROS in the guard cells were found to induce stomatal closure and/or inhibit of stomatal opening, eventually decreasing stomatal conductance, which is considered a plant protective mechanism **(Hill and Littlefield, 1969)**.

There are several different mechanisms, by which O_3 induces stomatal closure in different plant species **(Kangasjärvi et al., 2005)**. This response may protect plants against short-term O_3 peaks, or long term mild ozone stress, if accompanied by effective O_3 scavenging

mechanisms in the apoplast. However, stomatal closure is restrictive to gas exchange; therefore, O_3-induced stomatal closure counteracts CO_2 absorption and O_2 release required for photosynthesis, as well as, transpiration needed for evaporative cooling under heat stress. For example, O_3-induced stomatal closure in Siebold's beech during early summer reduced ozone influx and allowed the maximum photosynthetic capacity to be reached, but was not sufficient to protect the photosynthetic system in late summer and fall **(Hoshika et al., 2013)**.

E-O_3 is also known to inhibit stomatal closure of sensitive plants by inducing ethylene production that suppresses ABA role in stomatal closure **(Wilkinson and Davies, 2009; Wilkinson and Davies, 2010; Wilkinson et al., 2011)**. Varieties with less ethylene production under O_3 stress will have better stomatal control of O_3 flux.

1.1.2.4. ROS detoxification by ascorbate (ASC) in the apoplast and cell wall repair and protection

Early detection and quick responses upon O_3 penetration are essential for tolerance. It requires the combination of stomatal control of ozone flux, adequate total capacity for scavenging of ROS, and re-equilibrium of detoxification system. In addition, metabolic changes are required to allow enough supply of reducing power and carbon skeletons for repair and detoxification. These functions are required for O_3 tolerance despite the decrease in photosynthetic activity. It is clear that these acclimation responses are effective only against short-term peaks, and cannot continue for extended periods as those encountered under chronic exposure, because the continued O_3 flux will reduce carbon availability, in addition to the increase in demand for reducing power and energy. All these will leads to disequilibrium and to cell death **(Dizengremel et al., 2009)**. Increased cell wall lignification, and the up-regulation of the phenyl-propanoid pathway in leaves under long-term ozone exposure are some examples for repair and acclimation mechanisms **(Cabane et al., 2012)**.

There are some exceptions to the previous conceptualization; a comparison between the two tolerant/sensitive clones of white clover indicated the presence of other factors - than ascorbate - that play a role in ROS detoxification and cell wall repair in the apoplast **(D'haese**

et al., 2005). This was based on the lack of O_3 protection provided by higher ascorbate content in the sensitive clone (NC-S) than in the tolerant clone (NC-R) which had lower ascorbate.

1.1.2.5. Plasma membrane

Activation of phospholipases by O_3-induced H_2O_2 counteracts the oxidation of membrane unsaturated fatty acid and results in phosphatidic acid, which decreases H_2O_2-promoted programmed cell death **(Zhang et al., 2003)**. Oxylipins derived from oxygenation of polyunsaturated fatty acids of plasma membrane works as key signaling molecules that mediate O_3-stress control mechanisms. JA, the plant hormone is an example of oxylipin, that gradually controls O_3-induced programmed cell death in sensitive reactions making them gradually less sensitive **(Santino et al., 2013)**.

1.1.2.6. Containment of the visible O_3-lesions

Two different hormonal mechanisms can be suggested to be responsible for the containment of lesion spread. Ethylene itself can contribute to the containment of the ethylene-dependent O_3 lesion propagation since ethylene causes desensitization of the cells to its own action **(Wang et al., 2002)**. The ethylene receptor acts as a suppressor of ethylene signaling when it is not in contact with the hormone and this suppression is released after ethylene binds to the receptor. Thus, the O_3-induced synthesis of new ethylene receptor proteins could lead to decreased ethylene sensitivity and down-regulation of ethylene-dependent lesion spread. The down-regulation of ethylene synthesis seems to be slower or less efficient in the O_3-sensitive accessions **(Kangasjärvi et al., 2005)**.

In the oxidative cell death cycle, jasmonic acid (JA) and methyl jasmonate (MeJA) protect tissues from ROS-induced cell death and thus counteract the effects of SA and ethylene. O_3-induced cell death was inhibited when tobacco plants were pretreated with jasmonate with similar results found in Arabidopsis **(Örvar et al., 1997)**. Jasmonate treatment also reduced the amount of SA produced in response to ozone **(Rao et al., 2000)**. JA's antagonism to cell death is mediated at least partly through its effect on SA. JA also antagonizes ethylene

signaling. This interaction is mutually antagonistic, since ethylene also inhibited JA-induced gene expression **(Tuominen et al., 2004)**. JA affects ethylene signaling at the receptor level by decreasing ethylene sensitivity in a receptor-dependent manner. Therefore, JA induce up-regulation of an ethylene receptor isoform **(Schenk et al., 2000)**.

At the tissue level, the balance between ethylene, SA, and JA signaling probably shifts temporally and spatially, so that the SA- and ET-driven processes prevail in the first cells affected by O_3. Nevertheless, the further away from the site of initiation, the JA pathways become increasingly more induced to overcome the first processes and containment of cell death follows **(Kangasjärvi et al., 2005)**.

1.1.3. Wheat responses to O_3 Stress

1.1.3.1. Wheat Importance

Wheat is a key crop for global food security, and the staple food for 30% of the world population, with 711 million tonnes produced in 2013, around 44% of which produced in Asia **(FAO, 2016)**. It provides 21% of the food calories consumed globally and 20% of the protein to more than 4.5 billion people in 94 developing countries **(Braun et al., 2010)**. In some countries, such as those in North Africa, per capita consumption of wheat is as high as 240 kg per annum, provides nearly 55% of the carbohydrates **(Joshi et al., 2011)**. By 2050, the world will need 70% more food, nearly 4 billion tonnes of cereals, most of which are needed in developing countries **(Shiferaw et al., 2013; Tester and Langridge, 2010)**.

1.1.3.2. Wheat Evolution

Wheat is one of the oldest and most widely cultivated food crops worldwide. In 2013, it was grown on approximately 218 million hectares, representing 15.5% of the arable land in the world, nearly half of it in Asia **(FAO, 2016)**. It is adapted to a wide range of environments, from the equator to latitudes of 60°N and 44°S, and at altitudes ranging from sea level to 3000 m **(Singh et al., 2011)**. The widespread and adaptation of wheat could be attributed to its large sized (17 billion base pair) and complex genome.

Wheat has an allohexaploid genome, consists of three closely related, diploid subgenomes, AA, BB and DD **(Eversole et al., 2014)**. The A and B genomes diverged from a common ancestor around 7 million years ago. Genome D evolved from A and B genomes 1-2 million years later, through homoploid hybrid speciation **(Marcussen et al., 2014)**. Around 0.36-0.5 million years ago, a tetraploid (AABB) species evolved through hybridization between two diploid species, the first is an einkorn wheat, similar to modern *Triticum urartu* (genome AA), which resembles cultivated wheat - morphologically and in spike and seed development - much more than any of its other progenitors **(Ling et al., 2013)**. The second diploid species is an extinct or undiscovered goatgrass species, closely related to the *Sitopsis* section of *Aegilops* (genome BB) **(Haider, 2013)**. This tetraploidization lead to the primitive form of domesticated tetraploid wheat, closely related to *Triticum turgidum* subsp *durum*, (genomes AABB). The allohexaploid bread wheat (*Triticum aestivum* L., 2n = 6x = 42 chromosomes; genomic code AABBDD) has evolved around 10 thousand years ago in the Fertile Crescent **(Heun et al., 1997; Salamini et al., 2002).** It was a result of an allohexaploidization between the primitive tetraploid wheat and a goatgrass (*Aegilops tauschii*), which contributed genome DD, and led to the formation of bread wheat **(Marcussen et al., 2014)**.

This complex genome of wheat has evolved since the divergence at hexaploid level. Comparison of chromosome based sequences across the genomes showed dynamic gene gain, loss, and duplication since their divergence, without a high degree of transcriptional autonomy or global dominance for any of the subgenomes **(Mayer et al., 2014)**. This was accompanied by high sequence similarity of each of the subgenomes to wheat relatives, with retained structural conservation and limited gene loss **(Mayer et al., 2014)**. However, the tetraploid subgenomes (AABB) retrieved from hexaploid common wheat showed higher transcriptome expression **(Zhang et al., 2014)** and less DNA methylation when compared to cultivated and wild *T. turgidum* **(Liu et al., 2015a)**. These changes to the subgenomes as part of the allohexaploid and their subsequent adaptation might be what made wheat a very diverse crop.

1.1.3.3. Wheat Classes and Diversity

Wheat is a very diverse crop; varieties are visually classified according to the endosperm texture or hardness (soft or hard) and color (white or red), as well as, growth habit (winter or spring) **(Thomason et al., 2009)**. Hardness is determined by the strength of binding between the starch granules and the protein network surrounding them. Soft wheat has loose binding between starch and protein matrix, due to lower protein percentage, usually less than 10%, which makes it easy to grind, and gives soft-texture flour, suitable for high dense products baked to low moisture content, such as cookies and crackers. Hard wheat has stronger binding between starch and protein matrix, due to higher protein content (about 12%) which makes it more difficult to grind, and produces a coarse-texture flour, with more broken starch granules with high water uptake and provides more fermentable sugars for yeast, and therefore more suitable for yeast-leavened bread, such as pan bread **(Thomason et al., 2009)**.

The growth habit of wheat is determined by the vernalization requirement, and freeze and heat tolerance. Vernalization is extended exposure to cold temperatures at seedling stage that is required for flowering induction **(Roll-Hansen, 1985)**. Winter wheat is more freeze tolerant and requires vernalization to flower, however, it is less heat tolerant, and extended heat stress could result in devernalization, and failure to switch from vegetative growth to flowering. Spring wheat is freeze sensitive, and suffers from "winter kill" which is the death of some stems when exposed to freezing stress. However, it more heat tolerant and flowers without vernalization.

In the United States, there are five main classes of bread wheat, [in addition to durum wheat (*Triticum turgidum* subsp *durum*)] **(Thomason et al., 2009)**. These classes are:

1- Soft Red Winter Wheat (SRW): Suitable for high rainfall areas, such as the Mid-Atlantic region. It has low protein content (less than 10 %), usually used for cookies, crackers, and flat breads.

2- Hard Red Winter Wheat (HRW): Suitable for semi-arid regions, such as the Plains states. It has a wide range of protein content (averaging about 12%) percent, usually used for pan bread, rolls, and general-purpose flour.

3- Hard Red Spring (HRS): Suitable for the Midwest. It is used for croissants, rolls, bagels and pizza crust. It is also used to improve flour blends for bread and Asian noodles.

4- Soft White: Suitable for the Pacific Northwest, low protein content (less than 10 %), usually used for making cakes, muffins, cookies and pastries.

5- Hard White: Suitable for Central and Western states, low protein content (less than 10 %), usually used for making cakes, muffins, cookies and pastries. The newest wheat class of the US, closely related to the HRW, usually used in Asian noodles, hard rolls, bulgur, and yeast breads.

There are increasing efforts aiming at breeding in HRW wheat varieties adapted to the mid-Atlantic region, to provide local sources of bread flour, and save the consumers the transportation costs of transferring the HRW from the plains states **(Thomason et al., 2009)**.

1.1.3.4. Wheat sensitivity to O_3

Wheat is one of the most sensitive crops to O_3, at all growth stages **(Singh and Agrawal, 2010)**. Ozone effects on wheat plants include changes in antioxidant capacity **(Biswas et al., 2008a)**, changes in stomatal conductance **(Feng et al., 2012; Hassan, 2004; Pleijel et al., 2006)**, and reduced photosynthesis **(Biswas et al., 2013; Meyer et al., 2000; Wattal and Siddiqui, 2015)**. Ozone could also cause chlorotic and/or necrotic visible symptoms **(Mills et al., 2011)** and hastened leaf senescence **(Burkart et al., 2013; Gelang et al., 2000; Ojanpera et al., 1998)**. These effects are usually reflected on biomass, grain, and protein yield reductions **(Amundson et al., 1987; Feng et al., 2012; Hassan, 2004; Heagle et al., 2000; Mills et al., 2011; Ojanpera et al., 1998; Singh and Agrawal, 2010; Van Dingenen et al., 2009)**, the magnitude of these effects is dependent on the dose and genotype sensitivity.

Current O_3 levels during wheat growing season are high enough to cause average global yield reduction of 7-12 %, and this is expected to continue in the future to reach 9-18 % by 2030 **(Avnery et al., 2011a; Avnery et al., 2011b; Feng and Kobayashi, 2009)**. However, these effects of O_3 on wheat are not uniform across the world, as the highest yield reductions are usually experienced in developing and heavily populated countries, such as China and India, and these reductions are expected to continue and even increase **(Feng et al., 2012; Singh and Agrawal, 2010; Van Dingenen et al., 2009)**.

In general, wheat is known to be more sensitive than other cereal species **(Selldén and Pleijel, 1995)**. Oat (*Arena sativa* L) is considered relatively insensitive **(Pleijel et al., 1994)**, However, two spring oat varieties were found to have similar O_3 responses to two spring wheat varieties, and none of them appeared to be O_3 sensitive **(Hartikainen et al., 2012)**. On the other hand, Barley (*Hordeum vulgare* L) is considered an O_3-insensitive crop **(Mills et al., 2007; Selldén and Pleijel, 1995)**.

A meta analysis of the yield responses reported in 53 studies (1980 and 2007) did not find yield differences due to the growth habit **(Feng et al., 2008)**. However, these authors came to this conclusion despite the fact that winter wheat showed similar negative responses at 20% higher concentrations than spring wheat, which should indicate that winter wheat is more tolerant to O_3 than spring wheat. Meanwhile, spring wheat was more sensitive in terms of O_3 effects on chlorophyll content, which might be an indicator for greater visible symptoms, which was not considered in this particular comparison of the meta-analysis. The reported O_3-induced stomatal closure in spring wheat and the reduction in chlorophyll content are indicators of an over-reaction to O_3 stress through restricting gas exchange which might have resulted in lower photosynthesis and reduced chlorophyll maintenance. This is more likely than ROS-induced chlorophyll destruction. Differences among varieties have been reported within both spring **(Barnes et al., 1990; Pleijel et al., 2006; Velissariou et al., 1992)**, and winter wheat **(Biswas et al., 2008a; Biswas et al., 2008b; Biswas et al., 2013)**.

Because O_3 in known to induce senescence, and the role of antioxidant capacity of the tissue in tolerance, O_3 symptoms on wheat usually develop first on the older leaves, which are in the lower canopy, and from the tip of the leaf, where the older tissue is located. In addition, wheat yield is more sensitive to O_3 exposure during grain filling stage than younger stages **(Fiscus et al., 2005)**.

Reported trends in ground level O_3 concentrations in different regions around the world **(Cooper et al., 2014; Lee et al., 2014; Monks et al., 2015; Parrish et al., 2013)** indicate that O_3 will continue to be major challenge to wheat production all over the world. In China, O_3 levels are increasing O_3 throughout the entire wheat-growing season, and are expected to continue increasing until 2050. In the western USA, winter and spring are experiencing increasing O_3 levels, despite the decrease in summer, with peaks shifting more towards the important wheat grain-filling stage near the end of the season, in late spring. Increased levels in winter, without major changes in the already high concentrations in spring, suggest that O_3 stress on wheat grown in eastern USA is not decreasing. In Western Europe, ambient O_3 levels leveled off or decreased after 2000, at levels already high enough to impact wheat.

Yearly average baseline O_3 at rural or remote sites of the northern hemisphere (29-49 ppb) increase to even higher levels (37-59 ppb) with increasing altitude from sea level **(Cooper et al., 2014)** indicates that wheat is O_3 stressed nearly everywhere grown. This stress is even higher near polluted areas where high O_3 concentrations are common. Therefore, future wheat varieties with O_3 tolerance are critical for maintaining or increasing wheat yield.

O_3 also affects wheat interactions with abiotic stresses such as drought. For example, O_3-induced ethylene interferes with stomatal closure and causes reduced drought tolerance **(Wilkinson et al., 2011)**. This results in increased yield reductions under combined drought and O_3 stress when compared to drought alone **(Xu et al., 2007)**. Cross-tolerance to O_3 and drought was not observed in wheat **(Biswas and Jiang, 2011)**. In addition, O_3 affects wheat interactions with soil microorganisms, changing their processes and community structure. Five years of elevated O_3 stimulated soil N availability, but suppressed grain yields **(Chen et al.,**

2015). This was due to increased NH_4^+ availability and decreased microbial ability to retain NH_4^+, which increases nitrogen losses. However, these effects were less pronounced in O_3-tolerant than O_3-senstive cultivars. Soil microbial communities in wheat fields are indirectly affected by O_3 treatment, and these effects are dependent on the O_3 response of the wheat cultivar grown **(Li et al., 2012)**. When treated with O_3, the tolerant varieties had more influence on soil biota than sensitive varieties, which may have influenced soil organic matter decomposition, nutrient turnover, and probably greenhouse gas emission.

1.1.3.5. Factors limiting wheat breeding for O_3 tolerance

Despite the necessity and the potential for breeding O_3-tolerance wheat cultivars, the breeding efforts in this critical area for global food security are very limited for the following reasons:

1.1.3.5.1. The lack of awareness that plants are more sensitive to O_3 than humans are.

Although it has been known for several decades that plants are more sensitive to O_3 than humans are, the implementations are yet generally recognized. A clear sign of this problem is the lack of O_3 monitoring stations in agricultural areas in many parts of the world. Monitoring O_3 in agricultural rural areas is critical for quantifying effects on plants, animals, and human in those areas, as O_3 effects experienced in rural areas are higher than in urban areas **(Monks et al., 2015)**. Another sign is the absence of O_3 monitoring parameters and summaries routinely released that would reflect the effects on plants. Because wheat plants are exposed to O_3 throughout their entire growing season, they accumulate O_3 damage. Extended periods of exposure require estimating O_3 cumulative doses and communicating those estimates with growers. Current air quality standards consider O_3 levels below 50 to be within the good air quality category **(US EPA, 2016)**. However, 40 ppb is the threshold for plants, and known to cause significant yield reductions to sensitive crops such as wheat **(ICP Vegetation, 2011)**.

1.1.3.5.2. The misperception of the scale of the problem for wheat production.

Wheat is one of the most sensitive crops to O_3, at all growth stages **(Singh and Agrawal, 2010)**. Ozone effects on wheat plants are taking place at a global scale, however, they are more significant in developing countries, where food security is already endangered and more food production is needed **(Shiferaw et al., 2013; Tester and Langridge, 2010)**. O_3 transport ranges from local to hemispheric scales with increasing baseline O_3 worldwide, including areas where emission of precursors has been controlled and reduced. This dynamic indicates that E-O_3 will continue to be a problem on a global scale **(Cooper et al., 2014; Lee et al., 2014; Monks et al., 2015; Parrish et al., 2013)**.

1.1.3.5.3. The expectation that active selection for O_3 tolerance is not needed, as inadvertent or natural selection for O_3-tolerance is taking place, as a result of having diverse wheat varieties encountered by O_3 stress worldwide every season.

Unlike elevated CO_2 that undergoes a gradual increase in atmospheric concentrations due its long lifetime, E-O_3 is a diverse problem both spatially and temporally, due to its short lifetime, and the wide variety of factors controlling O_3 concentrations over time and space. These variations offset the consistent O_3 selection pressure required for conventional breeding procedures to make breeding progress **(Ainsworth et al., 2008)**. In addition, wheat is an annual crop, harvested and planted every year, which does not allow natural selection to occur as in the case of perennials **(Barnes et al., 1990)**. Furthermore, O_3 symptoms on wheat could be easily confounded by other stresses or misdiagnosed, which prevent growers and breeders from establishing the required connection between yield loss and O_3 damage. Without recognizing the cost of O_3 sensitivity, growers and breeders will not be selecting varieties to be grown or used for breeding based upon the O_3 responses. This problem was confirmed by the fact that modern wheat varieties are more sensitive than older ones **(Biswas et al., 2008b; Biswas et al., 2013; Pleijel et al., 2006)**. This is a result of selecting the new cultivars for high

stomatal conductance, and less mesophyll resistance to gas diffusion, and consequently higher photosynthesis **(Biswas et al., 2008b; Biswas et al., 2013)**, which enhances O_3 uptake resulting in greater O_3 sensitivity, especially when selection is conducted under inconsistent O_3 pressure **(Ainsworth et al., 2008)**.

1.1.3.5.4. The lack of information on the phenotypic diversity of O_3 responses among key breeding material.

Although differences in O_3 responses were documented among wheat varieties **(Biswas et al., 2013; Mills et al., 2011; Wilkinson et al., 2011)**, there is a lack of information on O_3 responses of key varieties currently used for agricultural production, and as parents for breeding future cultivars. Differential O_3 responses are documented among wheat varieties **(Biswas et al., 2008b)**, indicating the importance of identifying the relative O_3 responses of varieties to be used in breeding programs, studying O_3 effects on wheat performance, and the interactions with other phytobiome components. Winter wheat should be considered as a source of O_3 tolerance for spring wheat. There are reports from different parts of the world showing increased O_3-sensitivity in modern varieties of both spring **(Barnes et al., 1990; Pleijel et al., 2006; Velissariou et al., 1992)**, and winter wheat **(Biswas et al., 2008a; Biswas et al., 2008b; Biswas et al., 2013)**. However, there is limited information on the difference in O_3 responses between soft and hard red winter wheat cultivars, and the trends in O_3 response over time.

Screening the O_3 responses of breeding material that is being used in breeding programs for other significant traits (e.g. rust resistance, heat and drought tolerance) is an important approach to provide breeders with suitable breeding materials for O_3 tolerance. It will enable breeding varieties that are more resilient (tolerant to environmental stresses and resistant to pathogens at the same time). It will also compensate for the striking lack in efforts and resources currently devoted to breeding wheat for O_3 tolerance **(Ainsworth et al., 2008; Mills et al., 2011)**, by utilizing the genotyping data generated for identifying quantitative traits loci (QTLs) and markers associated with other traits. For example, screening plant materials used

by the Borlaug Global Rust Initiative (BGRI) **(McIntosh and Pretorius, 2011)** for breeding for O_3 tolerance could be a unique model for breeding for O_3 tolerance. Populations made by crossing breeding lines bearing novel rust-resistance genes, with rust universal susceptibles genotypes of contrasting O_3 response, are ideal material for such an effort.

1.1.3.5.5. The lack of information on the genetic nature and heritability of O_3 tolerance.

Despite the observed differences in O_3 responses among wheat varieties, and the current consensus that O_3 tolerance is a genetically controlled trait **(Barnes et al., 1999; Li et al., 2015; Singh and Jwa, 2013)**, there is a severe lack of information about the heritability of O_3 tolerance in wheat, and the genetic gain the tolerance delivers **(Burkart et al., 2013)**. Currently identified QTLs or markers associated with O_3 tolerance are limited to rice **(Frei, 2015; Kim et al., 2004)**, Arabidopsis **(Brosché et al., 2010)** and soybean **(Burton et al., 2016)**.

There is only one report **(Biswas et al., 2008a)** on the source of O_3 responses in hexaploid wheat, which attributed the origin of O_3 sensitivity in bread wheat to subgenome DD. However, to our knowledge, there are no reports on the genetic control of O_3 responses at the chromosome level. A screening of the 21 monosomic lines (each of which lack one chromosome) of any wheat variety against its full genome could correlate O_3 response (sensitivity of tolerance) to one chromosome. Ideal materials for this purpose are Chinese Spring and its 21 monosomic lines. Since they were first developed in 1953, these monosomic lines were used to associate wheat traits to specific chromosomes **(Sasaki et al., 1963)**. However, to our knowledge; such materials have never been used for identifying O_3 genetic control at the chromosome level.

Several studies used a doubled haploid population of the cross between Chinese Spring (low abscisic acid) and SQ1(low abscisic acid), in an attempt to identify QTLs associated with high yield under O_3 stress **(Quarrie et al., 2005a; Quarrie et al., 2006; Quarrie et al., 2007)**. However, the QTLs identified were generally associated with yield, but not specific to yield

under O_3 stress, therefore, they were uninformative to breeders. This might due to the lack of differential O_3 sensitivity of the two parents.

1.1.3.5.6. Missing the cross connections between O_3 responses of wheat cultivars and many other traits of interest.

O_3-induced response of wheat cultivars was shown to modulate O_3 effects on nitrogen use efficiency and nutrient availability in soil **(Chen et al., 2015)**, in addition to soil pH **(Li et al., 2013)**, and organic matter **(Lu et al., 2016)**. O_3 response also determines O_3 impacts on the structure and functionality of soil microbial communities **(Li et al., 2012; Li et al., 2013)**. O_3 sensitivity might affect plant interactions with the foliar microbiome, including major wheat pathogens as well as beneficial and biological control agents. E-O_3 was found to affect wheat necrotrophic diseases **(Tiedemann, 1992b)**, as well as biotrophic diseases, such as stem rust **(Heagle and Key, 1973a; Heagle and Key, 1973b)**, leaf rust **(Dohmen, 1987; Heagle, 1975; Pfleeger et al., 1999; Tiedemann, 1992a; Tiedemann et al., 1991; Tiedemann and Firsching, 2000)**, and powdery mildew **(Tiedemann, 1992a)**. Effects of O_3 on beneficial organisms and biological control are yet to be studied **(Mashaheet, 2016)**. This suggests that ignoring O_3 responses might result in reducing breeding efficiency for other traits. This was evident by the complex interactions between O_3 and drought responses of wheat **(Biswas and Jiang, 2011; Xu et al., 2007)**.

1.1.4. Parsimonious Strategies for Breeding Resilient O_3-Tolerant Wheat

Global food security requires specific selection strategies to improve yield in stressed environments **(Tester and Langridge, 2010)**. With the limited resources available, those strategies should make use of the current breeding efforts and materials. Wheat breeders around the world are combating several major limitations to wheat production including climate change, new races of rust diseases, in addition to the need for new varieties that meet the quality needed for local market, (e.g. breeding hard red winter wheat varieties that are adapted to eastern US). Breeding plants for O_3 tolerance is an attainable goal **(Barnes et al., 1999)**. However, the inconsistency of O_3-stress pressure in the field makes the current field-

plot evaluation practices unsuitable for breeding tolerant varieties. The limited breeding tools for O_3 tolerance hinder adding this trait to breeders' criteria of selection. Providing breeders with the scientific information on O_3 responses of the same material they currently use might be the most suitable and applicable procedure for breeding for O_3 tolerance.

An ideal and parsimonious procedure is to screen and select for O_3 tolerance in breeding materials already known for tolerance and resistance to abiotic and biotic stresses. Rust resistance and drought tolerance are examples for such traits. These traits have received great attention, especially in the last two decades. Many breeding populations for these two traits were mapped and their genotypic data could be utilized for O_3 genetic studies.

In large breeding programs, O_3 screening must be an outcome driven process. This is essential to keep the costs/benefit ratio reasonable. It also will avoid the tradeoffs between many competing priorities, such as the experiment size versus the detailed measurements, the number of replications versus the number of genotypes tested, the number of parameters measured and subsampling. There are different objectives for a plant breeder to pursue a germplasm screening, each of which requires different procedure. The following are some examples:

1.1.4.1. Exploring O_3 tolerance in Large Number of Wheat Breeding Materials

When screening a large number of diverse breeding lines, it is very helpful to begin with, a small of germplasm sub-groups that represent the diversity within the entire set. Then select genotypes that represent each sub-group (e.g. varieties with large contribution to the germplasm within the sub-group). It is also recommended to use varieties known as O_3-sensitive and O_3-tolerant, maybe from other programs, if previous information is not available. This will show where exactly this germplasm falls within the sensitivity-tolerance range. The genotype release date might be another way of sorting out the germplasm, or alternatively, used as a criterion for choosing genotypes within sub-groups. This should show differences among and within groups and - may be - the direction of unintentional selection for/against O_3

tolerance. In this type of experiment, the use of a wide range of ozone concentrations is recommended.

1.1.4.2. Identification of genetic markers

If the available germplasm includes any genetically mapped populations for genetic markers (e.g. SNPs), it will be useful to screen the parents of those populations against known O_3-sensitive and O_3-tolerant genotypes. At this stage, it is recommended to use a wide range of O_3 concentrations as well as multiple parameters and growth stages to elucidate the differences between the two parents. If the parents exhibit wide differences in O_3 response, there is an opportunity for identifying the QTL(s) controlling O_3 tolerance. Contrasting parents may justify a large screening for the entire population. In such a screening, only the differential O_3 concentrations and parameters determined from the preliminary experiment should be used, at appropriate replication and sampling regime. The optimum growth stage might differ from one population to another. For example, if the population is segregating for growth and development, seedling responses will be less confounded by phenological differences. In other words, it might be good to screen seedlings before they differentially grow. On the other hand, if there is no such segregation, then the growth stage at which the most differential O_3 responses were observed should be used.

1.1.4.3. Pre-breeding Screening

Knowing the O_3 responses of parents selected for crosses gives the breeder some idea about what relative range of O_3 responses in the progeny. If the target breeding area is O_3-stressed, starting with tolerant parents reduces the probability of selecting for sensitivity. Collaborators with access to O_3-screening facilities could screen potential parents, if such facilities are not available locally for breeders.

1.1.4.4. Pre-release Screening

In areas with high O_3 stress, making the crosses using parents with known O_3 responses gives the breeder some idea about the relative range of O_3 responses to expect in the progeny.

Starting the process with tolerant parents reduces the probability of selecting for sensitivity, in fact, the use of tolerant parents selects against O_3 sensitivity.

If the breeder is at the final phase of cultivar development, making the pre-release evaluations of varieties descendant from parents with unknown O_3 responses, a quick screening for the parents should provide some information on whether or not the selection for O_3 should be considered. If the two parents were found to have different O_3 responses, then the shortlisted varieties along with the two parents should be screened to assure O_3 tolerance in the final selection.

1.1.4.5. Validation of Seedling Screening with O_3-Yield Responses

If the breeder has utilized O_3 tolerant parents, making pre-release yield evaluations under O_3 stress is essential before claiming O_3 tolerance. Although wheat tolerance to O_3 visible symptoms is a desired trait that might affect wheat interactions with physical and biological environment, a yield benefit under O_3 stress must be demonstrated. This type of validation for yield superiority under O_3 stress should be conducted under open or semi-open field conditions.

The O_3 treatment used in such validation studies should be relevant to O_3 levels in the targeted area. The following treatments might correlate results obtained under controlled systems and results obtained under field conditions:

a. Ambient air (fenced chambers or plot).
b. Ambient air + EDU (fenced chambers or plot and the application of an antiozonant material such as ethylene diurea (EDU) to minimize the effect of O_3 for comparison with treatment (a)).
c. Non-filtered ambient air (paneled chambers or plots).
d. Charcoal filtered air (paneled chambers or plots to provide a “clean air” control for other treatments).
e. Near-ambient O_3 level (paneled chambers or plots).

f. Highest-relevant O_3 level (paneled chambers or plots).

g. Future O_3 level (paneled chambers or plots).

The comparison between treatments (a) and (b) will quantify the effects of ambient O_3 levels at the site. Whereas, the comparison between (a) and (c) will identify the effects of the exposure system structures on the O_3 response of the plants. Treatments d-g will be used to establish the O_3-yield curve.

1.1.5. O_3-Exposure Systems and Experimental Error Control in Wheat O_3-Screenings

Different levels of O_3 stress could be achieved through different O_3 exposure methods. All O_3 exposure methods aim at providing specific O_3 treatments, to the experimental unites of an O_3 exposure system. First, ambient air is passed through a charcoal filter (CF) to remove O_3. Then O_3 is generated using O_3-generator and mixed with CF-air to achieve the uniform targeted concentration in each treatment unit (chamber or a plot). Continuous monitoring and O_3 enrichment are essential to maintain treatment overtime.

1.1.5.1. O_3-Exposure Regimes

There are different procedures for delivering different levels of O_3 stress, and the change in magnitude of stress during the day. According to their magnitude, O_3 exposure regimes could be classified into:

a- Chronic exposure: Usually involves treatment with O_3 levels $\leq$ 120-150 ppb, and results in gradually developed symptoms such as chlorosis.

b- Acute exposure: Usually involves treatment with O_3 levels $\geq$ 120-150 ppb, and results in rapidly developed symptoms such as necrosis.

Diversity in O_3 responses between and within species, and even within plant canopy position, tissue age, and growth stages make it difficult to draw clear lines between the two

categories **(Fiscus et al., 2005)**. Perhaps each genotype or even plant tissue (certain leaf position and age) has its own limits for sub-symptomatic effect, chronic visible symptoms, and acute visible symptoms, and biomass and yield reductions. These limits may change overtime with growth and development.

O_3 exposure regimes could also classified according to the temporal changes in concentrations. The following are some of the most commonly used O_3 exposure regimes:

a- Steady-state Exposure:

Concentrations will be maintained at steady target that does not change over time and applied for certain number of hours during daytime. Suitable for obtaining average treatments without exposing plants to significantly high peaks.

b- Predefined Smooth Diurnal Profile:

Treatment will change throughout the day following a predefined diurnal profile that mimics the average diurnal ambient O_3 profile with low concentration in the morning that gradually increase after sunrise to reach the beak in the afternoon or evening, then declines overnight. The treatment in this case is referend to as the 12hr diurnal profile average, not the 24hrs average, assuming that the nocturnal stomatal conductance is negligible. However, nighttime stomatal conductance is not zero and may even be higher than expected for stomata that do not respond normally to ozone treatment. For example, O_3-induced ethylene production might result in a significant inhibition in stomatal closure, hence increasing nocturnal stomatal O_3 flux. This regime should use a diurnal profile that mimics natural O_3 profile at the site of interest. For example, at low altitude, O_3 concentrations usually decline at night, however, at high altitudes, nighttime ozone concentrations show less fluctuation and less nighttime decline.

c- Ambient-derived Exposure:

Treatments are assigned as proportions of ambient concentration collected at time of exposure. It mimics the fluctuations in O_3 concentrations. However, during periods of low

ambient O_3, the O_3 proportional treatments may not exceed the threshold levels required to observe differential responses between the two concentrations.

d- Fluctuating-Predefined Diurnal Curve:

Applying a random effect within a certain range (±10% of target) to the smooth predefined curve would result in a randomly fluctuating curve that would mimic ambient fluctuation, yet allow differential treatments.

e- Actual Ambient Concentrations:

A selected actual ambient O_3 data from a certain location, during a specific reason, in which O_3 concentrations were high enough to provide differential treatments if proportion procedure is applied. Unlike the use of current O_3 levels as a reference (which may not be high enough to cause differential stress), this regime uses real O_3 data previously recorded, which assures differential O_3 stress by the end of the season.

In general, an exposure regime that mimics O_3 natural fluctuations without losing differential treatments would be ideal. The predefined diurnal profile was found to cause more effects on photosynthesis than the steady-state exposure **(Meyer et al., 2000)**. This difference could be due to the higher peaks, or due to the changes in concentrations over time, which requires the plant to change and adapt its stomatal conductance overtime. Therefore, a fluctuating O_3 curve would be expected to cause more effects than a smooth predefined diurnal profile.

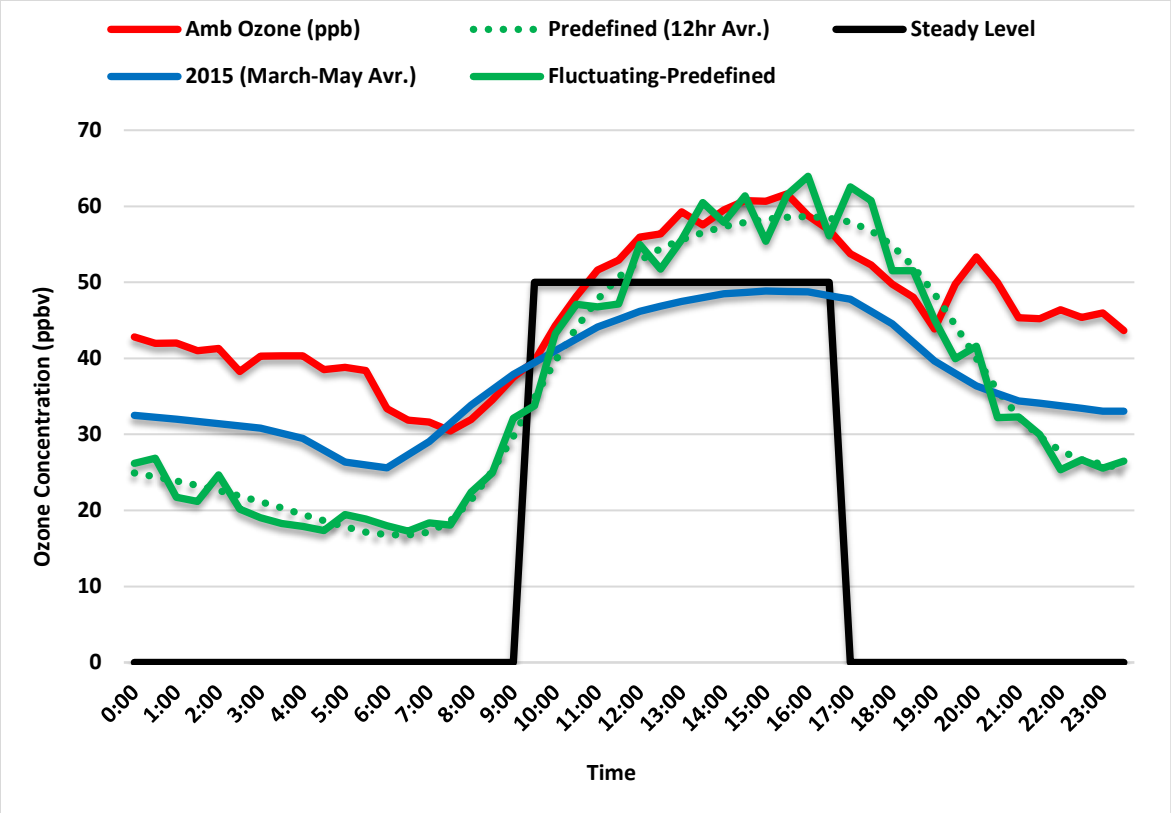


Figure (1): Changes in O_3 concentrations overtime using different O_3 exposure regimes. Average diurnal profile of the 2015 active growing season (March-May), and April 21, 2016 (50 ppb 12 average) are given as examples for short- and long-term ambient levels. Ambient data were monitored at the USDA-ARS Plant Science Unit field site, 5 km south of Raleigh, NC, U.S.A. Elevation was 110 m above sea level, and provided by Samuel Ray & Walt Pursley.

1.1.5.2. O_3-Exposure Systems

There are different systems that accommodate the treated plants, monitor O_3 concentrations, and generate and deliver O_3 to maintain the targeted treatments. Some systems controls other major environmental factors such water supply, temperature, relative humidity, light density and CO_2. The following are some of the most commonly used O_3 exposure Systems:

1.1.5.2.1. Continuous Stirred Tank Reactors (CSTRs)

CSTRs are cylindrical chambers covered with Teflon film, inside a charcoal filtered greenhouse. Chambers have single-pass air ventilation system, designed for rapid mixing of gases **(Heck et al., 1978; Rogers et al., 1977)**. The air comes mixed with O_3 from the top of the chamber, and continuously stirred with a fan positioned at the chamber ceiling, then a negative pressure blower system removes the air from the bottom after passing through the plant canopy. Relative humidity is monitored and controlled, by adding water vapor under pressure to the air as it enters the chamber. Temperatures are monitored and the entire greenhouse temperature is controlled, but there is no control for individual chambers. The supplemental light source is located over the chamber to compensate for the green house and chamber shading effects and allow for manipulation of day length.

Figure (2): Continuous Stirred Tank Reactors (CSTRs) inside a charcoal filtered greenhouse. Plant Research Unite, USDA-ARS, Raleigh, NC, USA.

1.1.5.2.2. Outdoor-plant Environment Chambers (OPECs)

OPECs are outdoor chambers covered with Teflon **(Flowers et al., 2007)** and designed for closed air circulation system. Air is pumped through an inlet duct in one side of the chamber, where it is mixed with moisture released from sprinklers under pressure. The air is directed upward to avoid exerting pressure on the plants from the side, and to allow more air mixing at the top of the chamber. The air is recycled through the outlet duct on the opposite side of the chamber. The air entering the chamber is adjusted to the set temperature and then passed through a charcoal filter to remove O_3. Afterwards, O_3 and CO_2 are added as needed and mixed with air before it passes through a dehumidifier to remove excess water to meet the relative humidity target, and the air enters the chamber again to repeat the cycle.

Figure (3): Outdoor-Plant Environment Chambers (OPECs). Plant Science Unite Field, USDA-ARS, Raleigh, NC, U.S.A.

1.1.5.2.3. Open-top Chambers (OTCs)

OTCs are cylindrical shaped chambers of a metal structure supporting two levels of polyvinyl chloride (PVC) panels providing isolation from horizontal wends. The top panel is solid, whereas the lower panel is consists of a double panel with perforations in the internal layer through which a fan box blower system forces charcoal filtered air into the chamber and out the open top **(Booker et al., 2009)**.

Figure (4): Open-top Chambers (OTCs). Plant Science Unite Field, USDA-ARS, Raleigh, NC, U.S.A.

1.1.5.2.4. Air Exclusion System (AES)

AES plots are regular field plots surrounded by two double-walled panels on the long sides only, with fences on the two short sides. The panels are similar to OTC's bottom panels and are placed in parallel to form a 3m x 10m treatment area. Charcoal-filtered (CF) air is provided by fan boxes equipped with heat exchangers to provide optional elevated temperature treatments **(Burkey et al., 2016)**.

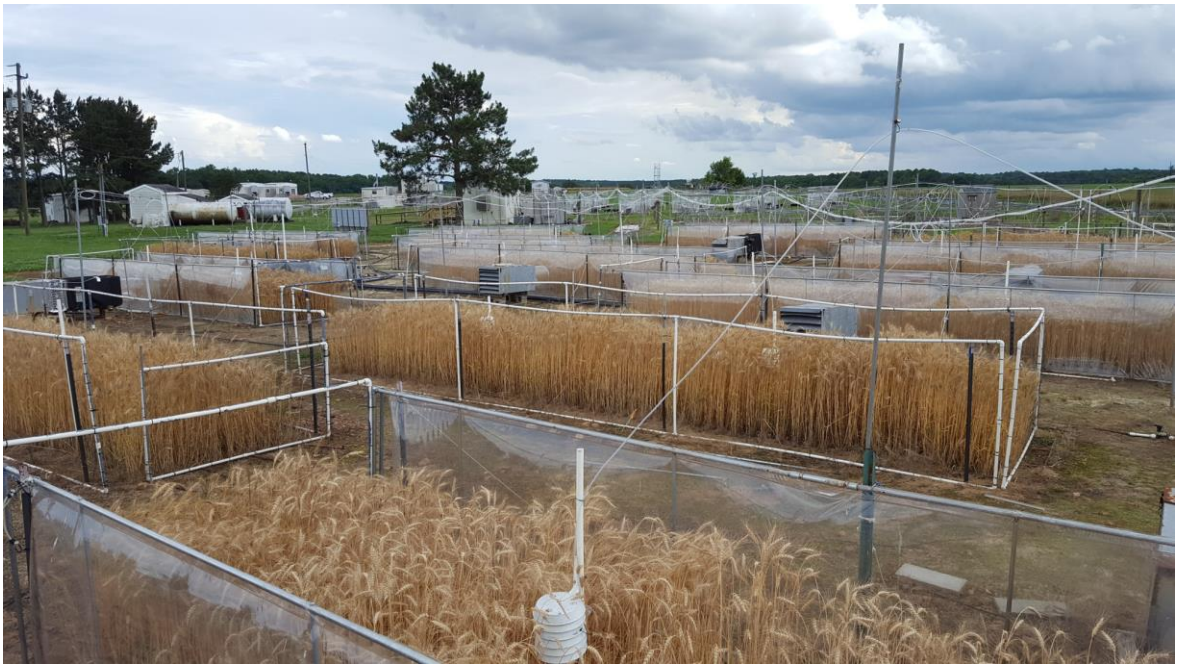


Figure (5): Air Exclusion System (AES). Plant Science Unite Field, USDA-ARS, Raleigh, NC, U.S.A.

1.1.5.2.5. Free-air Gas Concentration Enrichment (FACE)

FACE is developed to provide minimum interruption to the soil-plant-air-light continuum, using a computer simulation to control gas dispersion from circles of pipework sources. However, it a large scale design that requires high O_3 generation, spatial and computational requirements **(McLEOD, 1995)**. Furthermore, FACE does not provide isolation against ambient air, which means that control O_3 treatments are limited to that provided by ambient air which in some locations may be above the threshold for plant response. Ideally, the FACE system is established in a remote area with relatively low O_3 levels.

Fluctuations in wind direction and speed as well as ambient O_3 level make it difficult to achieve the consistent O_3-stress pressure under such completely open system. The results obtained from FACE and OTC were found to lead to similar conclusions despite the differences between the approaches **(Morgan et al., 2006)**. Wheat plants tested using the FACE approach require at least 1 m buffering zone to avoid edge effect **(Long et al., 2005)**, a rule that could be applied to all systems.

1.1.5.3. Standardization of Visible Injury Scores

1.1.5.3.1. Standardization of Tissue Scored

Wheat has a complex and dynamic canopy, which makes it difficult to assign O_3 scores on a whole canopy basis, hence, O_3 injury is scored in a leaf basis as shown in Figure 6). The canopy of a single wheat plant consists of a main stem that emerged from the germinated seed and primary tillers that develop from the base of the main stem, usually each of which is the bud of one of the leaves. Similarly, in low-density situations, the primary tillers in turn develop secondary tillers, in a similar fashion to the main stem.

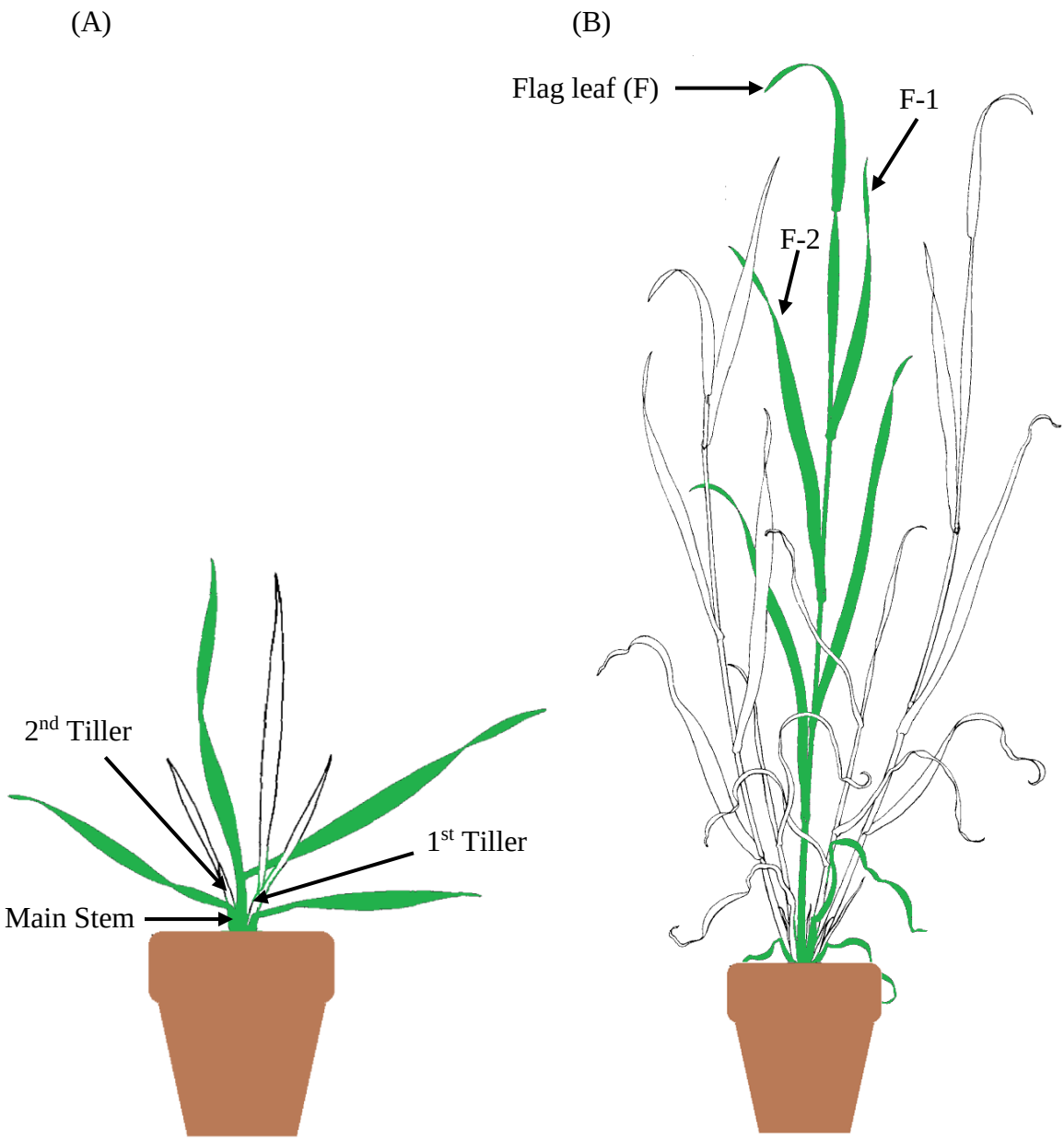


Figure 6: Wheat canopy structure at seedling stage [13, 22 **(Zadoks et al., 1974)**] and adult-plant stage (18, 46 Zadoks). Modified from **(Tottman, 1987)**.

Leaves on the main stem are easier to identify and to track. Once a tiller is developed in the bud of a leaf, usually the tiller pushes the leaf away from the main stem, making the identification of leaf origin more difficult. For tissue standardization, the main stem and leaf order might need to be tagged at the beginning of tillering stage. To assure plant material uniformity, starting with excess numbers of plants (20-30%) increases the chances of achieving uniformity, by excluding the top and bottom 10-15% percent in the developmental stage distribution.

Wheat leaves in a wheat canopy can be identified through combinations of position (using the top of the bearing stem as reference), age (time after being unfold), and order (using the base of the bearing stem as a reference). To compare ozone scores across individual plants and treatments, all three criteria must be taken into account. A suitable procedure is tagging the main stem for tracking the leaf order. Leaves on the main stem, starting from the third leaf to the flag leaf at the top, represent all leaf positions, order and age within the canopy. Tracking leaf order is usually begun at the seedling stage. The top-leaf position is occupied by the newly emerged leaf until the flag leaf (last leaf to develop on a stem) emerges. After flag leaf emergence, the leaf position is usually referred to as Flag leaf (F), Second-top leaf (F-1) and Third-top leaf (F-2).

Data for O_3 injury are not usually collected from the first leaf on the main stem. Emergence of the third leaf on the main stem mostly coincides with the emergence of the first tiller. The third leaf on the main stem typically very close in age to the first leaf on the first tiller. At the seedling stage, scoring the third and fourth leaf and maybe the fifth on the main stem allows for assessment of a wide range of leaf order, age, and position. At adult-plant stage, data are usually collected from the flag leaf of the main stem because of the large contribution of flag leaves to the yield. To acquire data on leaves of different age at the adult plant stage, there are two procedures:

a- Collecting data from F, F-1, and F-2 on the main stem. This offers a good representation of leaf age, position and order, with high certainty. However, the sample contains only

one flag leaf with high contribution towards yield. This method might be more suitable for injury screening purposes, and characterizing O_3 impacts on different canopy layers.

b- Collecting data from flag leaves of main stem, first tiller, and second tiller. This offers a sample of three leaves that are significant contributors to yield. However, it provides less representation of age and order diversity. In addition, all leaves will be at the top of the canopy. This approach could be more suitable for injury/yield penalty associations.

1.1.5.3.2. O_3 Injury Scoring System

Due to the canopy complexity, O_3 scores should be gives in a leaf basis, with respect to leaf position, order and age. Leaves on the main stem (the third leaf and above) are the easiest to track. O_3 injury scores are the percent of the leaf area showing chlorosis or necrosis in response to O_3 stress, starting at the leaf tip, where the older cells are located and progressing towards the developmentally younger cells at the base of the leaf blade. In some cases, wheat leaves might show bronze color, similar to symptoms observed mainly on dicots such as snap beans or soybean.

Because wheat leaves are thin, the control of light environment at scoring is critical for consistent scoring procedure. A visible injury scoring space with constant light environment is recommended. Scoring O_3 symptoms in a greenhouse with the sun light changing in angle and density is not a suitable procedure. O_3 symptoms progress overtime following completion of O_3 exposure, so statistical blocking is essential to avoid confounding the genotype or treatment effects by the drift in scores if the scoring process requires several days.

When the number of plants is small, leaves could be scanning and O_3 injury could be assessed using image analysis software, such as APS Assess 2.2. Otherwise, a scoring guide with images of wheat leaves showing different levels of O_3 symptom and the corresponding percent injury assigned will increase data quality and reduce the estimation error. Another approach is having O_3 injury scored by different people and use the average score.

1.2. Disease Triangle for Wheat Rust under Elevated Ground-level O_3 Concentrations

1.2.1. Causal Agents and Spread of Wheat Rust Pathogens

Wheat is attacked by three rust diseases, caused by three fungi. Stem (Black) rust (Sr) is caused *Puccinia graminis* f. sp. *tritici* (*Pgt*), Leaf (Brown) rust (Lr) is caused by *Puccinia triticina* Eriks. (*Pt*), and Stripe (Yellow) rust (Yr) is caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*) **(Morgounov et al., 2012)**. Rust fungi are classified in the phylum Basidiomycota, class Urediniomycetes, order Uredinales, and family Pucciniaceae. This family contains 17 genera and approximately 4121 species, the majority of which are in the genus *Puccinia* that contains all three rust fungi **(Leonard and Szabo, 2005)**.

Stem rust is more adapted to higher temperatures than the other rusts, historically causing losses in Africa, the Middle East, Asia, South West Pacific, Europe, and the Americas **(Leonard and Szabo, 2005)**. Being a major breeding target of the green revolution and the deployment and durability of stem rust resistance gene (*Sr*) 36, in addition to the eradication of the alternate host, barberry (mainly *Berberis vulgaris*), kept stem rust controlled for several decades, and reduced the need for breeding efforts to identify and incorporate new rust resistance genes **(Morgounov et al., 2012)**. This stabilized situation was challenged by the resurgence of stem rust in middle and east Africa after the emergence of a new race called Ug99, that is virulent on *Sr31* a widely adapted resistance gene, imposing a major threat to most of wheat germplasm around the world **(Pretorius et al., 2000)**. Since then, race Ug99 evolved to a lineage with 13 variants and spread to 13 countries from South Africa to Egypt in the north and Iran in the east, and it is has the potential to spread to other major wheat production area around the world **(Fetch et al., 2016)**.

Leaf rust is the most frequent and widely spread rust of wheat due to its wide range of virulence and environmental adaptation **(Bolton et al., 2008)**. Leaf rust attacks wheat wherever it is grown, but the yield losses are usually small unless the epidemic is sever, and covers a large geographical area **(Morgounov et al., 2012)**. It is an important disease in major wheat

production areas around the world including, the Americas, Eastern Europe, Central and Southeast Asia and North Africa.

Stripe rust was an important wheat disease in cooler and moist environments, such as high elevations and latitudes and in cooler years, due to its low temperature requirements **(Chen et al., 2014)**. However, the new *Pst* races adapted to warmer temperatures that emerged after 2000, and the introduction to Australia and South Africa, showed that this fungus is capable of thriving under higher temperatures **(Morgounov et al., 2012)**. The early infection is increasingly resulting in severe yield losses since 2000, as recorded in the United States, South Africa, China, Central and west Asia, and Australia **(Morgounov et al., 2012)**.

The global spread of wheat, O_3 stress and rust diseases indicate the importance of studying their interactions and the cross connection between wheat responses to one of these stressors that might mediate wheat response to one of the other stress factors. This also indicates the importance of incorporating both disease resistance and O_3 tolerance as major components of resilience in future wheat cultivars.

1.2.2. General Cycle of Rust Diseases and Possible O_3 Effects

Each of the wheat pathogens and the diseases they cause, their specific requirements, host range, alternative hosts and symptomology were discussed in detail in a series of pathogen profiles, each of which focused on one of the rust pathogens [Sr **(Leonard and Szabo, 2005)**, Lr **(Bolton et al., 2008)**, and Yr **(Chen et al., 2014)**]. However, all three rusts share the same general standard disease cycle and stages, summarized here with the possible O_3 interactions.

All rust pathogens are now considered as macrocyclic, heteroecious rust pathogens. Macrocyclic means that the pathogen is capable of forming the complete set of five types of rust spore stages, and heteroecious means they are formed on two hosts (primary- and alternative host). Urediniospores and teliospores are formed on grass hosts, where the urediniospores act as the recurrent inoculum in rust epidemics, whereas the teliospores are a resting structure formed towards the end of the season or under unsuitable conditions. No

sexual reproduction takes place on wheat. Teliospores germinate later forming basidiospores of different mating types that infect the alternative host [mainly barberries (*Berberis* spp.)] in the vicinity. On barberry, the other two stages are formed, and sexual reproduction takes place. Pycniospores formed in a pycnium of a certain mating type fertilizes a receptive hypha in another pycnium of a different mating type, forming new combinations of fungal hyphae that produce aeciospores spores. When aeciospores formed on *Berberis* are transmitted to wheat, they germinate and infect the susceptible plants, and the urediniospores are produced as a recurrent inoculum causing the rust epidemic on the plant. The following is a summary of the different stages of the rust disease cycle in the disease epidemic on wheat, and the possible O_3 effects on each stage:

1.2.2.1. Dissemination

Dissemination is the release of primary inoculum (i.e. urediniospores) from the fungal structures (pustules). Urediniospores from the pustules are released once the epidermis of the host is ruptured under the pressure of spores developed underneath. O_3 stress is known to affect plant cuticle **(Kontunen-Soppela et al., 2007)**, and might have effects on the strength of the epidermal cover of the pustule and to accelerate or delay pustule rupture and spore release, which in turn might impact disease spread and progression rate. To our knowledge, there is no study that tested such hypothesis. However, at least two reports showed that O_3 affects the structure and texture of cuticular waxes **(Karnosky et al., 1999; Percy et al., 2002)**.

1.2.2.2. Transmission

Transmission is the transfer of spores (inoculum) from diseased plants (source of inoculum) to a healthy tissue (on the same plant or another plant). Rust spores are airborne, transmitted via air currents long distances, or within water droplets for short distance. Factors such as spore size, mass, texture and shape, determine the wind speed required for spores to become airborne and the travelling distance in the air and final deposition. Because spores on the leaf surface are exposed to O_3, there could be some direct O_3 effects on properties such as spore texture. O_3 also could have indirect effects on the spores by affecting the nutrient

availability in the host tissue, which might affect spore size, mass or shape. To our knowledge, there is no study that tested this hypothesis.

1.2.2.3. Inoculation

Inoculation is the landing of spores (the inoculum) on the host tissue (infection court), or the process through which the inoculum comes in contact with the infection court. Through this process, spores are either precipitated from or deposited by air on the leaf surface. The properties of the leaf surface are main factors in this process, which might be affected by O_3 stress **(Kontunen-Soppela et al., 2007)**. O_3 was found to affect cuticle roughness, and to increase aspen leaf rust by 5 folds **(Karnosky et al., 1999)**, which might in turn affect the spore exchange between leaf surface and leaf boundary layer. To our knowledge, there is no study that tested this hypothesis on wheat.

1.2.2.4. Spore Germination

Spore germination starts with water imbibition, and the pathogen recognition of the host under the suitable conditions, which results in activating the growth and extension of the germination structure to the port of entry. Spore germination in rust requires viable spores, a wet leaf surface for several hours (mainly from dew formation or rain) and overnight darkness, at a suitable temperature. Leaf wetness might be affected by the rate of water condensation or drying out. This would be determined by leaf surface properties and microclimate interaction with canopy structure. O_3 is known for altering cuticular-wax structure, by changing the ratio of hydrocarbons to fatty acids, which represents the ratio between the most and the least hydrophobic cuticular wax components, respectively **(Karnosky et al., 1999; Percy et al., 2002)**. The resulting increase in leaf wetting would facilitate free water film formation and provide more suitable environment for rust spore germination.

The key step for germination is the recognition of the susceptible host tissue. Usually pathogen spores only germinate on their susceptible hosts, by recognizing the biochemical signature of the tissue surface. Elevated concentrations of both O_3 and CO_2, as well as their

combination were found to increase the synthesis of fatty acids, which stimulate host recognition by the pathogens **(Percy et al., 2002)**.

Germinating spores extend a germination tube that recognizes the topology of the leaf surface and grow perpendicular to the long sides of the epidermal cells and either locates the stoma (the entry-point) or growth ceases as resources are exhausted. Once the germination tube recognizes the stoma, it forms an appressorium over the stomatal opening, and the fungal growth ceases until the morning. O_3 effects on cuticle roughness observed under electron microscopy and the corresponding topology changes are thought to be a major reason for aspen leaf rust increase by five folds **(Karnosky et al., 1999)**.

Spore viability, spore size and nutrient stocks might be affected directly by O_3 stress during or after spore formation, or indirectly by O_3 impacts on nutrients availability in the host tissue during spore formation. However, there is no even significantly high O_3 concentrations (60-240 ppb) did not affect viability of non-stressed or O_3-stressed spores **(Heagle and Key, 1973a; Heagle, 1975)**.

1.2.2.5. Penetration

Penetration is the process by which the pathogens enters into the host tissue. Rust fungi enters through stomata. This stage takes place in the morning, if the leaf surface is still wet, and the appressorium is not desiccated. When the light induces stomatal opening, *Pgt* **(Leonard and Szabo, 2005)** and *Pst* **(Chen et al., 2014)** recognize the decrease in CO_2 concentration at the stomata and sends out a penetration peg from the lower surface of the appressorium, through the opened stoma, into the sub-stomatal cavity below the epidermis. *Pt* does not require light and is not affected by CO_2 concentrations, as stomata close in response to appressoria formation, and the infection peg pushes through **(Bolton et al., 2008)**. In the sub-stomatal cavity, the infection peg forms a sub-stomatal vesicle, from each end of which an infection hypha may be produced and sent to one of the adjacent cells. Once the tip of the infection hyphae contacts the cell wall of the host's cell, it forms a haustorium mother cell. So far, the pathogen has managed to penetrate into the internal part of the leaf, but there is no

direct contact between the pathogen and the host's cell membrane, as the host's cell wall is still separating the two.

Prolonged O_3 exposure might also result in stomata blockage due to via affecting cuticular waxes **(Karnosky et al., 1999)**. O_3 is also known to interfere with stomatal closure, and O_3 overnight peaks are not uncommon. These overnight peaks usually coincide with high O_3 concentrations in the morning, which is the time of penetration of rust pathogens. O_3-induced stomatal closure or delayed penetration means that the fungal structure may be heated and dehydrated by sunlight and the penetration is aborted. However, *Pt* might not be affected by stomatal closure, as it actively pushes the penetration peg even through closed stomata.

Sub-symptomatic, pre-inoculation O_3 exposure had no effect on stem rust penetration to wheat leaves, whereas, injurious pre-inoculation treatments reduced penetration **(Heagle and Key, 1973a; Heagle, 1975)**. On the other hand, post-infection O_3 exposure had no effect on penetration **(Heagle and Key, 1973a; Heagle, 1975)**.

1.2.2.6. Infection

Infection is the establishment of a direct biological contact between the pathogen and the host's cell membrane, and securing nutritional flow from the host's cell. This occurs by sending an infection peg that penetrates the cell wall of the host cell by both enzymatic dissolution and physical pressure. However, it does not penetrate the plasma membrane of the host cell. Then, the tip of the penetration peg grows into a haustorium in the periplasmic space between the plasma membrane and the cell wall of the host cell. Depending on the initial spore's resources, 1-3 haustoria may be formed in different adjacent cells.

If the spore originally formed under O_3 stress, the endogenous nutrient stock in the spore might have been affected by O_3, which might have indirect effect on the infection structure formation. However, even significantly high O_3 concentrations (e.g. 60-240 ppb) did not seem to have such effect on O_3-stressed spores **(Heagle and Key, 1973a; Heagle, 1975)**.

If penetration is taking place under high O_3, penetration structures could also be affected directly. The host cell wall structure and functionality might be affected by O_3 stress, which might have impact on its interaction with the penetration peg. O_3-stressed cell walls might be easier for the pathogen enzymes to process, and might take less physical force from the infection peg to penetrate. On the other hand, O_3-generated ROS in the apoplast might have negative effects on pathogen extracellular enzymes and effectors.

In addition, the formation of appressoria, vesicles and haustorium mother cells are induced by one six-carbon derivative, one of several six-carbon derivatives of the plant lipoxygenase (LOX) pathway (trans-2-hexen-1-ol), a pathway known to be induced in plants by both pathogen attack **(Leonard and Szabo, 2005)**, and O_3 stress **(Vaultier and Jolivet, 2015)**. In resistant hosts, resistance compounds are deposited around the infection peg location to prevent the penetration of the cell wall or the forming the appressoria or the direct contact of the appressoria with the plasma membrane of the host cell. The plants' ability to timely produce and deliver these compounds might be affected under O_3 stress. This resistance strategy also depends on the early recognition of the pathogen, which might depend upon receptor proteins on/in the plasma membrane. O_3 effects on the plasma membrane and protein denaturation due to the O_3-induced ROS might also interfere with this process.

Establishing the nutritional relationship between the pathogen and the host is a very complicated process, which takes place through a battle for dominance on the haustorium/plasma membrane interface (usually separated by extra-haustorial matrix). The performance of an O_3-impaired plasma membrane might affect the plants ability to prevent nutrient acquisition through haustoria. Furthermore, low to moderate O_3 stress could cause sub-symptomatic (sub-symptomatic) effects, such as membrane disintegration, leading to electrolyte leakage into the apoplast **(Zheng et al., 2011)**. This could provide more nutrients for the pathogen in the apoplast that will not involve the haustoria. These types of effects were found to alter wheat interactions with necrotrophic pathogens by increasing disease severity **(Tiedemann and Pfähler, 1994)**. However, the effects of such mild stress on biotrophic pathogens, such as wheat rusts, are yet to be investigated.

Sub-symptomatic, pre-inoculation O_3 exposure had no effect on stem rust infection to wheat leaves, whereas, injurious pre-inoculation treatments reduced infection **(Heagle and Key, 1973a; Heagle, 1975)**. On the other hand, post-infection O_3 exposure had no effect on infection **(Heagle and Key, 1973a; Heagle, 1975)**.

1.2.2.7. Invasion

Invasion is the ability of the pathogen to spread from the infection locus to surrounding host tissue. In rusts, this requires the utilization of the host's resources after a successful infection. In a susceptible host, the plant fails to recognize the pathogen inoculation, penetration or infection on early enough to suppress the invasion. On the other hand, resistant host recognizes the pathogen and timely activates resistance mechanisms to prevent each of these invasion requirements from happening. Many of the rust resistance genes encode for nucleotide-binding site leucine-rich repeat (NBS-LRR) protein domains, which are usually resistance protein (RP) located on the apoplastic side of the plasma membrane **(Kolmer, 2005)**. These domains are known as signal receptors especially for diseases.

Incompatible reactions (resistance) occur when the resistance gene interacts with a specific avirulence gene from the rust pathogen, which initiates a specific signal that induces resistance defense mechanisms. Contrarily, compatible reactions (susceptible) and infections occur in the absence of an avirulence gene, or the failure of an RP to detect the avirulence gene product (pathogen effector). Because of their locations, the both the NBS-LRR proteins and the pathogen's effectors are vulnerable to O_3-induced ROS in the apoplast. This might result in protein denaturation and function loss.

There are several resistance mechanisms, among which the hypersensitive response (HR). In HR responses, the host recognizes the pathogen and initiates a programmed cell death response in the surrounding cells adjacent to the infection locus. This is carried out usually by intensive ROS production that results in killing the host cells that would provide the pathogen with nutrients to continue the invasion. The second result is that HR is a signaling process that

indicates that the host is under a pathogen attack, therefore, inducing a systemic priming effect. These two mechanisms give the HR a doubly effective defense against pathogens.

There are two major requirements for the HR response to succeed in stopping the pathogen without extreme damage: (1) the timely activation of the ROS production machinery at the infection site, and (2) the timely activation of an effective ROS scavenging system in the tissue surrounding the targeted HR zone. O_3 stress has the potential to interfere with both requirements for a successful HR response. First, O_3 stress response metabolism involves directing plants resources towards increasing antioxidant activities. Under moderate O_3 stress, reversal of ROS-scavenging physiology into ROS-producing physiology might be slower than initiating ROS-production from the normal equilibrium state, which could be a potential benefit to the pathogen. On the other hand, if the O_3 stress is high, O_3-induced ROS could lead to induction of a more rapid HR response. Second, O_3 stress might interfere with HR containment by the tissue surrounding the HR zone, as HR containment in O_3 stressed tissue might be more difficult comparing to normal tissue.

Near-threshold O_3 treatment (36.0-38.8 vs. 46.4-55.5 ppb) was found to increase poplar leaf rust by fourfold due to decreased phenolic glycoside concentrations (important defense compounds in poplar) and increased leaf witting **(Karnosky et al., 1999; Percy et al., 2002)**. This suggests that O_3 could also enhance rust diseases by directly suppressing the chemical defenses of the host. Symptomatic pre-inoculation O_3 exposure restricts stem rust invasion to wheat leaves, resulting in smaller colonies, whereas post-inoculation injurious treatments did not restrict invasion until 4 days after infection **(Heagle and Key, 1973a; Heagle, 1975)**.

1.2.2.8. Sporulation

Sporulation is the formation of a spreading or resting structure that would secure the ability of the pathogen to spread and thrive through the season and survive between seasons. In rusts, after a successful invasion at the infection locus, the fungus forms a dense sporulation-mat of hyphae beneath the host epidermis, on which sporophores grow and produce masses of urediniospores that causes the epidermis to expand and then rupture, forming the major rust

sign (pustule). The color of the urediniospores is what defines the rust diseases (black, brown, yellow). O_3 effects on the host might determine the resistance of epidermis to rupture and expose spores, or the time it takes the fungus to acquire enough nutrients to start producing spores. O_3 could also affect the total amount of nutrients available to the pathogen to utilize for spore production, hence the number of spores produced and the rate of spore production, or the available nutrients stored in each spore.

There is enough evidence that relatively high O_3 treatments (60-240 ppb) significantly reduced stem rust spore production on injured wheat leaves **(Heagle and Key, 1973a; Heagle, 1975)**, but not on non-injured leaves **(Heagle and Key, 1973b)**.

1.2.3. Disease Components of Wheat Rusts

Stem rust diseases are quantified using different parameters, each of which could be used as an indicator of specific aspects of the rust/wheat/time interactions. The following are some of the parameters frequently used **(McIntosh et al., 1995; Pask et al., 2012; Roelfs et al., 1992)**:

1.2.3.1. Incubation Period

In rust, the incubation period is the time from inoculation to the appearance of first visible symptoms (flecking at infection loci where the pustules will develop later) or appearance of pustules (as pustules might appear directly without flecking, which is typical in fully susceptible reactions). O_3-stressed tissue might show flecking sooner than normal tissue (shortened latent period), as chlorophyll content might have been affected prior to infection.

1.2.3.2. Latent Period

In rust, latent period is the time from inoculation to the appearance of 50% of the total number of ruptured pustules developed in a certain area. This is an indicator of the total sporulation process. In some situations, the traditional calculation of latent period is used as the time from inoculation to the appearance of the first ruptured pustule. This is an indicator as to when the diseases starts to be contagious. Increased nutrient availability caused by O_3-

induced electrolyte leakage into the apoplast might enable the fungus to acquire nutrients for more rapid sporulation which might shortened the latent period. Alternatively, O_3-induced decreased photosynthesis may result in fewer resources available to the fungus and delay or prevent sporulation. In addition, O_3 effects on the cuticle characteristics might accelerate or delay pustule rupture.

1.2.3.3. Disease Severity and Percent Sporulation Area

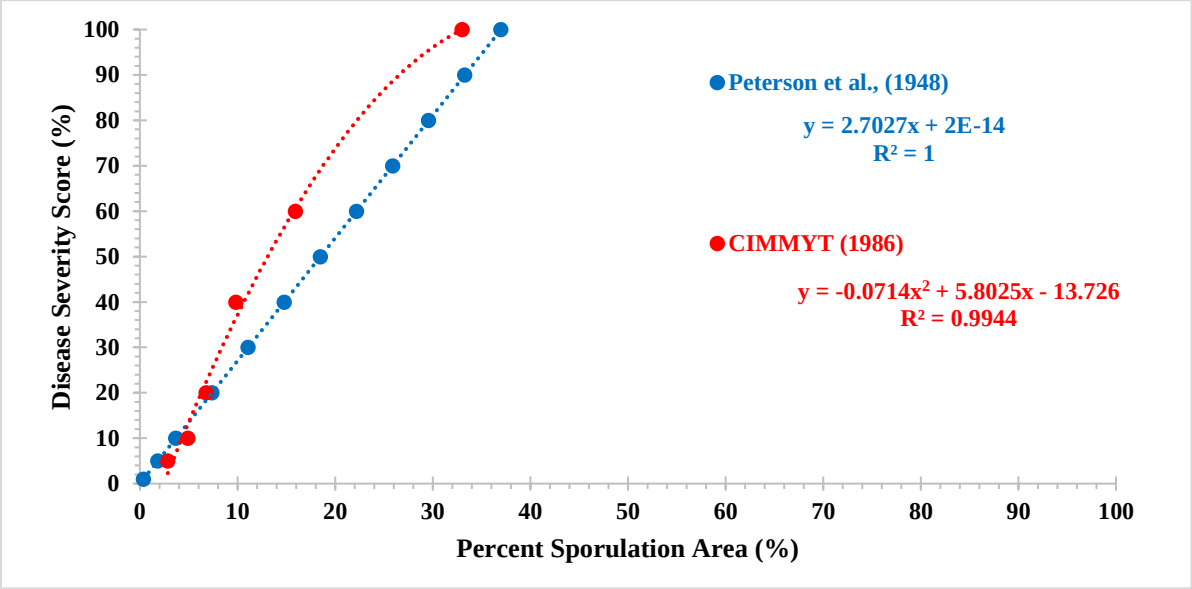
Disease severity is the percentage of disease area relative to the total tissue area examined **(Mayee and Datar, 1989)**. In rust diseases, disease severity is assessed using a relative scale of the ratio between the observed sporulation area and the maximum sporulation area possible of the observed infection type. There are two scales commonly used as shown in Figure (7):

a- Modified Cobb Scale: Assuming a simple linear relationship between disease severity scale and the actual sporulation area, with a maximum disease severity of 100% corresponding to a maximum percent sporulation area of 37% of the diseased tissue **(Pask et al., 2012; Peterson et al., 1948; Roelfs et al., 1992)** .

b- CIMMYT Rust Scoring Guide Scale: Assuming a non-simple linear relationship between the disease severity scores and the actual percent sporulation area, assuming a maximum disease severity of 100% corresponding to of 33% sporulation percentage of the diseased tissue **(CIMMYT, 1986)**.

Percent sporulation area is usually assessed using imaging or scanning the diseased tissue, and running the images through image analysis software. Currently, the American Phytopathological Society (APS) offers two software programs developed and customized for this purpose: (1) Assess 2.2, which is software compatible with Windows operated devices, and (2) The Leaf Doctor, an iPhone application compatible with OS operated devices.

(a)



(a)

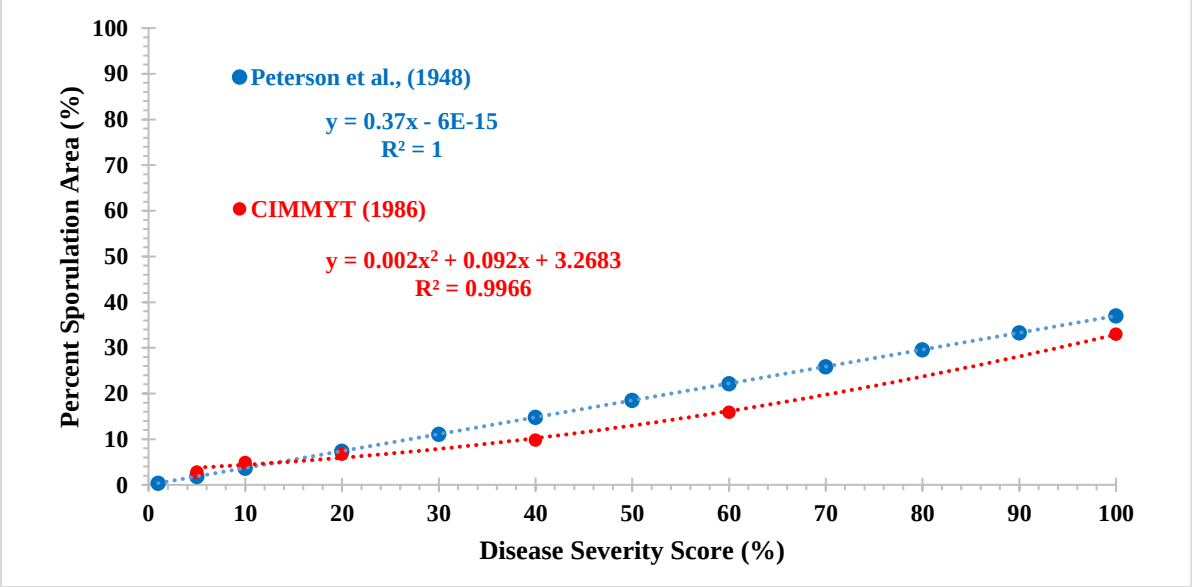


Figure (7): Simple **(Pask et al., 2012; Peterson et al., 1948; Roelfs et al., 1992)** and polynomial linear relationship **(CIMMYT, 1986)** between Disease Severity Scores and actual Percent Sporulation Area.

Disease severity and percent sporulation area could be affected by O_3 in several ways. Effects of O_3 on germination, penetration, infection, and/or sporulation might be unidirectional which would be reflected as net changes disease severity and sporulation area. Alternatively, O_3 might have multidirectional effects on different host stages; in this case, disease severity and percent sporulation area may, or may not show a net effect.

1.2.3.4. Disease Incidence (Prevalence)

Disease incidence is the proportion of diseased plants or plant units (e.g. leaves or stems) per sample **(Mayee and Datar, 1989)**. It does not take into account the disease severity on the diseased tissue. The disease incidence might be affected by O_3 by two mechanisms: First, if O_3 induces disease on certain leaf or tiller category that was not expected to show disease at all (could be postulated under sub-symptomatic O_3 stress). Second, if O_3 completely inhibits disease on tissue that was expected to show disease, probably under high O_3 stress causing necrotic O_3 effects.

1.2.3.5. Receptivity (Pustule Density)

Receptivity is the number of uredinia per area. It is an indicator of the inoculation density and the infection success percentage. O_3 effects on receptivity could be similar to its effects on disease severity and percent sporulation area.

1.2.3.6. Spore Production

Spore production could be measured as the number of spores or spore weight per pustule or area. Because rust spores are airborne, it is difficult to quantitatively collect them under natural conditions. O_3 could affect spore production in two ways: (1) effects on the host plant and resource availability; and (2) after the pustule ruptures, the fungal mat and sporophores in the pustule are no longer protected from ambient O_3 by the cuticle. However, the spore masses on the pustule surface might receive most of the O_3 exposure.

1.2.3.7. Reaction/Infection Types

Wheat/rust reaction types could be categorized into nine different phenotypes, based up on the presence of uredinia, flecking, chlorosis, and pustule size **(McIntosh et al., 1995)**:

0 Immune: No Sporulation, and no visible symptoms are observed in response to inoculation

; Near Immune: No Sporulation, with visually observed flecking, which are small chlorotic or necrotic areas resulting from HR.

1 Very Resistant (R): Small circular uredinia, surrounded by necrosis, circular-shaped.

2 Moderately Resistant (MR): Small-medium uredinia, with green islands, surrounded by necrosis or chlorosis (oblong-shaped in stem rust).

3 Moderately Susceptible (MS): Medium-sized uredinia, sometimes with chlorosis (small-diamond shaped in stem rust).

4 Susceptible (S): Large-sized uredinia without chlorosis

X Heterogeneous, with randomly distributed uredinia of different sizes, on the same leaf.

Y Heterogeneous, with large-sized uredinia at leaf tip.

Z Heterogeneous, with large-sized uredinia at leaf base.

A better designation of reaction types could be achieved by adding more details about the pustule size and distribution, as well as chlorosis and necrosis to the previous nine phenotypes **(McIntosh et al., 1995)**:

– Uredinia size is minimum for infection type.

= Uredinia size is normal for infection type.

+ Uredinia size is larger than normal for infection type.

++ Uredinia size at maximum for infection type.

C Chlorosis is more than normal for infection type.

N Necrosis is more than normal for infection type

, Discrete infection types on one leaf.

Once ruptured, pustules are another port of entry for O_3 molecules. The most affected areas by this new O_3 route are the sporulation mat and the surrounding fungal and host tissue at the infection locus. O_3-induced chlorosis or necrosis in those areas might change the infection type. Differential O_3 responses of host tissue (e.g. leaf tip vs. leaf base) might result in, or probably explain some of the mixed phenotypes resulting from one race, and their different patterns on the leaf blade (e.g. X, Y and Z).

1.2.3.8. Average Coefficient of Infection (ACI)

Coefficient of infection is calculated as the product of multiplying the disease severity field scores recorded according to the modified Cobb scale **(Peterson et al., 1948)** by a numerical coefficient of infection, assigned based up on the field infection type **(McIntosh et al., 1995)**. The coefficients of infection are assigned as following: Resistant (0.2); Moderately Resistant (0.4); Intermediate (0.6); Moderately Susceptible (0.8); Susceptible (1.0) **(Stubbs et al., 1986)**.

1.2.4. Traditional Disease Triangle of Stem Rust under O_3 Stress

The disease triangle **(Stevens, 1960)** is a key fundamental concept in plant pathology that shaped our understanding of plant diseases. This concept is being used to illustrate plant diseases at all levels, such as, public talks, introductory and advanced phytopathology courses, and complex scientific discussions. Several modifications were proposed to better illustrate the phenomenon of plant disease in quantitative, temporal, and social contexts **(Agrios, 2005; Francl, 2001; Scholthof, 2007).** In this part, a new variant of the original disease triangle will be discussed. It is a model that better illustrates the components determining the net effects of each of the three major disease factors, while maintaining the simplicity of the original disease triangle.

In the traditional disease triangle illustrated in Figure (8), the disease occupies the area of a triangle, with the host, the pathogen, and the environment all located at the triangle vertices **(Stevens, 1960)**. The biotic disease phenomenon will not take place if any of the three requirements is absent. The disease pressure is a relative indicator of favorability of the non-host parameters to the disease. For example, high disease pressure is the combination between favorable environmental conditions and high inoculum amount and viability.

A commonly used variant of the disease triangle is obtained by placing the three components on the three sides of the triangle instead of the vertices **(Agrios, 2005; Francl, 2001; Scholthof, 2007)**. The length of each side (in relation to the others) indicates the relative net impact of the factor on the disease (i.e., the area inside the triangle). For example, in Figure (9), the environment side of the triangle is longer, which means it has more influence on the disease at this time point than both the host and the pathogen. Despite showing the overall effect of the factor (environment), this illustration does not provide enough information about the components deriving this effect. In addition, the traditional disease triangle does not tell us much about the mechanism of the effects. These effects could be either due to environmental effects on the host or on the pathogen, or on both. Furthermore, it does not show how this mechanism is changing overtime.

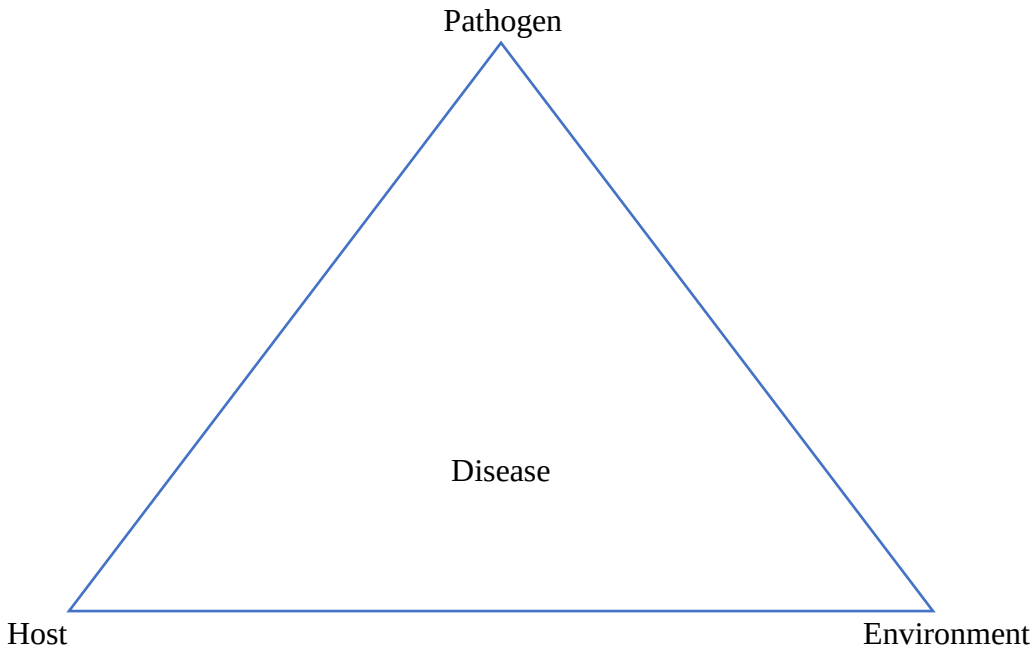


Figure (8): Traditional disease triangle with the host, pathogen and the environment on the triangle vertices.

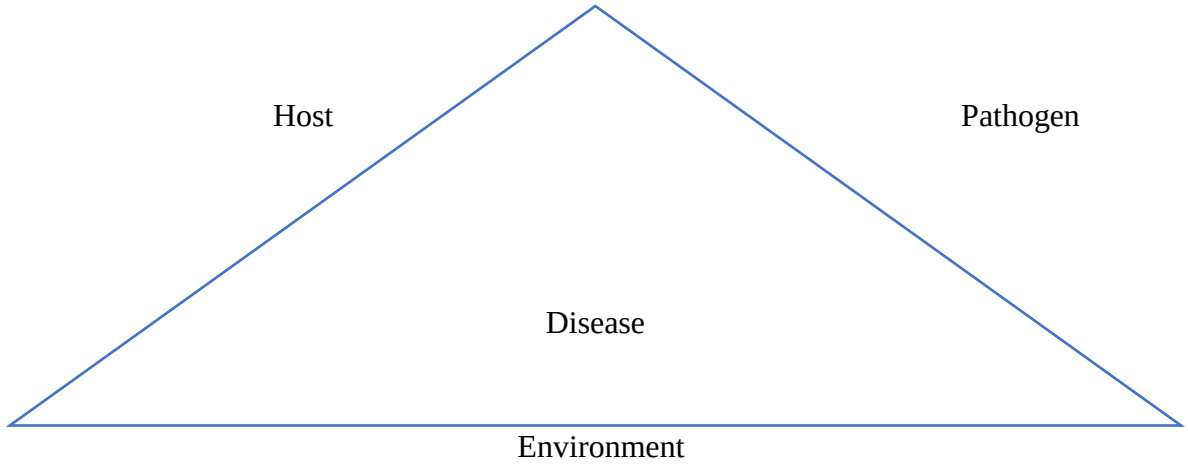


Figure (9): Disease triangle with the host, pathogen, and the environment on the three sides of the triangle, showing more weight to the environmental effects (i.e. longer side). The host and the pathogen are equally represented (similar side lengths).

All the proposed variants of the disease triangle carried over this deviation from the original model. Therefore, they lack the complexity needed to show the components of each of the three factors. A three-dimensional vertices model would solve this problem with a simple, easy to comprehend modification. It would also offer a more robust illustration, without sacrificing simplicity.

1.2.5. Three-dimensional vertices model for wheat rust under elevated O_3

This model returns to the first illustration in the traditional disease triangle. Therefore, it keeps the major components of the triangle at the vertices. However, it uses three dimensions at each vertex to represent the main three components (sub-factors) determining the net effect of the assigned major disease triangle constituent as shown in Figure (10). By reserving the Z-axis at each of the three vertices to the time factor, the model is also suitable for demonstrating the temporal aspects of detailed interactions in complex pathosystems. In this respect, this illustration is very similar to the disease pyramid **(Agrios, 2005)**, and the disease prism **(Francl, 2001)**. However, this presentation adds more details by considering the sub-factors of each of the three major factors. The following is a detailed discussion of the three basic components of this model.

1.2.5.1. The sub-components of the host's effect

The effect of the host on the disease is determined by the following sub-factors factors: The host resistance/susceptibility to the pathogen; the host sensitivity/tolerance to the environmental factor(s); the time point and the corresponding host related variables (e.g. growth stage). We can represent each of these three sub-factors with one dimension at the host's vertex of the disease triangle. Each dimension could present the host sub-factor itself, in correspondence to another host related variable. This will give us the ability to illustrate two variable on one axis.

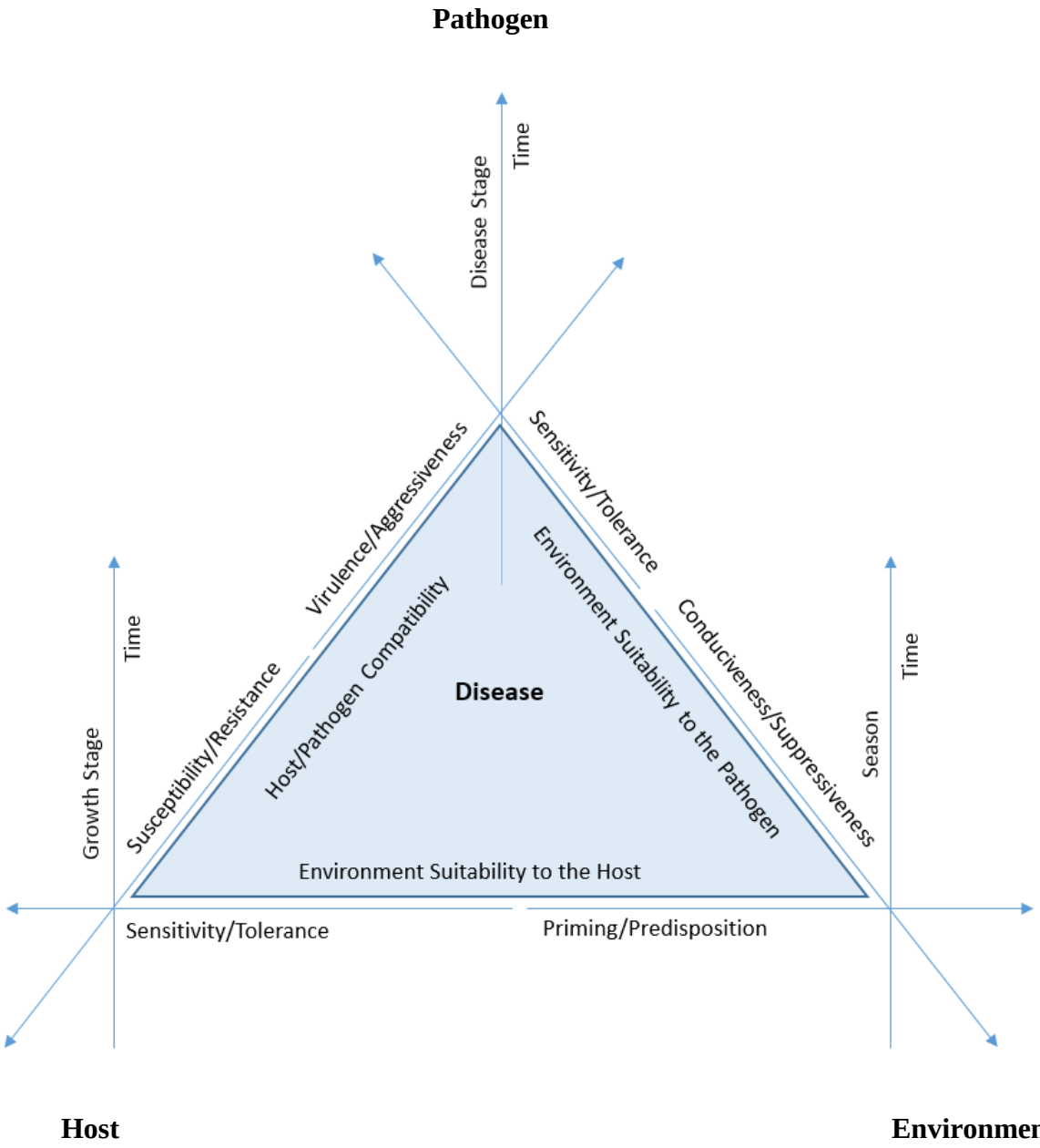


Figure (10): Three-Dimensional Vertices Model, a robust disease triangle variant, illustrating sub-components of each of the three major disease factors (host, pathogen, and environment).

1.2.5.1.1. The host resistance/susceptibility to the pathogen (rust):

This is the X-axis of host. The host's response to the pathogen could range from immune (might theoretically include non-host responses) to fully susceptible. Along with the virulence/aggressiveness of the pathogen, this sub-factor determines the host/pathogen infection type. This is usually a genetically controlled interaction, governed by the resistance gene(s) in the host, and the presence or absence of the corresponding avirulence gene(s) in the pathogen, which determines the disease compatibility. A non-compatible reaction means the host being fully resistant (immune), whereas, a compatible reaction means that the host is susceptible, with partial resistance being a part of the susceptibility continuum.

Some rust resistance genes are known for their growth stage and environmental sensitivity. The change in plant resistance over time could be easily illustrated by changing the intersection between the host's resistance/susceptibility axis and the time axis. Adult-plant resistance is known to be effective at adult-plant stage, but not at seedling stage. The pathogen itself is not changing; however, the host/pathogen compatibility outcome is changing because of the host.

Effects of O_3 on major gene resistance (MGR) versus adult-plant resistance (APR) and susceptibility is yet to be investigated. Resistance genes encode for different resistance proteins with different sites of actions; some of them are intracellular, on plasma membrane, and others in the apoplast. These different sites of actions might imply different exposure to O_3 stress, hence, different vulnerability to O_3 effects.

1.2.5.1.2. The host sensitivity/tolerance to the environmental factor(s):

This axis represents the tolerance of the host to the environmental stresses with predisposing effects (increasing host susceptibility), or host's sensitivity to the environmental factors with priming effects (increasing host resistance). This also reflects the indirect effects of the environment on the pathogen, by negatively or positively affecting the host, causing

predisposing or priming effects, respectively, which in turn result in increasing or reducing the host vulnerability.

Wheat adult-plants and older leaves are known for being more sensitive to O_3 than seedlings and younger leaves, including potential age differences in response along the continuum from the base to the tip of each leaf blade. Wheat yield is more sensitive to O_3 when plants are exposed after flowering. Leaf area of wheat plants was more sensitive to O_3 at anthesis that at stem elongation **(Feng et al., 2008)**. To better illustrate and infer O_3 effects on rust diseases of wheat, the tissue age and relative sensitivity to O_3 must be clarified and considered. For example, sub-symptomatic O_3 treatments were found to increase disease severity of necrotrophic pathogens **(Tiedemann and Pfähler, 1994)**. However, the effects of such concentrations on rust diseases are yet to be investigated. Current knowledge is based upon the effects at high O_3 levels where foliar injury was severe. Several studies **(Dohmen, 1987; Heagle and Key, 1972; Pfleeger et al., 1999; Tiedemann and Firsching, 2000)** showed that relatively high O_3 concentrations significantly reduced leaf rust, due to leaf necrosis and the reduced infection court available for the pathogen infection, invasion and reproduction.

1.2.5.1.3. The time and its corresponding host-related variables

The time axis for the host shows days or weeks from planting to harvesting. It could be used to illustrate the time at which the disease is occurring, the environmental stress is encountered and/or the data is being collected. On this axis, the corresponding host's growth stage could be presented. The growth stage determines the type of tissue (functionally, structurally and spatially) the pathogen is attacking, or the environment is affecting (priming or predisposing). The canopy structure changes overtime and determines the microclimate conditions. In addition, the growth stage determines the available time for the pathogen and the environment to accumulate effects on the host.

When we put all three host sub-factors together, they generate a three-dimensional host effect model that is simple yet robust. The intersection point of the three dimensions represents the net host's effect on the disease at this time point, with its components.

1.2.5.2. The sub-components of the pathogen's effect

The pathogen's role in the disease could be represented by the following sub-factors: The pathogen's virulence/aggressiveness to the host; the pathogen's sensitivity/tolerance to the environmental factor(s); the time point and the corresponding pathogen related variables. We can represent each of these three sub-factors with one dimension at the pathogen's vertex of the disease triangle. Each dimension presents the pathogen's sub-factor itself, in correspondence to another pathogen related variable. This will give us the ability to illustrate two variabless on one axis.

1.2.5.2.1. The pathogen's race(s) virulence/aggressiveness to the host

This is the X-axis of the pathogen. The pathogens ability to infect and cause disease on the host could range from avirulent (might theoretically include non-pathogen organisms), virulent-non-aggressive, to virulent-aggressive. This pathogen axis is the other determinant of the host/pathogen infection type in rust diseases. This is usually a genetically controlled interaction, governed by the resistance gene(s) in the host, and the presence or absence of the corresponding avirulence gene(s) in the pathogen, which determines the disease compatibility. O_3 may or may not have effects on different host/pathogen compatibility combinations. However, current knowledge is based up studies focused only on the compatible (virulent pathogen races).

The pathogens produce different effectors to suppress host resistance and/or modulate host functions in their favor. Depending of the site of action of those effectors, they may be more vulnerable to O_3 effects. Minute changes in the pathogen effectors change their functions and their detectability (perception by host receptors), which means that O_3 effects on the effectors could result in more or less disease depending on the magnitude and type of change.

1.2.5.2.2. The pathogen's sensitivity/tolerance to the environmental factor(s)

The pathogen and the host might have different environmental limiting factors, for each of their stages. For example, the pathogen needs free water for spores to germinate, certain temperature, humidity and light conditions for penetrations, infection and sporulation. Another example is ROS production which is a major part of plant defense mechanisms. The pathogens are expected to be more adapted to ROS than the host is. There is limited data on rust pathogens sensitivity to O_3 stress, especially within a host tissue.

1.2.5.2.3. The time and its corresponding host-related variables:

The time at which the disease is occurring, relative to the host's growing season and the environmental seasonal context. On this axis, the corresponding pathogen's disease stage could also be presented. Certain stages of rust diseases are sensitive to certain factors, such as free water or dew formation at inoculation. In addition, the order of biological and environmental stresses might affect the outcome of the interaction between these two stresses on the host. For example, ruptured pustules on plant leaves prior to O_3 stress might increase O_3 dose and symptoms. On the other hand, O_3 treatment causing foliar injury prior to the inoculation might suppress the rust disease.

1.2.5.3. The sub-components of the environment's effect

The environment in which the host is grown also affects the disease through it three sub-factors: The environment's conduciveness/suppression of the pathogen; the environment's priming/predisposition to the host; the time point and the corresponding environment-related variables.

1.2.5.3.1. The environment's conduciveness/suppressiveness to the pathogen

This is the X-axis of the pathogen's effect. Each of the disease stages might have a different limiting environmental factor. For example, in all rust pathogens, free water on the leaf surface is required for spore germination. However, light is required only for *Pgt* and *Pst* but not for *Pt*. High light intensity for the first 3-4 days after inoculation is conducive to Sr, whereas low light intensity during the same period is conducive to Yr.

1.2.5.3.2. The environment's priming/predisposition to the host

Some environmental factors could make the plant or susceptible to disease while others predispose them to become more resistant. E-O_3 could have both types of effects. O_3 treatment can cause stomatal closure, which if occurs in the early morning (e.g. overnight high O_3 peaks) will interfere with *Pgt* and *Pst* penetration. O_3 also is known to induce ethylene production, which in turn suppresses stomatal closure and which would facilitate penetration.

1.2.5.3.3. The time and its corresponding environment-related variables

Seasonal, diurnal patterns and cycles and their timing to both plant growth stages and disease stages are key components of the environmental effects on rust diseases. For example, diurnal and seasonal O_3 peaks coincide with plant's activity peaks during the daytime and after anthesis later in the season. High O_3 concentrations at anthesis had significant impacts on the LAI comparing later in the season, but had no effect on LAI when applied at stem elongation stage **(Feng et al., 2008)**. The duration and the level of the environmental stress determine the direction and the magnitude of the stress effects, on both the pathogen and the host.

1.3. Research Objectives

1.3.1. Ozone Responses of the Rust Near-Universal Susceptible Panel of Wheat Genotypes, and their Applications in Breeding and Phytobiome Research

a. Identify the O_3 responses of eight rust near-universal susceptible genotypes, at different O_3 concentrations, in two exposure systems (a) continuous stirred tank reactors (CSTRs), and (b) out-door plant environment chambers (OPECs)
b. Identify O_3-sensitive rust susceptibles to be used for characterizing O_3 tolerance simultaneously with rust resistance.
c. Identify O_3-sensitive/tolerant pairs for studying O_3 effects on different rust diseases and races, and the wheat phytobiome.
d. Identify O_3-sensitive/tolerant pairs that could be used for biomonitoring O_3 levels, and testing of rust inoculum simultaneously.

1.3.2. Identification of the Genetic Control of Ozone Tolerance in Wheat (Triticum aestivum L. cv. Chinese Spring) at the Chromosome Level.

a. Identify the individual contribution of each of the 21 chromosomes of Chinese Spring, and their importance for the observed O_3-tolerance of this cultivar.

1.3.3. Relative O_3-Responses of Some Winter Wheat Genotypes, and Their Suitability for Breeding Resilient Varieties.

a. Analyze O_3 monitoring data collected during wheat active growing season (March-May), over the last 10 years (2006-2015), at the field plots of the USDA-ARS Plant Research Unit in Raleigh, NC.
b. Investigate the relative O_3 responses at the seedling stage of 24 winter wheat genotypes and compare with two varieties of spring wheat, as well as two winter varieties of oat and barley, and one winter variety of triticale.

c. Investigate any general trends of O_3 sensitivity of tested varieties of winter wheat, and compare with trends in sensitivity of hard and soft winter wheat.
d. Compare O_3 responses of hard and soft wheat varieties, and identify suitable sources for O_3 tolerance in both categories that could be used as parents in simultaneous breeding for hardness and O_3 tolerance.
e. Identify varieties with suitable O_3-responses to be used in simultaneous breeding for O_3 tolerance and rust resistance, and identify the genetic control and markers associated with both traits.
f. Identify O_3-sensitive varieties with different rust reaction types to be used in investigating the effects of O_3 stress on different types of rust-resistance in wheat.
g. Investigate the O_3-yield responses of two winter wheat varieties with differential visible injury responses.

1.3.4. Dissecting the Effects of O_3 and CO_2 Concentration, Exposure Timing and Duration on the Disease Components of Stem and Leaf Rusts on Winter Wheat.

a. Investigate the effects of different O_3 concentrations on stem rust-winter wheat interactions.
b. Investigate the effects of near-ambient O_3 and elevated CO_2 exposure timing and duration on stem rust-winter wheat interactions at the seedling stage.
c. Investigate the effects of near-ambient O_3 and elevated CO_2 exposure on stem rust-winter wheat interactions at the adult-plant stage.
d. Investigate the effects of near-ambient O_3 and elevated CO_2 exposure on interactions between stem rust and winter wheat varieties with different rust responses (susceptible, moderately-susceptible, moderately-resistant, and resistant), at the adult-plant stage.

2. Ozone Responses of the Rust Near-Universal Susceptible Panel of Wheat Genotypes, and their Applications in Breeding and Phytobiome Research.

2.1. Abstract

Sustainable increase in wheat production is inevitable for attaining global food security. This sustainability requires resilient wheat cultivars that can cope simultaneously with major biotic and abiotic stresses, while maintaining healthy phytobiomes, and high yield. The current global efforts, aiming at breeding wheat resistant cultivars to the rapidly evolving rust pathogens, could also be utilized for breeding tolerant cultivars to the elevated ambient ozone levels. These two are concomitant threats and limiting factors to wheat production worldwide that are not addressed simultaneously, despite of the necessity and the potential, under current limited resources.

This research identified the O_3 responses of a key set of wheat genotypes used in breeding wheat for rust resistance. This is an essential step towards breeding rust-resistant, O_3-tolerant cultivars having higher grain yield. It is also a critical step for phytobiome research elucidating the effects of O_3 on different rust races, and their interactions with other wheat phytobiomes, including natural detrimental, beneficial and biological control agents. The tested set included seven bread wheat genotypes (Chinese Spring, Line E, Little Club, LMPG-6, McNair 701, Morocco and Thatcher), and one durum wheat lines (Rusty). Seedlings were treated with different O_3 concentrations, in two O_3 exposure systems. Visible injury and biomass data were collected.

Visible injury results showed consistent differential O_3 responses between tested varieties. Thatcher and LMPG-6 were the most O_3 sensitive, whereas Chinese Spring and to a lesser extent, Little Club, were the most tolerant among the eight genotypes tested. Biomass data showed similar results for bread wheat genotypes. However, the durum wheat line 'Rusty' that was ranked moderately susceptible based upon the visible injury, was the most sensitive in terms of biomass production. These results suggest that LMPG-6 could be used as an O_3-sensitive genotype, and Chinese Spring as an O_3-tolerant genotype in wheat stem rust studies.

Similarly, Thatcher is a suitable O_3-sensitive that could be used with either Little Club or Morocco for studying O_3 effects on different races of wheat leaf rust.

2.2. Introduction

Wheat (*Triticum aestivum* L.) is a key crop for global food security, and the staple food for 30% of the world population **(FAO, 2016)**. Sustainable increase in wheat production is inevitable for attaining global food security **(Shiferaw et al., 2013; Tester and Langridge, 2010)**. This sustainability requires better understanding of wheat phytobiome **(American Phytopathological Society, 2016)**, including the plant community, microbiome and macrobiome communities **(Dicke, 2016)**, soil and climate. This improved understanding will increase our ability to address major biotic and abiotic plant stresses simultaneously, with respect to their interactions with other phytobiome components. In addition, it will better elucidate challenges and opportunities available for plants in their environment.

To meet the challenge of producing 70% more food, including nearly 4 billion tonnes of cereals by 2050 **(Shiferaw et al., 2013)**, the current available limited resources must be used more effectively **(Ledford, 2015)**; It is necessary to utilize the available resources in some extensively studied areas, to achieve improvement in other major but less supported areas. The research presented here focuses on the crossroads between breeding wheat for the rapidly evolving rust pathogens, and elevated tropospheric ozone levels (E-O_3), also known as surface O_3. These are two major concomitant threats to wheat production worldwide **(Joshi et al., 2011; Singh and Agrawal, 2010)**, yet, they are not addressed simultaneously in breeding programs. Moreover, breeding wheat for rust is a global success model, whereas, breeding wheat for O_3 tolerance is not currently a priority.

The Borlaug Global Rust Initiative (BGRI) is a unique model for success **(McIntosh and Pretorius, 2011)**, through which global efforts to combat emerging rust races resulted in the identification of new major rust resistance genes, and adult-plant resistance in wheat germplasm worldwide. Chromosome and loci locations and associated molecular markers were identified for many of these genes, mainly by crossing the breeding material bearing the

novel source of resistance to a susceptible genotype to make mapping populations. By phenotyping and genotyping these populations, associated markers and gene or QTL locations were identified **(Yu et al., 2014)**. Some of these genes were cloned **(Steuernagel et al., 2016)**. However, few studies have examined ozone tolerance in wheat **(Ainsworth et al., 2008; Mills et al., 2011)**. Perhaps a reason for this research gap is the misperception between stratospheric and tropospheric O_3.

Unlike the protective O_3 naturally formed in the stratosphere, surface O_3 in the ground level layer of the troposphere is a secondary air pollutant and a greenhouse gas **(Booker et al., 2009)**, formed by photochemical reactions between nitrogen oxides (NO_x) and non-methane volatile organic compounds (NMVOC) **(Jacob, 1999)**. O_3 is the most phytotoxic air pollutant **(Sandermann et al., 1998)**, due to its high oxidizing power to plant physical compartments, such as cuticle and cell wall, as well as cell membranes **(Iriti and Faoro, 2007)**. Moreover, it has a diffusion coefficient through plant leaves similar to CO_2 **(Fares et al., 2012)**, however, it has 9.06% higher solubility in water than CO_2 **(Haynes, 2015)**. Once dissolved in the apoplastic fluid, O_3 generates primary reactive oxygen species (ROS). If not detoxified, these O_3-originated ROS react directly in the apoplastic fluid with the cell wall and/or plasma membrane, inducing a self-propagating oxidative burst of biogenic ROS that spreads to the surrounding tissue, similar to the hypersensitive response resulting from incompatible host/pathogen interactions **(Pellinen et al., 1999; Vahisalu et al., 2010)**.

Under low to moderate external O_3 concentrations, the influx of O_3 and ROS generated during the early phase will be detoxified by the apoplastic antioxidants capacity. However, under high external O_3 concentrations currently encountered, high stomatal conductance, low mesophyll resistance to gas diffusion (traits wheat breeders are currently selecting for, to increase CO_2 flux and photosynthesis), the influx of O_3 during the early phase exceeds the apoplastic antioxidant capacity to detoxify ROS formed from O_3 degradation **(Pellinen et al., 1999; Vahisalu et al., 2010)**. This over flow of ROS in the apoplast could also occur due to O_3 deposition to wet leaves, as the effective O_3 flux into the leaf will be increased, causing acute symptoms, at O_3 levels that are typically expected to only cause chronic symptoms

(Altimir et al., 2006). On the other hand, some plants might over-react to O_3 by extreme stomatal closure, which significantly decreases stomatal conductance. O_3-induced visible injury and the significant stomatal closure will negatively affects plants performance and interactions with their phytobiome.

Wheat is one of the most sensitive crops to O_3, at all growth stages **(Singh and Agrawal, 2010)**. Ozone effects on wheat plants include biochemical changes such as antioxidant capacity **(Biswas et al., 2008a)**, gas exchange and stomatal conductance **(Feng et al., 2012; Hassan, 2004; Pleijel et al., 2006)**, and reduced photosynthesis **(Biswas et al., 2013; Meyer et al., 2000; Wattal and Siddiqui, 2015)**. O_3 could also cause chlorotic and/or necrotic visible symptoms **(Mills et al., 2011)** and hasten leaf senescence **(Burkart et al., 2013; Gelang et al., 2000; Ojanpera et al., 1998)**. The effects of ozone reduce plant biomass, grain yield, and protein content **(Amundson et al., 1987; Feng et al., 2012; Hassan, 2004; Heagle et al., 2000; Mills et al., 2011; Ojanpera et al., 1998; Singh and Agrawal, 2010; Van Dingenen et al., 2009)**. The magnitude of these reductions is dependent on the dose and genotype sensitivity. Current O_3 levels during wheat growing season are high enough to cause average global yield reduction of 7-12 %, and this yield reduction is expected to reach 9-18 % by 2030 **(Avnery et al., 2011a; Avnery et al., 2011b; Feng and Kobayashi, 2009)**. However, the effect of O_3 on wheat yields is not uniform across the world, as the highest reductions are usually experienced in developing and heavily populated countries, such as China and India **(Feng et al., 2012; Singh and Agrawal, 2010; Van Dingenen et al., 2009)**.

Unlike the gradual global increase in CO_2, E-O_3 is a more diverse problem both spatially and temporally. Spatial variations in E-O_3 result from the relative location to sources of the precursors, and the ratio between them (NO_x/VOCs) **(Khoder, 2009)**. Whereas, the temporal variations result mainly from the meteorological effects, such as stagnant weather conditions (inversion layer formation), and changes in precursor emission (e.g. weekend phenomenon) **(Khoder, 2009)**. These variations offset the consistent O_3 selection pressure needed for conventional breeding procedures to make breeding progress **(Ainsworth et al., 2008)**. In China Europe, modern varieties of wheat were found to be more sensitive to O_3 than

older varieties **(Biswas et al., 2008b; Biswas et al., 2013; Pleijel et al., 2006)**. This may be a result of selection for high stomatal conductance, and less mesophyll resistance to gas diffusion, needed for higher CO_2 flux and increased photosynthesis **(Biswas et al., 2008b; Biswas et al., 2013)**. However, this is a recipe for O_3 sensitivity, especially when conducted under inconsistent O_3 selection pressure **(Ainsworth et al., 2008)**.

Despite the observed differences in O_3 responses among wheat varieties, there is a lack of information about its heritability, and the potential of O_3 tolerance to contribute to genetic gain **(Barnes et al., 1999; Burkart et al., 2013)**. To obtain such information, the relative importance of genotype and genotype by environment interaction (GxE) to O_3 tolerance is needed. The influence of the environment should include those factors that modify stomatal conductance, such as CO_2 **(Lu et al., 2016; Manning and v. Tiedemann, 1995)**, drought **(Afzal et al., 2015; Biswas and Jiang, 2011; Herbinger et al., 2002; Quarrie et al., 2005b; Xu et al., 2007)**, and water vapor pressure deficit **(Altimir et al., 2006; Cape et al., 2009)**. Similarly, biological factors affecting cuticle integrity, such as diseases and insects may also modify the phenotype observed. To effectively select for ozone tolerance, molecular markers and genomic location for the QTLs are needed **(Quarrie et al., 2005b; Quarrie et al., 2006; Quarrie et al., 2007)**.

Wheat genomes A and B are considered the source of O_3 tolerance, whereas the D genome may be responsible for O_3 sensitivity **(Biswas et al., 2008a)**. O_3 injury and biomass data showed that chromosome 7A is critical for O_3 tolerance in the cultivar Chinese Spring, whereas chromosome 5D was correlated to biomass O_3 sensitivity, but not visible symptoms **(Mashaheet et al., 2016b)**. However, no QTLs or specific markers for O_3 tolerance have been identified yet.

The differential response of wheat cultivars to ozone also influence other important factors, such as nitrogen use efficiency and nutrient availability in soil **(Chen et al., 2015)**, soil pH **(Li et al., 2013)**, and soil organic matter **(Lu et al., 2016)**. The structure and functionality of soil microbial communities may be influenced by the plants' ozone response

(Li et al., 2012; Li et al., 2013). The ozone response might also affect the foliar microbiome, including major wheat pathogens, as well as beneficial and biological control organisms. Ozone was found to affect wheat diseases such as, stem rust **(Heagle and Key, 1973a)**, leaf rust **(Dohmen, 1987; Heagle, 1975; Pfleeger et al., 1999; Tiedemann, 1992a; Tiedemann et al., 1991; Tiedemann and Firsching, 2000)**, and powdery mildew **(Tiedemann, 1992a)**. O_3 mainly decreased these biotrophic pathogens by restricting the host tissue available for them to survive. However, in each of these studies, the relative host sensitivity to O_3 was not considered, and the effects of the host responses to O_3 on different rust races singly or in mixtures was not investigated. In addition, all these studies used relatively high O_3 concentration that are irrelevant to wheat growing season and areas in major parts of the world **(Mashaheet et al., 2015)**.

Near-universal, rust susceptible wheat genotypes having differential O_3 responses are key to understanding ozone-rust interactions. These genotypes are susceptible to most rust races, and therefore allow most races to infect unimpeded **(Ali, 1994)**.,The leaf rust resistance gens *LrMo* in the cultivar Morocco and *LrLC* in the cultivar Little Club are common to most races of leaf rust **(Ali, 1994)**. Because 'Morocco' and 'Little Club' are used to maintain and increase rust inoculum, worldwide, they are typically used as the susceptible parents in inheritance of resistance studies. However, there is no information available on the response of near-universal susceptible genotypes to O_3. This study was designed with the following objectives:

a. Screening a panel of 8 near-universal, rust-susceptible genotypes for O_3 response, at different O_3 concentrations, in two exposure systems (a) Continuous Stirred Tank Reactors (CSTRs), and (b) Outdoor Plant Environment Chambers (OPECs).

b. Identify O_3-sensitive susceptibles to be used for characterizing O_3 tolerance simultaneously with rust resistance.

c. Identify O_3-sensitive/tolerant genotype pairs for studying O_3 effects on different rust diseases and races, and wheat phytobiome.

d. Identifying an O_3-sensitive/tolerant genotype pairs that could be used for biomonitoring O_3 levels, and detect rust inoculum simultaneously.

2.3. Materials and Methods

2.3.1. Plant materials

For the CSTR study, seven genotypes were tested. The genotypes and their USDA-National Plant Germplasm System accession number were 'Chinese Spring' **(Zeven, 1971)**, 'Line E' **(Luig, 1983)**, 'Little Club' (CItr 4066), 'LMPG-6' **(Knott, 1993)**, 'McNair 701' **(Middleton et al., 1973)**, 'Morocco' (PI 431591), and 'Rusty' (PI 639869). In the OPEC exposure system, the cultivar 'Thatcher' (CItr 10003) was added to the same seven genotypes. For both exposure systems, seedlings were grown in charcoal filtered (CF) air in the greenhouse for 30 days after planting. Plants at Zadok growth stage of 21-23 were selected and the fourth leaf on the top of the main stem were tagged, and then moved to CSTRs or OPECs.

2.3.2. Ozone treatment

After being moved to the exposure systems, plants were acclimated for two days in CF before treated with O_3. In the CSTRs, five O_3 treatments (CF, 50, 70, 90 and 110 ppb) were used for 5 days (7 hr/day, at 25°C and 60% RH). The experimental design involved 15 chambers in three blocks. Each treatment was randomly assigned to one chamber per block. One plant per genotype per chamber was used. In the OPECs, plants were exposed for 14 days to one of four O_3 treatments using a predefined diurnal O_3 profile (CF, 50, 70, 90 ppb, 12hrs average of 24hr diurnal profile, at 25/16°C day/night and 50% RH). The experimental designed included eight OPECs, divided into two blocks were, four chambers each. Treatments were randomly assigned to OPECs within block. Two plants per genotype were randomized to each OPEC.

2.3.3. O_3 Injury Assessment

Two days after the end of exposure, the plants in the CSTR experiments were visually assessed under constant light conditions for percent O_3 injury on the fourth leaf of the main stem. In the OPECs, all leaves on the main stem of plants were evaluated. However, only 3rd, 4th and 5th leaves were considered for statistical analysis, as the first and second leaves showed some evidence of senescence. A 0-100% scale was devised based on the percent of the leaf exhibiting chlorosis and necrosis, as shown in Figure (11). This scoring guide was established by scanning detached leaves on a flat bed scanner and analyzed using Assess 2.2, the APS image analysis software (APS, Saint Paul, Minnesota, USA).

2.3.4. Dry Matter Accumulation Rate

In the OPECs, shoot dry weights were obtained before treatment for three plants per genotypes and after treatments for all the treated plants, by drying samples in paper bags to constant weight under forced air (55°C). Biomass data was not attempted in the CSTR experiments because of the short period of exposure (5 days). Different dry mater parameters were calculated:

- Dry Matter Accumulation Rate (DMAR)

$$DMAR = \frac{(Initial\ Dry\ Weight - Final\ Dry\ weight)}{Exposure\ Duration}$$

- Adjusted Dry Matter Accumulation Rate (ADMAR)

$$ADMAR = \frac{(DMAR)}{Initial\ Dry\ Weight} \times 100$$

This parameter was calculated to account for the differences in the initial weight among the different entries tested.

- CF-Referenced Relative Dry Matter Accumulation Rate (RDMAR)

$$RDMAR = \frac{(ADMAR\ for\ individual\ plant)}{Average\ ADMAR\ for\ their\ entry\ under\ CF} \times 100$$

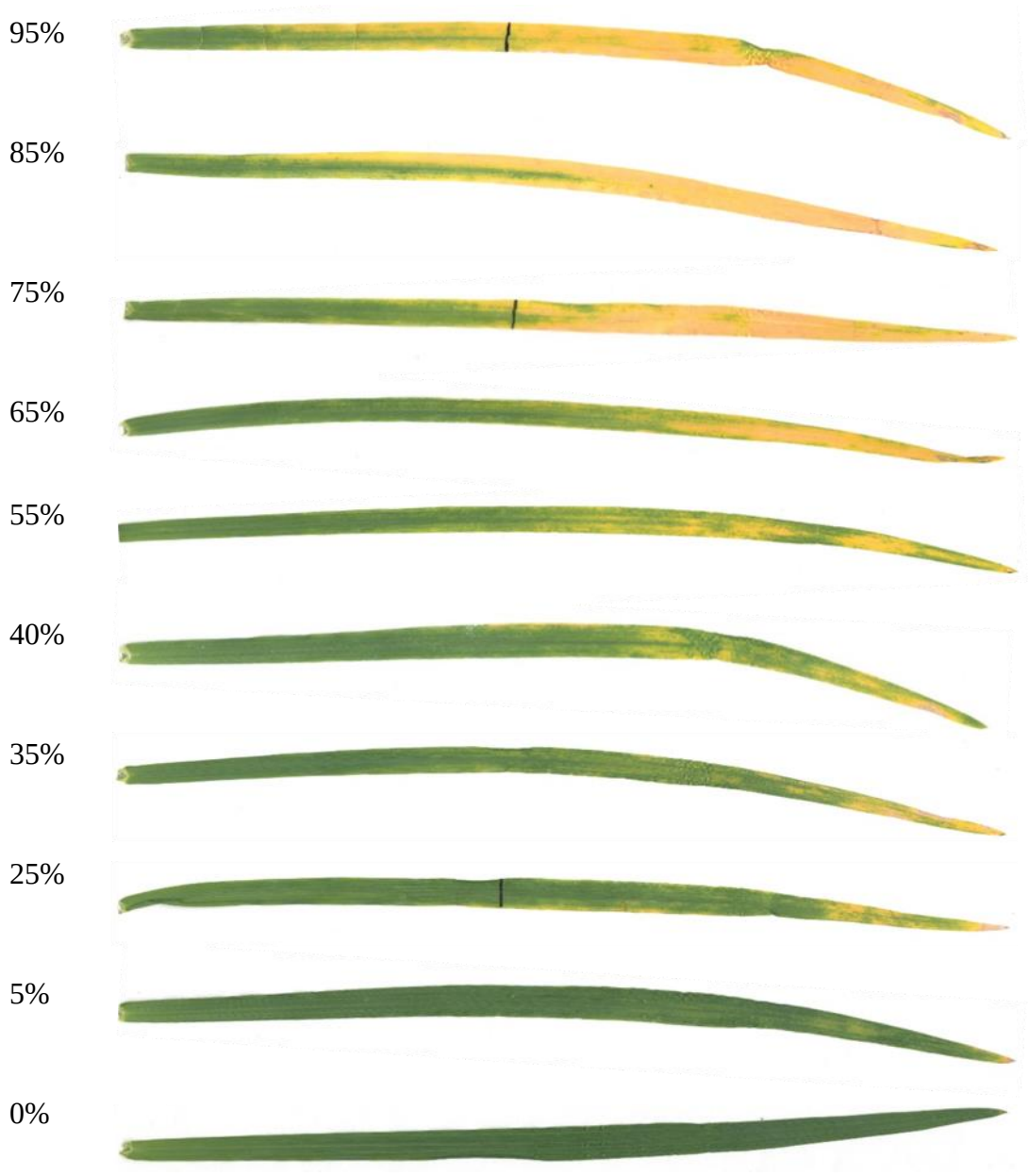


Figure (11): Ozone Injury Scoring Guide. O_3 visible symptoms exposed to O_3 in Continuous Stirred Tank Reactors (CSTRs). Percent injury were assessed using scanned detached leaf images, analyzed using APS Assess 2.2 (APS, Saint Paul, Minnesota, USA).

This parameter accounts for two types of differences among genotypes: (1) the differences in initial weight, and (2) the differences in dry matter accumulation rate under CF. This parameter is ideal for estimating the effect of increased O_3 levels on the biomass accumulation rate, and compare it across entries.

2.3.5. Statistical Analysis

Using the Glmmix procedure in SAS 9.4 (SAS Inc., Cary, NC, USA), averages were separated according to Tukey's post-hoc test.

2.4. Results

2.4.1. Ozone Injury

2.4.1.1. Seedling Screening in CSTRs

At an ozone exposure of 50 ppb, there were no significant differences in visual injury between the genotypes, as shown in Figure (12). However, in comparing the genotypes, at an O_3 exposure of 70 ppb, LMPG-6 had 78.3% injury, which was significantly greater than McNair 701 at 31.7%; Rusty at 15.0%; Little Club at 13.3%; Morocco at 11.7%; Chinese Spring at 9.2%; and Line-E at 6.0%. Little Club and Chinese Spring had significantly less O_3 injury than LMPG-6 at 90 ppb. At the highest exposure of 110 ppb, Chinese Spring had the least amount of O_3 injury (20.8%), which was significantly less than Morocco (70.0%); McNair 701 (78.3%); Rusty (86.7%); Line E (88.3%); and LMPG-6 (98.3%).

In comparing the ozone concentrations within each genotype, Chinese Spring showed no significant difference in foliar injury from 50 ppb (0.6%) to 110 ppb (20.8%). LMPG-6 was the only genotype that had significantly greater injury at 70 ppb compared to 50 ppb. LMPG-6, McNair 701, Rusty, Line E, and Morocco all had significantly greater injury at 110 ppb compared to 50 ppb, as shown in Figure (12).

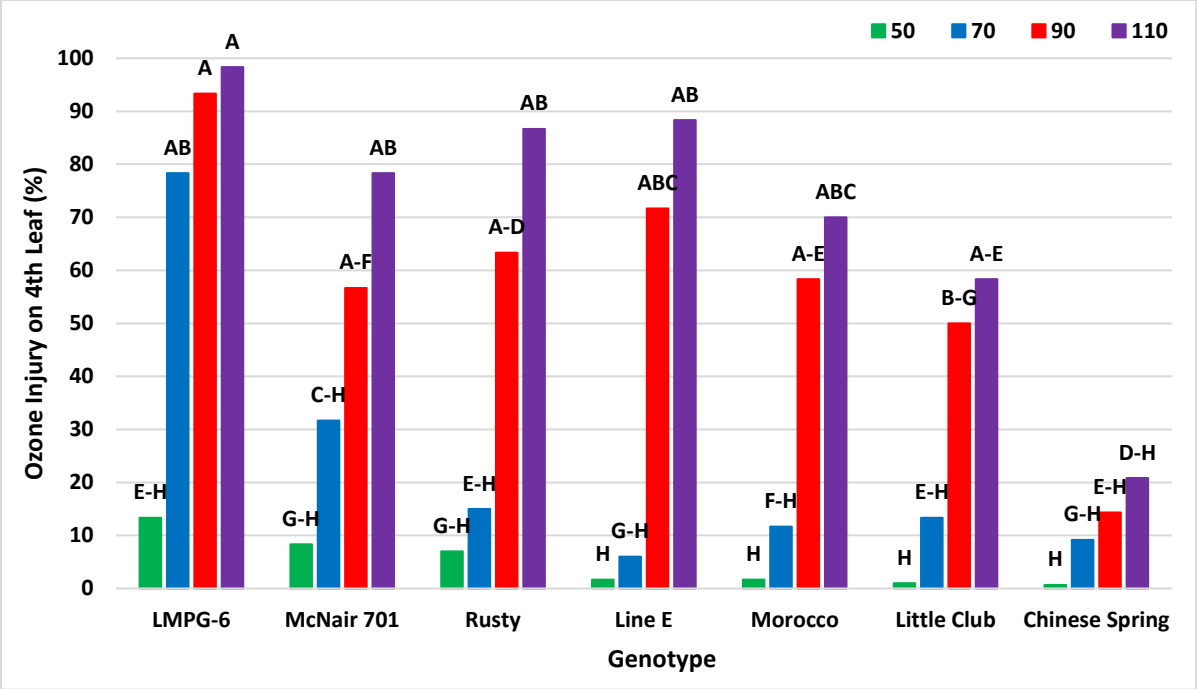


Figure (12): O_3 injury on the fourth leaf of seven rust near-universal susceptible genotypes, at four different O_3 levels, exposed for 5 days (7hrs/day, at 25°C and 60% RH) in CSTRs. Injury were visually estimated, under constant light conditions according 0-100% scale. Mean separation by Tukey's post-hoc test.

2.4.1.2. Seedling Screening in OPECs

In the OPECs, genotypes were exposed at 3 ozone concentrations, 50, 70, and 90 ppb, as shown in Figure (11). At an exposure of 50 ppb, Thatcher and LMPG-6 had significantly greater ozone injury than the other 5 genotypes. At 70 ppb, ozone injury to Morocco (35.1%), Little Club (34.1%) and Chinese Spring (14.4%) was significantly less than the injury on Thatcher (64.1%), LMPG-6 (54.7%), McNair 701 (49.8%), Rusty (47.8%), and Line E (47.3%). Within all the genotypes, greater percentages of ozone injury were found at 90 ppb compared to 50 ppb.

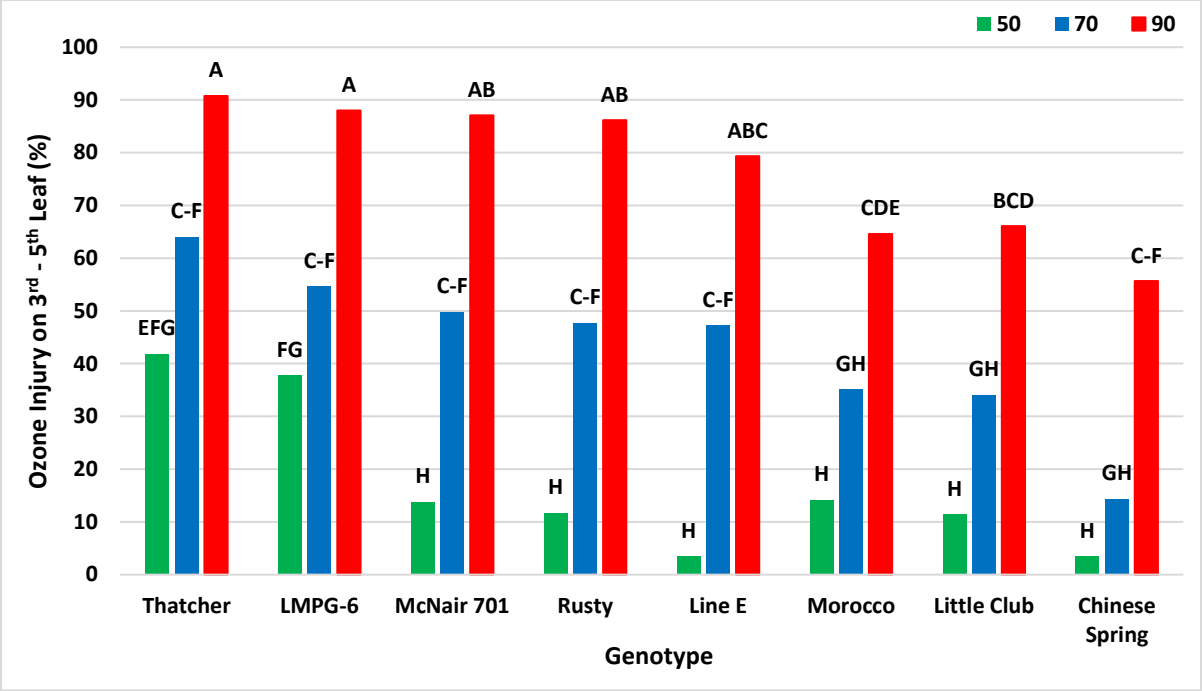


Figure (13): O_3 injury on the 3rd-5th leaf of eight rust near-universal susceptible genotypes, at three different O_3 levels, exposed for 14 days (12hrs/day, at 25°C and 50% RH) in OPECs. Injury was visually estimated, under constant light conditions according 0-100% scale. Mean separation by Tukey's post-hoc test.

As shown in Table (1), the genotypes can be ranked by relative ozone injury. Chinese Spring can be considered to be ozone tolerant, and LMPG-6 and Thatcher as ozone sensitive. The other five genotypes had moderate ozone injury, compared to the sensitive and tolerant genotypes. Based up on their ranking, and could be used with either little Club (tolerant) or Morocco (moderately sensitive) as a contrasting pair to study the effects of O_3 responses on different races of leaf rust. LMPG-6 (sensitive) or McNair 701 (moderately sensitive) could be used with Chinese Spring as contrasting pairs to study O_3 effects of different stem rust races. Surprisingly, the durum wheat variety, Rusty was ranked moderately sensitive. Durum wheat is known to be more tolerant to O_3 than bread wheat **(Biswas et al., 2008a)**.

Table (1): Relative ozone responses of eight rust near-universal susceptible wheat genotypes, conducted under different ozone levels, for different exposure periods, in two different systems: Continuous Stirred Tank Reactors (CSTRs) and Outdoor Plant Environment Chambers (OPECs). Varieties were ranked according to their overall responses in two screening studies.

Genotype	Screening in CSTRs*	Screening in OPECs#	Ranking	Rust Susceptibility
Thatcher	n/a	65.56 (A)	Sensitive	Leaf Rust
LMPG-6	70.83 (a)	60.14 (AB)	Sensitive	Stem Rust
McNair 701	43.75 (b)	50.25 (BC)	Moderately Sensitive	Stem Rust
Rusty	43.00 (b)	48.56 (BC)	Moderately Sensitive	Stem Rust
Line E	41.92 (b)	43.39 (C)	Moderately Sensitive	Stem Rust
Morocco	35.42 (b)	37.94 (C)	Moderately Sensitive	Leaf Rust
Little Club	30.67 (b)	37.19 (CD)	Moderately Sensitive	Leaf Rust
Chinese Spring	11.25 (c)	24.51 (D)	Tolerant	Stem Rust

*, Average visible injury on the 4th leaf of the main stem under four O_3 concentrations (50, 70, 90, 110 ppb, 7h d^{-1}, for 7 days), in CSTRs.

#, Average visible injury on the 3rd-5th leaf of the main stem under three O_3 concentrations (50, 70, 90 ppb, 12h average, predefined diurnal profile, for 14 days), in OPECs.

2.4.2. Dry Matter Accumulation Parameters

Although the genotypes were planted at the same day, and were selected for emergence time and growth stage uniformity, as well as canopy structure, they differed in their initial dry matter prior to the inoculation, as shown in Figure (14). These genetic differences in biomass might affect the dry matter accumulation rate. This hypothesis was tested by investigating the regression lines of the dry matter accumulation rate on the initial dry weight.

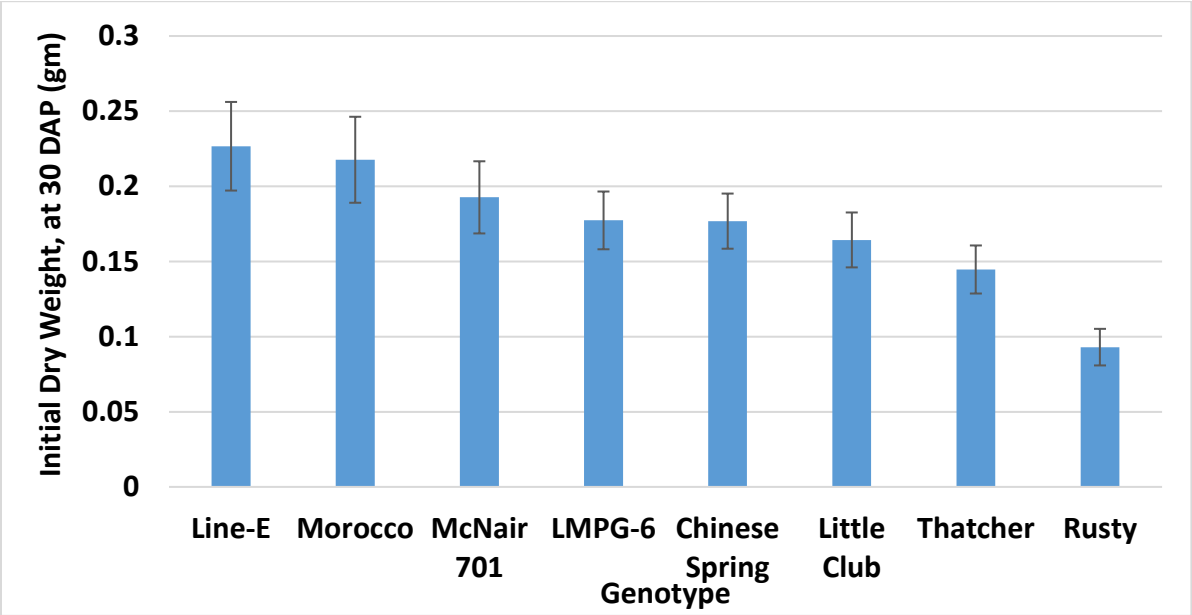


Figure (14): The initial dry weight (gm) of the eight genotypes at 30 days after planting (DAP), and prior to O_3 treatment in OPECs.

The initial dry weight showed negligible effect on the dry matter accumulation rate of the eight genotypes in charcoal filtered air, as shown in Figure (15). However, initial dry weight showed greater effects on the dry matter accumulation rate under the three O_3 exposure concentrations in the OPECs. This indicated that smaller plants had lower dry matter accumulation rates than larger plants under elevated O_3 concentrations, whereas plant size had no effect on dry matter accumulation rates in charcoal filtered air. This differential dry matter accumulation rate under different O_3 concentrations is mainly derived by durum wheat line 'Rusty', which could be observed when we separate the seven bread wheat genotypes as shown in Figure (16). These finding suggested the correction of dry mater accumulation rates to the initial dry weight, as applied in calculating the relative dry matter accumulation rate (RDMAR).

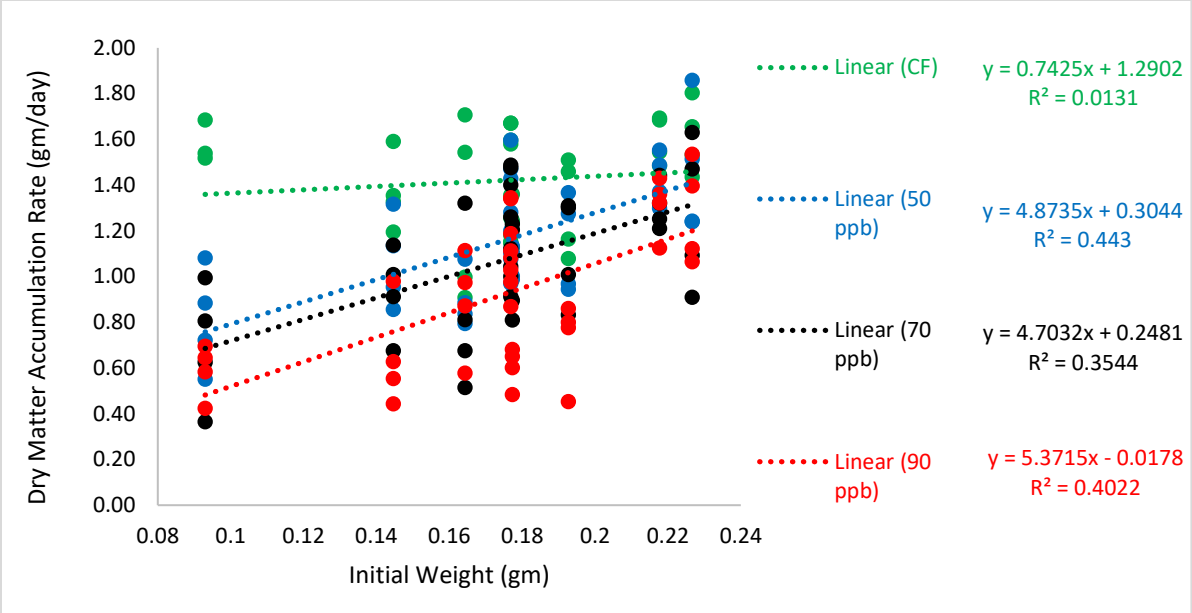


Figure (15): The regression line of the dry matter accumulation rate on the initial weight of the eight genotypes under different O_3 treatments.

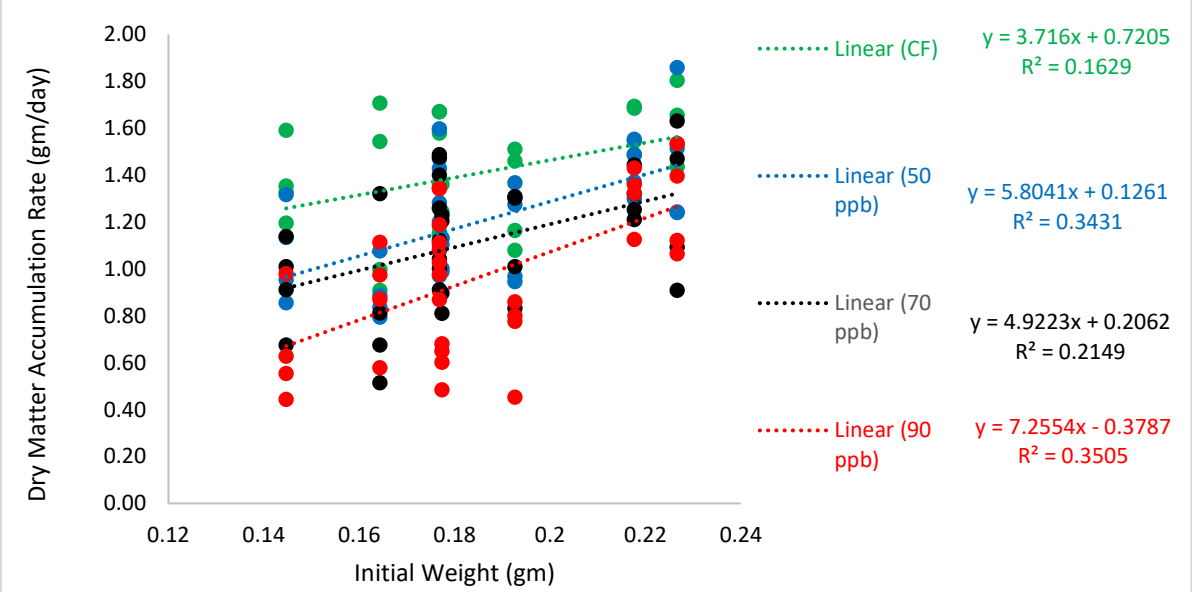


Figure (16): The regression line of the dry matter accumulation rate on the initial weight of the seven bread wheat genotypes under different O_3 treatments.

There was a reduction in the rate of biomass accumulation caused by increasing ozone concentrations for all the genotypes, as compared to charcoal filtered air as shown in Figure (17). However, for every 1% increase in O_3 concentration, the dry matter accumulation rate for Chinese Spring was decreased 0.23%, whereas Rusty was decreased by 0.75%. LMPG-6 had a reduced biomass accumulation rate of 0.59% and the rate of Thatcher was at 0.62%.

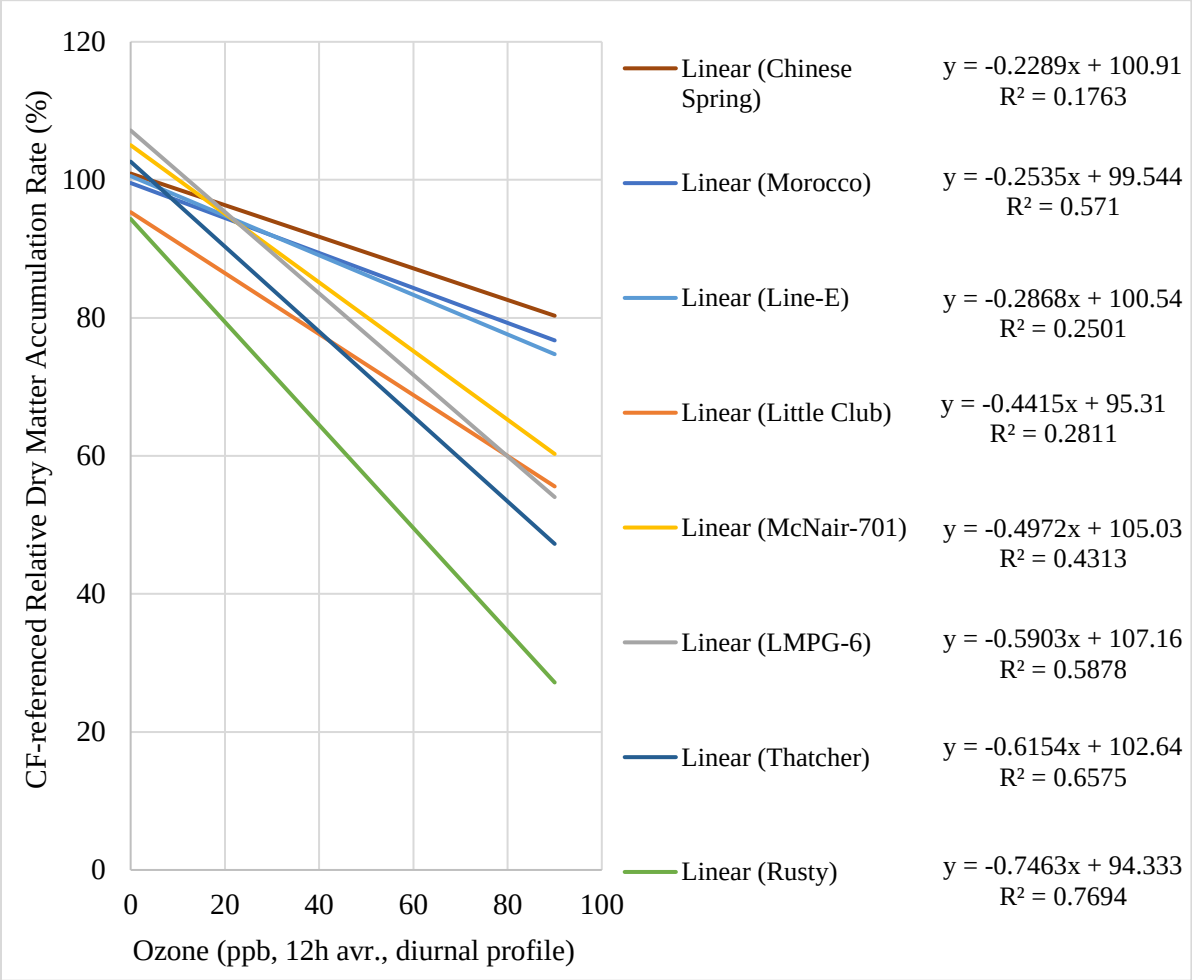


Figure (17): Relative dry matter accumulation rate of eight rust near-universal susceptible genotypes at different O_3 levels (12hrs/day, at 25°C and 50% RH, for 14 days in OPECs), referenced to their RDMAR in Charcoal filtered air (O_3= zero ppb).

Although it does not consider the initial weight, CF-referenced post-treatment dry matter parameter resulted in a similar ranking as shown in Figure (18). Chinese Spring were the most tolerant genotype, with only 0.2% reduction in dry matter accumulation rate, for every 1 ppb O_3 increase, whereas the similar decrease in O_3 resulted 0.77% in the dry matter accumulation rate of Rusty.

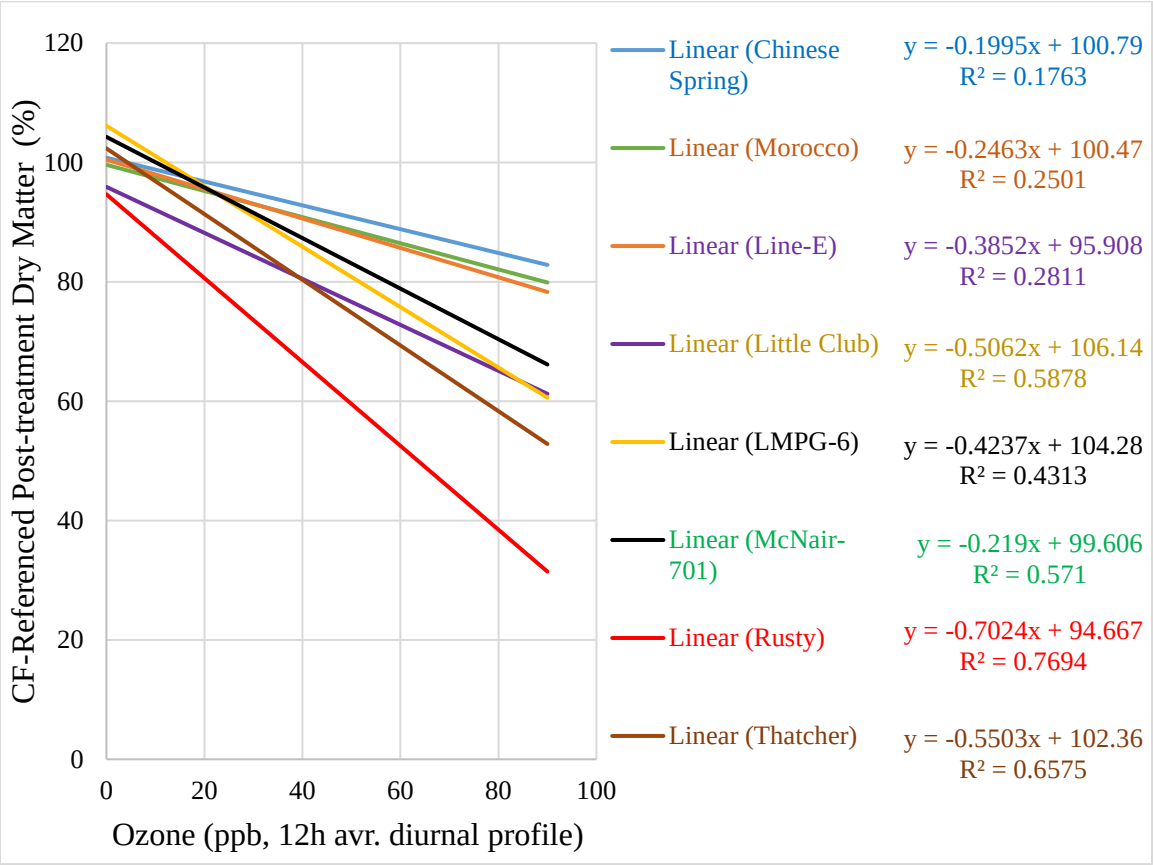


Figure (18): Regression lines of post-treatment dry matter of eight rust near-universal susceptible genotypes referenced to the genotype average dry matter in Charcoal filtered air (O_3= zero ppb). Tested O_3 treatments were CF, 50, 70 and 90 ppb (12hrs/day, at 25°C and 50% RH, for 14 days in OPECs).

2.5. Discussion

The goal of this study was to determine the O_3 responses of the key rust near-universal susceptible panel of eight wheat genotypes. This is the first step towards: (1) identifying the genetic control of O_3 tolerance in wheat, (2) elucidating O_3 effects on different rust races and their consequent effects on the wheat phytobiome, and (3) identifying suitable genotypes for biomonitoring O_3, with using the rust-susceptible lines that can detect rust spores. In order to achieve this goal, the panel was screened in two different O_3 exposure systems (CSTRs and OPECs), and their visible injury and biomass responses were investigated.

The results indicated that O_3 exposure differentially affected these eight genotypes. This suggested that such key genotypes are suitable for the aforementioned applications. Although differences in O_3 responses were documented among wheat cultivars **(Biswas et al., 2013; Mills et al., 2011)**, more information on key genetic stock material was needed. Hence, this research provided the foundation for utilizing the differential responses of key wheat genotypes in for wheat breeding and rust investigations.

Chinese Spring was the most tolerant genotype to both O_3-induced symptoms and biomass reductions. As a result, this central variety for genetic studies of wheat **(Mayer et al., 2014)**, which is usually used as a parent that lacks many agronomic traits **(Quarrie et al., 2005b)**, could be used as a source of O_3 tolerance, based up on the evidence provided here. In addition, the wide diversity of available genetic stocks in Chinese Spring **(Quarrie et al., 1994)** makes it an ideal for identifying the location O_3 tolerance in wheat. Currently, O_3 tolerance is attributed to the AB genome, whereas the sensitivity has been found in the D genome **(Biswas et al., 2008a)**. Because of the availability of the 21 monosomic lines in Chinese Spring, it could be used to correlate O_3 tolerance or insensitivity at the chromosome level.

The cultivar Thatcher and the breeding line LMPG-6 were the most O_3-sensitive genotypes. Because Thatcher is a near-universal susceptible to wheat leaf rust **(Darino et al., 2015)**, Thatcher could be used for characterizing the two traits simultaneously. Near-isogenic lines (NILs) for most of the leaf rust (*Lr*) resistance genes are currently available in the

Thatcher background **(Kolmer, 2015; Kolmer, 2005; McIntosh et al., 1995). These NILs** could be used to investigate possible pleotropic effects of the *Lr* genes on O_3 response. This pleotropic effect could be hypothesized because of the central role that ROS plays in both O_3 stress, and the hypersensitive response mediating resistance conferred by many major-effect resistance genes. The Thatcher NILs could also be used to investigate the effects of O_3 on the efficacy of single leaf rust resistance genes. The resistance proteins encoded by those *Lr* genes are most likely encountered by the non-specific ROS burst, and their integrity and functionality might be affected.

Moreover, Thatcher caries a complex adult-plant resistance to stem rust **(Hiebert et al., 2016)**; therefore, it can be used for studying O_3 interaction with this type of stem rust resistance, when compared to LMPG-6, the O_3-sensitive, and stem rust susceptible genotype. One of the mapping populations in the Hiebert et al study, was made from the cross of RL6058 (GSTR 433) by Chinese Spring. RL6058 is a Thatcher backcross line having the rust resistance gene *Lr34*. So, this mapping population could be used to investigate ozone tolerance. Although it would first need to be determined, whether *Lr34* has an effect on ozone tolerance in Thatcher.

Studies investigating O_3 effects on leaf rust races and their phytobiomes could use Thatcher as a sensitive genotype, and use Morocco or Little Club as moderately tolerant genotypes, as all three genotypes are widely used as universal susceptibles for leaf rust **(Ali, 1994)**. The genotypes LMPG-6 and Chinese Spring are susceptible to most races of stem rust, and could be used as simultaneous rust and O_3 bioindicators.

These pairs of genotypes with contrasting O_3 responses are useful tools for studying the biological and physical components of the wheat phytobiome. Elevated O_3 affects wheat interactions with soil microorganism, changing their processes and community structure, in addition to carbon allocation. Elevated ozone decreased the fungi/bacteria ratio, and the magnitude of this change was dependent on the O_3 response of the wheat cultivar **(Li et al., 2012)**. These effects may have also influenced soil organic matter decomposition, nutrient turnover, and possibly greenhouse gas emission **(Li et al., 2012)**. Using rust susceptible

varieties in such studies could show the indirect effects of O_3 on different rust races by changing their microbiome context.

O_3-sensitive cultivars showed increased yield reductions under combined drought and O_3 stress when compared to drought alone **(Xu et al., 2007)**. This is likely due to O_3-induced ethylene production that interferes with stomatal closure and causes reduced drought tolerance **(Wilkinson et al., 2011)**. Cross-tolerance to O_3 and drought was not observed in wheat **(Biswas and Jiang, 2011)**. Five years of elevated O_3 stimulated soil N availability, but suppressed grain yields **(Chen et al., 2015)**. This was due to increased NH_4^+ availability and decreased microbial ability to retain NH_4^+, which increases Nitrogen losses. However, these effects were less pronounced in O_3-tolerant than O_3-senstive cultivars. Elevated O_3 also increased soil pH **(Li et al., 2013)**, which could alter several soil chemical, physical and microbial structure. Rust susceptible varieties with known O_3 sensitivity could be used to investigate the interplay of several soil factors belowground, and the rust populations on the shoots above ground

In conclusion, the results presented in this research could accelerate breeding for O_3 tolerance using existing resources, as well as, it would facilitate simultaneous breeding for leaf rust and/or stem rust resistances and O_3 tolerance using the same breeding materials. A potential next step could be screening existing mapping populations involving the ozone tolerant and ozone sensitive genotypes found in this study, to better understand any relation between O_3 tolerance and specific leaf or stem rust resistance genes. This is could be conducted on a global scale through new approaches, such as “Delivering the Genetic Gain in Wheat”, the next phase of the BGRI. Another important step is to implement the results discussed here for deciphering the multifactorial interactions of different phytobiome components under elevated O_3. The multidisciplinary approach offered by phytobiome studies, and the open discussion it initiated between academia, industry and the public entities are optimum for maximizing the benefits of such information.

3. Identification of the Genetic Control of Ozone Tolerance in Wheat (*Triticum aestivum* L. cv. Chinese Spring) at the Chromosome Level.

3.1. Abstract

Ambient air ozone (O_3) pollution is a major challenge to wheat production worldwide, especially in heavily populated developing countries, where more food production is needed. Breeding O_3-tolerant wheat varieties is the most suitable adaptation in the absence of a concerted international effort to reduce the emission of O_3 precursors. Identifying the genetic control of O_3 tolerance and associated markers is an essential first step towards breeding O_3-tolerant varieties that maintain high yield under current and anticipated future increases in O_3 levels.

Very little is known about the genetic control of O_3 responses in wheat. O_3 tolerance is currently attributed to subgenomes A and B, whereas the sensitivity is thought to be inherited from subgenome D. However, there is no information available at chromosome or loci levels. The individual chromosome contribution could be identified by screening the 21 monosomic lines (each of which lack one chromosome) of any wheat variety against its full genome, and correlate its O_3 response (sensitivity or tolerance) to one of the chromosomes. An ideal variety for this purpose is Chinese Spring, the corner stone for genetic studies of wheat.

This paper investigated individual chromosome contribution to the genetic control of O_3 response of the Chinese Spring variety, by screening its 21 monosomic lines, and comparing them to the response of the full genome. Plant material was screened at the seedling stage, under different O_3 treatments, in two different gas exposure systems. Visible injury results suggested that the observed O_3 tolerance is correlated with chromosomes 7A, with no evidence for sensitivity factors associated with any subgenome D chromosome. Mapping population from the cross between Chinese Spring and O_3-sensitive varieties, (e.g. Thatcher) could determine the exact location(s) of O_3 tolerance gene(s) and identify the associated genetic marker(s).

3.2. Introduction

Tropospheric Ozone (O_3) is a secondary air pollutant and a global warming gas in ambient air, the concentrations of which have been increasing since the industrial era, and are expected to continue to increase in the future **(IPCC, 2014a)**. It is formed by photochemical reactions between non-methane volatile organic compounds (NMVOCs) and nitrogen oxides (NO_x) **(IPCC, 2014b)**. After being formed in ambient air, O_3 molecule could be inhaled by human and animals, causing respiratory problems. In addition, it could diffuse into plant leaves, where it is considered the most phytotoxic air pollutant **(Sandermann et al., 1998)**.

Ozone has a high oxidizing power (+2.07 eV) **(Iriti and Faoro, 2007)**, a high diffusion coefficient through plant leaves (similar to CO_2), and a high solubility in water (10 times higher than CO_2) **(Zuccarini, 2009)**. Therefore, O_3 molecules penetrate directly into the leaf through opened stomata and immediately dissolve in the apoplastic fluid, generating primary Reactive Oxygen Species (ROS) in the apoplast **(Vaultier and Jolivet, 2015)**. If not detoxified, these non-biogenic ROS react directly in the apoplastic fluid within the cell wall and/or plasma membrane, inducing a self-propagating oxidative burst of biogenic ROS that spreads to the surrounding tissue **(Pellinen et al., 1999; Vahisalu et al., 2010)**. The resulting oxidative stress causes visible injury, biomass and yield reduction in sensitive plants including major crops, such as wheat, soybean, corn and rice, threatening global food security.

Wheat (*Triticum aestivum* L.) is one of the most sensitive crops to elevated O_3 at all growth stages **(Singh and Agrawal, 2010)**. Currently, wheat yield reduction due to elevated O_3 is estimated to be 7-12% of the yield worldwide, and is expected to reach 9-18% by the year 2030 **(Avnery et al., 2011a; Avnery et al., 2011b; Feng and Kobayashi, 2009)**. Breeding wheat for O_3 tolerance is essential for increasing yield under O_3 stress (especially in developing countries), and is very essential adaptation needed for sustainably producing 70% more food by 2050 **(Shiferaw et al., 2013; Tester and Langridge, 2010)**.

Wheat is also grown in about 15.5% of arable land, making it one of the most widely grown crops worldwide **(FAO, 2016)**. It encounters elevated O_3 concentration nearly

everywhere it is grown. This wide spread of O_3 stress was expected to evolve modern wheat varieties towards increased O_3 tolerance, however, evidence support the opposite trend **(Biswas et al., 2008b)**. By selection for higher stomatal conductance and less mesophyll resistance to gas diffusion **(Barnes et al., 1999)**, a recipe for higher O_3 sensitivity. Therefore, active selection is critical for increasing O_3 tolerance in wheat. However, breeding efforts are limited by the lack of information on the genetic control for the trait, the absence of associated genetic marker associated, and the complexity of the wheat genome.

Wheat genome is an allohexaploid (2n = 6x = 42 chromosomes; genomic code AABBDD), consists of three closely related diploid subgenomes, AA, BB and DD **(Eversole et al., 2014; Marcussen et al., 2014)**. There is only one report **(Biswas et al., 2008a)** on the source of O_3 responses in hexaploid wheat, which attributed the origin of O_3 sensitivity in bread wheat to subgenome DD. However, to our knowledge, there are no reports on the genetic control of O_3 responses at chromosome level. A screening of the 21 monosomic lines (each of which lack one chromosome) of any wheat variety against its full genome could correlate its O_3 response (sensitivity or tolerance) to one of its chromosomes. An Ideal material for this purpose is Chinese Spring and its 21 monosomic lines. Since they were first developed in 1953, these monosomic lines were used to associate wheat traits to specific chromosomes **(Sasaki et al., 1963)**. To our knowledge, such materials have never been used for identifying O_3 genetic control at the chromosome level.

Chinese Spring is a central variety for genetic studies of wheat **(Mayer et al., 2014)**. Since it is agronomically unsuitable **(Quarrie et al., 2005b)**, it is often used as a reference for mapping desired traits. It is frequently crossed to a contrasting genotype, and the resulting mapping populations are phenotyped and genotyped to identify the genetic control and associated genetic markers **(Salamini et al., 2002)**. Due to the low content of abscisic acid in Chinese Spring, it was crossed with two high abscisic content genotypes (Ciano 67 and SQ1), and mapping populations were used to identify QTLs to associated to high abscisic acid content **(Quarrie et al., 1994)**. A similar procedure was used to identify QTLs for high grain weight in “Rye Selection 111” **(Kumar et al., 2006)**.

Stomata are the entry point for O_3, and they control stomatal conductance and gas exchange. The absence of each Chinese Spring chromosome was found to affect the characteristics of the stomatal apparatus, such as number, length and width of stomata, as well as the distance between stomata or between stomatal rows **(Davydov, 1999)**. However, the conclusions drawn from this research should be contingent on the suitability of the disomic control used as a substitute for the cultivar itself. Indeed, this depends on the nature of chromosome substitution led to the formation of the disomic control. Yet, it showed an example for the use of monosomic lines in determining the effect of individual chromosomes on different traits.

In a series of studies **(Quarrie et al., 2005b; Quarrie et al., 2006; Quarrie et al., 2007)**, a doubled haploid population from the cross between Chinese Spring and SQ1 were used to identify QTLs associated with yield under different stresses including elevated ambient O_3. However, no data were presented to show any differential O_3 response between the two parents, or the genetic gain expected in the population **(Quarrie et al., 2007)**. Consequently, no QTLs specific to elevated O_3 were designated.

This paper is the first attempt to identify the effect of each chromosome of Chinese Spring variety on its O_3 response. In a previous screening, Chinese Spring was found to be the most O_3-tolerant among eight genotypes tested **(Mashaheet et al., 2016c)**. Therefore, screening the monosomic lines of Chinese Spring could correlate its O_3 tolerance to one of its 21 chromosomes. Chinese Spring is also the variety chosen by the International Wheat Genome Sequencing Consortium to sequence the whole genome of wheat, the first draft of which is already published **(Mayer et al., 2014)**. This will allow a full genome reference to precisely map any identified markers and genes, and facilitate the incorporation of that marker in breeding programs.

In order to achieve this objective, seedlings of Chines Spring cultivar (full genome) and its 21 monosomic lines were screened in two different O_3 exposure systems: Continuous

Stirred Tank Reactors (CSTRs) and Outdoor-plant Environment Chambers (OPECs), and O_3 injury and biomass data were collected.

3.3. Materials and Methods

3.3.1. Plant Materials

For both studies, seedlings of Chinese Spring variety and its 21 monosomic lines were grown in charcoal filtered (CF) air in the greenhouse for 30 days from planting. Plants at growth stage of 21-23 in Zadok's scale were selected for growth stage and canopy status, the fourth leaf on the top of the main stem were tagged, and then moved to CSTRs or OPECs.

3.3.2. Ozone treatment

In both systems, plants were acclimated for 2 days in CF before treatement with O_3. In CSTRs five O_3 treatments (CF, 50, 70, 90 and 110 ppb) were used for 5 days (7hrs/day, at 25°C and 60% RH). The experiment design involved 15 chambers in three blocks. Each treatment was randomly assigned to one chamber per block. One plant per genotype per chamber was used. In OPECs, Plants were exposed for 14 days to one of four O_3 treatments using a predefined diurnal O_3 profile (CF, 50, 70, 90 ppb, 12hrs average, at 25/16°C day/night and 50% RH). Eight OPEC chambers divided into two blocks were used. Each treatment was randomly assigned to one chambers per blocks. Two plants per genotype were randomized within each chamber.

3.3.3. Ozone Injury Assessment

For plants treated in CSTRs, two days after the end of exposure, percent O_3 injury on the fourth leaf of the main stem was visually assessed following a 0-100% scale, whereas, all leaves on the main stem of plants treated in OPECs were evaluated. However, only 3^{rd}-5^{th} leaves were considered for statistical analysis, as the first and second leaves showed some signs of senescence, and not all plants had leaves above the fifth.

3.3.4. Dry Matter Accumulation Rate

In OPECs, shoot dry weight was measured before treatment (6 plant for Chinese Spring, and 3 plants per monosomic line) and after treatments for all treated plants. Biomass

data was not attempted in the CSTRs study because of the short period of exposure limited biomass gain. Three dry mater parameters were calculated:

- Dry Matter Accumulation Rate (DMAR)

$$DMAR = \frac{(Initial\ Dry\ Weight - Final\ Dry\ weight)}{Exposure\ Duration}$$

- Adjusted Dry Matter Accumulation Rate (ADMAR)

$$ADMAR = \frac{(DMAR)}{Initial\ Dry\ Weight} \times 100$$

This parameter was calculated to account for the differences in the initial weight among the different entries tested.

- CF-Referenced Relative Dry Matter Accumulation Rate (RDMAR)

$$RDMAR = \frac{(ADMAR\ for\ individual\ plant)}{Average\ ADMAR\ for\ their\ entry\ under\ CF} \times 100$$

This parameter accounts for the differences in initial weight and the differences under CF. It was suitable for estimating the effect of increased O_3 on the biomass accumulation rate, and compare it across entries **(Velissariou et al., 1992)**.

3.3.5. Statistical Analysis

Using the Glmmix procedure in SAS 9.4, averages were separated according to Dunnett test, using the cultivar with complete genome as the control.

3.4. Results

3.4.1. Ozone Injury

In CSTRs, the lack of chromosome 7A was correlated with significant increase in O_3 visible symptoms scored on the fourth leaf as illustrated in Figure (19), which suggests that chromosome 7A is essential for Chinese Spring tolerance to O_3 visible symptoms. The absence of any other chromosome did not cause any significant differences from the full genome. None of subgenome D chromosomes seems to be responsible for sensitivity, as their absence did not cause any decrease in O_3 injury.

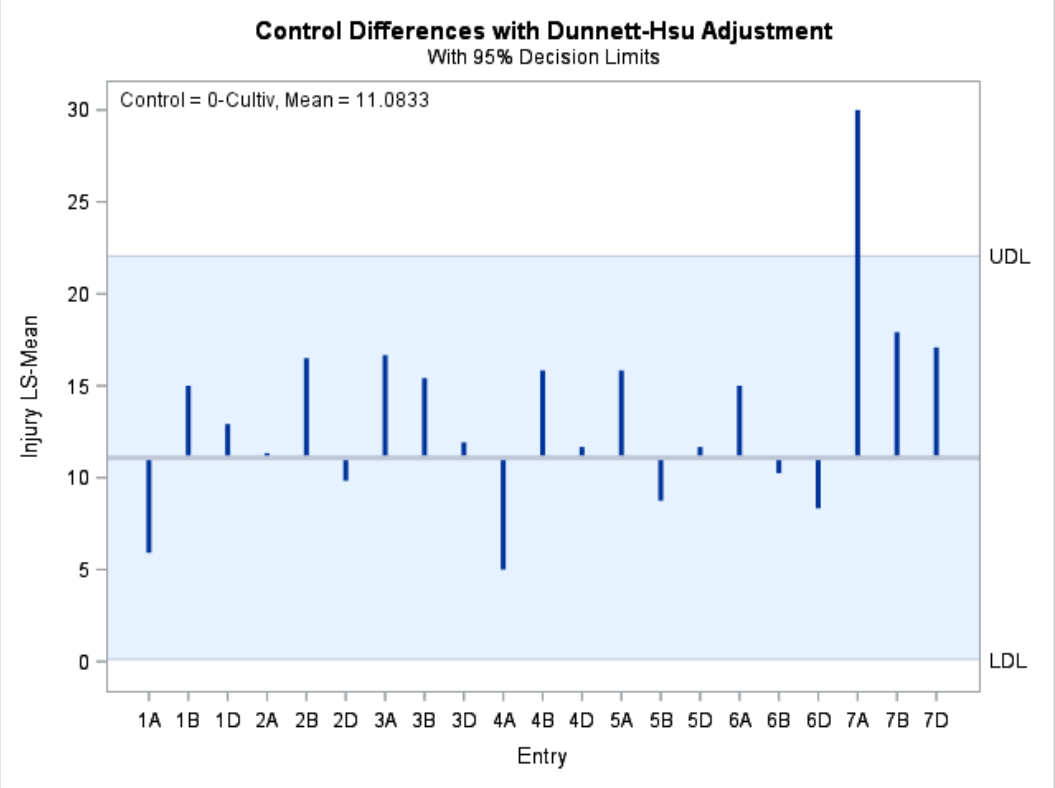


Figure (19): O_3 injury on the fourth leaf of Chinese Spring variety and its 21 monosomic lines, at four different O_3 levels, exposed for 5 days (7hrs/day, at 25°C and 60% RH) in CSTRs. Upper and lower decision limits (UDL and LDL, respectively) at α=0.05 are determined using Dunnett-Hsu adjustment.

Ozone injury data in OPECs supported the results obtained in CSTRs as illustrated in Figure (20). The absence of chromosome 7A also correspond to the only significant change in O_3 injury. This further supports chromosome 7A correlation to the tolerance of Chinese Spring to O_3 visible symptoms. Subgenome D chromosomes did not cause any significant decrease in O_3 symptoms when they were absent.

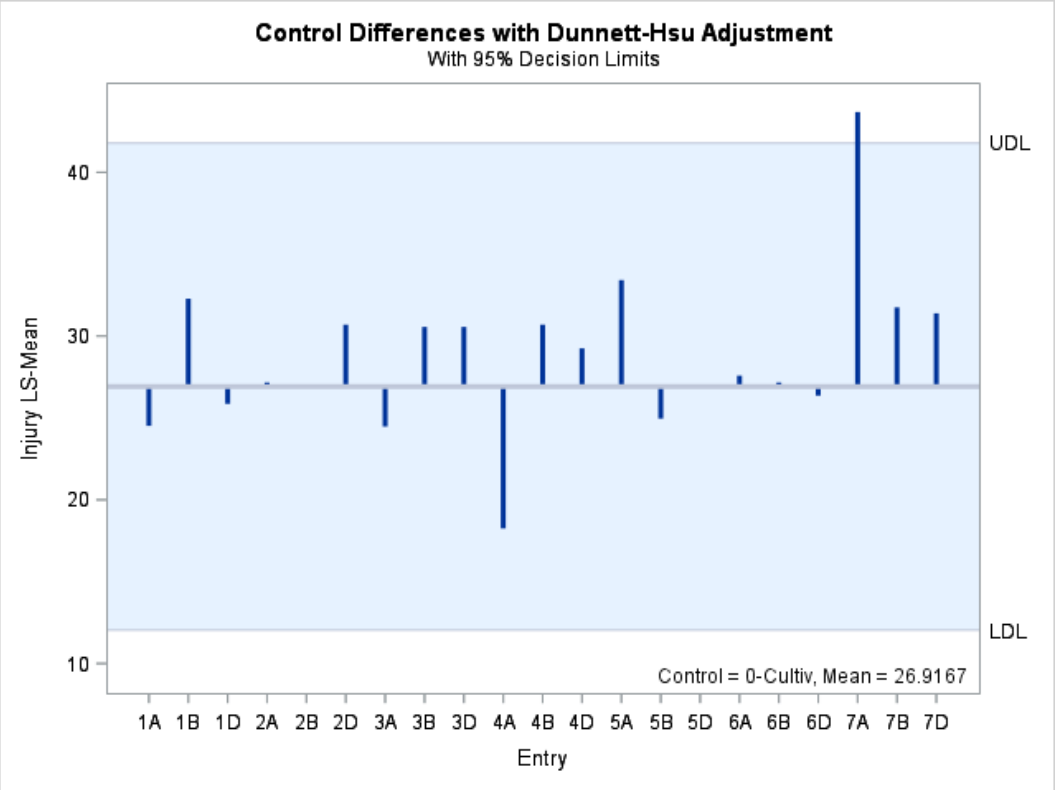


Figure (20): O_3 injury on the 3rd-5th leaf of Chinese Spring variety and its 21 monosomic lines, at three different O_3 levels, exposed for 14 days (12hrs/day, at 25°C and 50% RH) in OPECs. Upper and lower decision limits (UDL and LDL, respectively) at α=0.05 are determined using Dunnett-Hsu adjustment.

3.4.2. Dry Matter Accumulation Rate

The absence of chromosome 1A and 7D caused significant decrease in the initial shoot dry weight at 30 after planting, as shown in Figure (21). The presence of significant differences in initial shoot dry weight among entries, indicated that O_3 effects on dry matter accumulation rates must be adjusted to the initial weight before treatment (RSDAR).

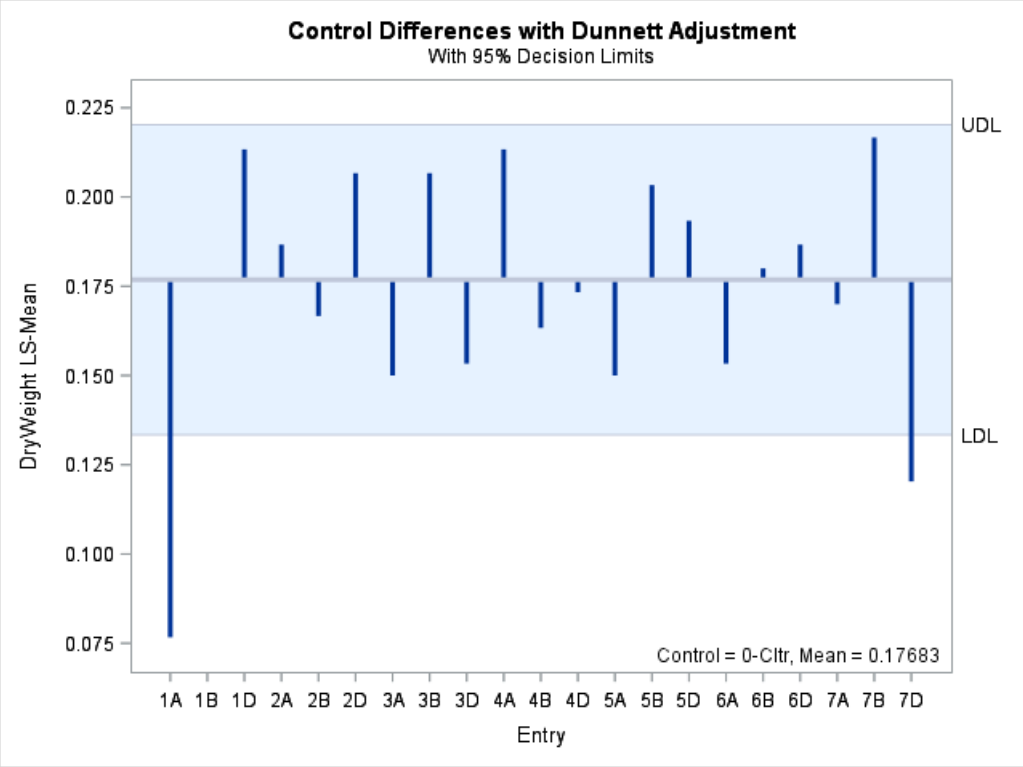


Figure (21): Mean shoot dry matter of 21 monosomic lines of Chinese Spring wheat at the initial time point 30 days after planting, compared to the cultivar with complete genome (Cultivar). Upper and lower decision limits (UDL and LDL, respectively) at α=0.05 are determined using Dunnett adjustment.

The dry matter accumulation rate was affected by the initial dry weight. This indicated that smaller plants had lower dry matter accumulation rates than larger plants under all

treatments used, as shown in Figure (22). These finding suggested the correction of dry mater accumulation rates to the initial dry weight, as applied in calculating the relative dry matter accumulation rate (RDMAR).

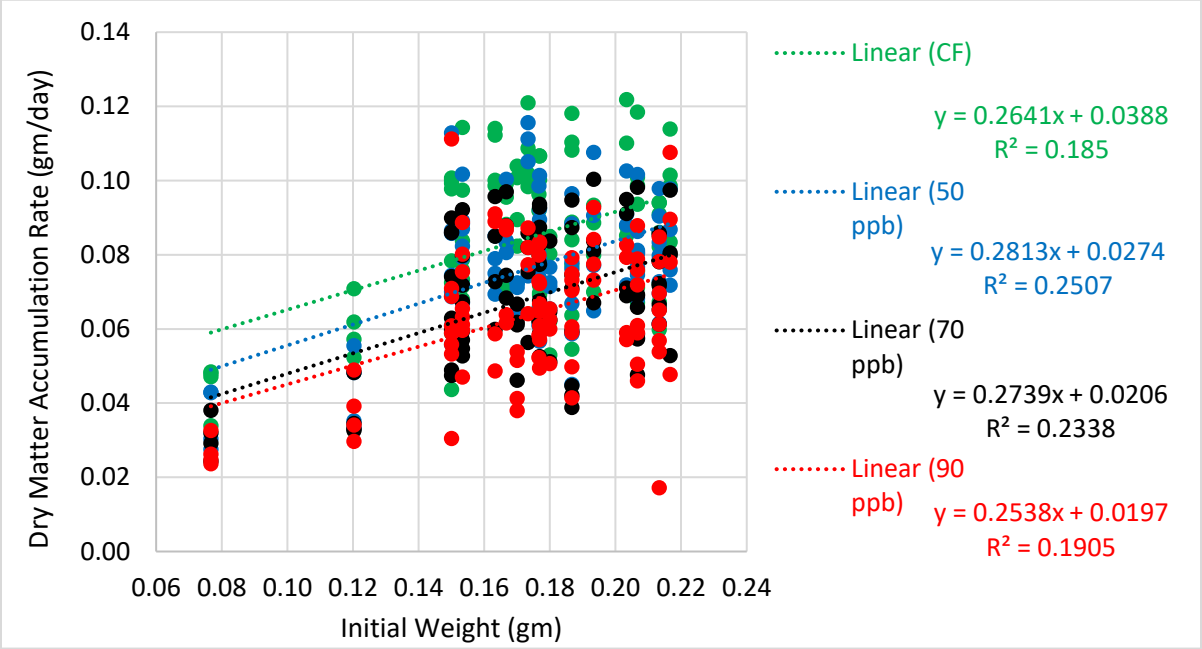


Figure (22): The regression line of the dry matter accumulation rate on the initial dry weight of the Chinese Spring and its 21 monosomic lines under different O_3 treatments.

By referencing to the ADMAR at CF, and the calculation of CF-referenced ADMAR, we were able to compare slope of the curve of different entries as illustrated in Figure (23). The absence of chromosome 7A had the highest effect on shoot dry matter accumulation rate. For every increase of O_3 concentration by one ppb, this monosomic line showed 0.45% less RDMAR than CF, which resulted in about 50% lower RDAR at 90 ppb O_3 after 14 days of exposure. On the other hand, the absence of chromosome 5D resulted in shoot dry matter insensitivity to O_3. The RDMAR curve was almost flat, suggesting that chromosome 5D is critical for the biomass sensitivity of Chinese Spring to O_3.

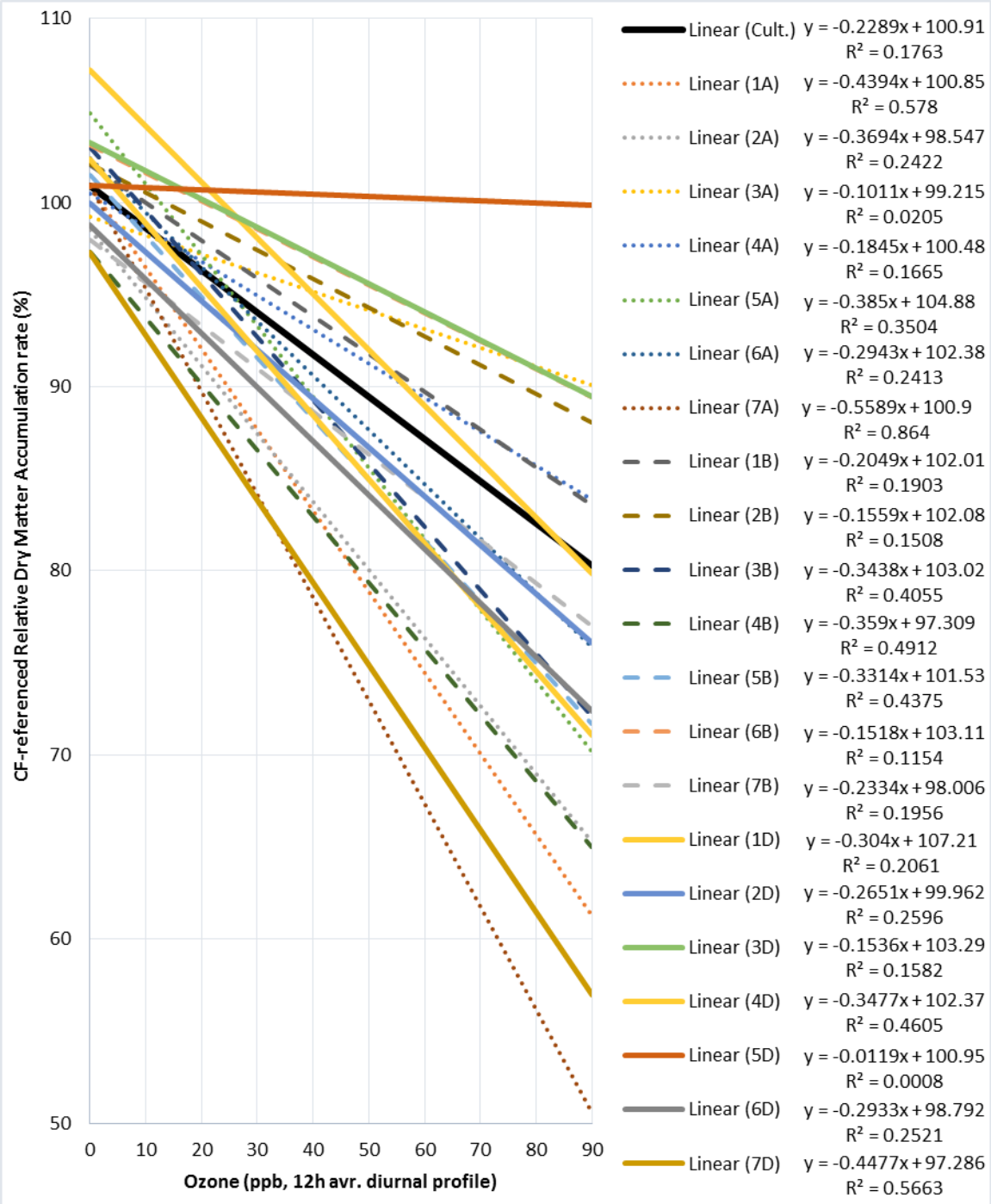


Figure (23): Relative dry matter accumulation rate of Chinese Spring wheat and its 21 monosomic lines at different O_3 levels (12hrs/day, at 25°C and 50% RH, for 14 days in OPECs), referenced to their RDAR in Charcoal filtered air (O_3= zero ppb).

3.5. Discussion

The goal of this study was to determine the individual chromosome contribution towards O_3 tolerance in Chinese Spring wheat. This objective was achieved by screening the cultivar with complete genome and its 21 monosomic lines under different O_3 treatments, in two different gas exposure systems. Characterizing the chromosome basis of O_3 response is a first step towards achieving a better understanding at DNA level, especially when the final full genome sequence of this variety is published as expected next year, or even with the first draft currently available **(Mayer et al., 2014)**.

O_3 injury and biomass data showed that chromosome 7A is critical for O_3 tolerance in CS, whereas chromosome 5D was correlated to biomass O_3 sensitivity, but not visible symptoms. These findings are in agreement with previous report **(Biswas et al., 2008a)** attributing O_3 tolerance in hexaploid wheat to subgenomes AABB, and sensitivity to subgenome DD. **Biswas et al., (2008)** came to this conclusion by comparing wild and cultivated winter wheat varieties, including those closely related to modern wheat progenitors. They found that durum wheat (AABB) was the most O_3-tolerant, followed by *T. monococcum*, a successive sister of *T. urartu*, the more likely origin of genome AA **(Marcussen et al., 2014)**. Here we provided direct evidence using hexaploid wheat that chromosome 7A is essential for tolerance to O_3, using both O_3 injury and biomass accumulation rate. Meanwhile, **(Biswas et al., 2008a)** found that *Aegilops tauschii*, a goatgrass and the donor of subgenome DD, was the most O_3-sensitive progenitor. In this paper, the presented results narrowed that down to chromosome 5D, which was correlated with biomass O_3 sensitivity, but not visible injury symptoms.

To our knowledge, there is only one study that invested the differences in the characteristics of the stomatal apparatus as a result of individual chromosome absence in each of the monosomic lines of the Chinese Spring **(Davydov, 1999)**, which might explain some of the changes in O_3 responses. The study showed that the absence of chromosome 7A was associated with the least stomatal length amongst all monosomic lines, which indicates that the increased sensitivity reported here is likely derived by other mechanism(s) that is/are not

affected by the size of stomata. These mechanisms of increased sensitivity in the absence of chromosome 7A could include lower O_3 detoxification or reduced ability of injury containment. The absence of chromosome 5D associated with biomass insensitivity to O_3 in this study, was shown by **(Davydov, 1999)** to significantly reduce the number of stomata on both leaf surfaces, and increase stomatal width on the lower surface only. The number of stomata does not seem to have a significant impact on O_3 responses in Chinese Spring, because the absence of chromosome 5A caused more reduction in the number of stomata (by 29.9% and 53.4% on the upper and lower leaf sides respectively) comparing to the disomic control **(Davydov, 1999)**. However, the results presented here showed that the absence of chromosome 5A had no significant impact on O_3 responses.

Although wheat subgenomes have evolved after divergence, at all ploidy levels (diploid, tetraploid and hexaploid) **(Mayer et al., 2014)**, studying wheat progenitors still provides useful information. Since their divergence, wheat subgenomes undergone dynamic gene gain, loss, and duplication **(Mayer et al., 2014)**, but this did not seem to affect the conclusion drown about O_3 responses using progenitors. Each of the hexaploid wheat subgenomes showed high sequence similarity to the wild progenitor, with retained structural conservation and limited gene loss **(Mayer et al., 2014)**. Therefore, the diversity in hexaploid wheat responses to O_3 might be explained by the observed transcriptional dependence evolved between subgenomes, without global dominance of any subgenome **(Mayer et al., 2014)**.

Chinese Spring was selected for this study for two reason: (1) the central role it plays in wheat genetic studies and because it was found to be O_3 tolerant **(Mashaheet et al., 2016c)**, and (2) the availability of its 21 monosomic lines. Nevertheless, the same procedure used here with Chinese Spring could be applied to other varieties with available monosomic line stocks, such as Thatcher, where sensitivity could be correlated to any of the chromosomes. More advanced techniques such as chromosome substitution and the formation of disomic wheat lines with sensitive background genome with individual chromosome replacement from Chinese Spring is another procedure that could be used to narrow down the genetic control of O_3 tolerance in Chinese Spring. For example, the replacement of chromosome 5D in Chinese

spring with the same chromosome from another variety could confirm the role of 5D in O_3 sensitivity. An ideal disomic will be a Thatcher disomic with chromosome 7A from Chinese Spring. Thatcher was found to be sensitive to O_3 **(Mashaheet et al., 2016c)**. The use of the sensitive Thatcher background genome with chromosome 7A would verify the role of chromosome 7A as a source of tolerance if the resulting disomic become more tolerant. The results discussed in this research makes this procedure is more feasible. The results presented in this paper were obtained at the seedling stage, and further studies at adult plant stage are needed.

A next step towards identifying O_3 tolerance or sensitivity loci is to study mapping population of Chinese Spring chromosomes 7A and 5D respectively. Parents of mapping populations for O_3 responses should be selected based upon their O_3 responses. A doubled haploid population from the cross between Chinese Spring and SQ1 were used to identify QTLs associated with yield under different stresses including elevated ambient O_3 **(Quarrie et al., 2005b; Quarrie et al., 2006; Quarrie et al., 2007)**. However, no QTLs specific to elevated O_3 were designated **(Quarrie et al., 2007)**. This might be due to the lack of differential O_3 response between the two parents. Mapping populations of the cross between CS and key O_3 sensitive varieties might be more informative. An ideal candidate for such purpose is the hard red spring wheat variety Thatcher. It also was found to be very sensitive to O_3 (unpublished data). It is a key wheat variety, known for its susceptibility to leaf rust, widely used in mapping resistance genes for leaf rust **(Bolton et al., 2008)**.

4. Relative O_3-Responses of Some Winter Wheat Genotypes, and Their Suitability for Breeding Rust and Ozone Tolerant Varieties.

4.1. Abstract

Elevated ground level ozone concentrations and the rapidly evolving rust diseases are major threats to wheat production worldwide. Breeding hard red winter wheat cultivars that are adapted to eastern United States is currently performed. Identifying the O_3 responses of wheat materials used in breeding programs is the first step towards breeding resilient varieties. This study investigated the O_3 responses of 24 soft and hard red winter wheat varieties, in relation to some other varieties from different wheat classes and different cereal crops.

First, we summarized O_3 monitoring data collected, from a typical sub-urban location in Raleigh NC, USA, during wheat active growing season (March-May) over the last 10 years, and compared them to the recent reports on O_3 level trends in the region. Results indicated that the 12hr average O_3 concentrations were mostly above the threshold of 40 ppb for sensitive plants, with a trend towards decline over years and over moths within the year. However, hourly average data from the 2015 showed high variability in daily and extended cycles of high overnight, low daytime or typical O_3 peaks. These fluctuations indicated that O_3 stress could be a limiting factor to wheat in the region, and showed the importance of considering O_3 responses of breeding materials used in this area.

Second, we investigated the relative O_3 responses of 12 hard and 10 soft red winter wheat genotypes, in comparison with one hard white winter and two spring wheat genotypes, as well as two varieties of each of oat, triticale and barley. Plants were exposed at seedling stage to one of five O_3 treatments (CF, 50, 75 or 100 ppb for 8h d^{-1}) in CSTRs. O_3 injury results indicated that on average winter wheat was more tolerant than spring wheat, similar to oat, and more sensitive than triticale and barely.

Tested hard wheat genotypes were found to be more sensitive to O_3 than soft wheat, indicating the importance of considering the O_3 responses of parents used to increase improve

hardness. The tested red wheat genotypes exhibited a decrease in sensitivity to O_3-induced foliar injury over time of cultivar release, with a higher rate of decline after the year 2000.

Finally, Coker 9553 and MD01W28-08-11are two genotypes and breeding parents that showed differential O_3 responses at seedling stage in the first experiment, and were selected to investigate their yield responses under four O_3 (CF, 50, 75 or 100 ppb, 12h average) treatments in OTCs. Total yield results showed that under low O_3 Coker-9553 had higher yield, but yield declined under higher O_3 treatments. On the other hand, MD01W28-08-11 showed yield superiority under high O_3 concentrations, which makes it a good source of O_3 tolerance. Because these two parents have also contrasting adult-plant responses to stem rust races (including races in Ug99-lineage), they are a suitable pair for simultaneous breeding for both stressors.

4.2. Introduction

Breeding wheat (*Triticum aestivum* L.) cultivars with resiliency to biotic and abiotic stresses is essential for global food security. In North Carolina, as in most wheat growing areas of the U.S. east of the Mississippi River, the commonly-grown market class of wheat is soft red winter. Flour from soft red winter wheat is typically used for cakes, cookies, biscuits, and similar products. Niche markets of hard winter wheat (red or white) have become established in parts of North Carolina **(Sethi, 2015)**. Hard wheat flour is primarily used for bread and pastries. There are several diseases and insects that limit wheat production in the eastern U.S., including the potential for epidemic spread of rust diseases. In addition, trends in elevated ozone (O_3) concentrations in the eastern U. S. over the last few decades showed increased levels in winter, and no decrease in spring, despite the improvement achieved in summer, which indicated that O_3 levels are still high during the wheat growing season **(Cooper et al., 2014; Lee et al., 2014; Monks et al., 2015; Parrish et al., 2013)**. Furthermore, O_3 flux into wheat leaves are expected to be high under the high humidity and rainfall in the eastern U. S.

It could be advantageous to simultaneously breed wheat for ozone tolerance and rust resistance. If attempted, it will be limited by the lack of information on O_3 response in wheat germplasm, and genetic markers needed for markers-assisted selection. In fact, breeding wheat for O_3 tolerance alone is yet to be attempted on a rigorous scale **(Ainsworth et al., 2008; Mills et al., 2011)**. Some research suggests that modern varieties of wheat are more sensitive to O_3 than older varieties **(Biswas et al., 2008b; Biswas et al., 2013; Pleijel et al., 2006)**. This may be a result of selecting wheat for high stomatal conductance, and less mesophyll resistance to gas diffusion, and consequently higher photosynthesis **(Biswas et al., 2008b; Biswas et al., 2013)**. However, this is a recipe for O_3 sensitivity, especially when conducted under inconsistent O_3 selection pressure **(Ainsworth et al., 2008)**.

Wheat has been shown to be one of the most sensitive major crops to elevated ground-level O_3 (E-O_3) concentrations **(Mills et al., 2007)**. O_3 is one of nature's most powerful oxidizers, with the triatomic-oxygen molecule being extremely unstable and immediately oxidizes everything it encounters **(Haynes, 2015)**, including the external and internal tissues

of wheat **(Booker et al., 2009)**. Ozone has similar diffusion ability to CO_2 through plant leaves **(Fares et al., 2012)**, but O_3 has higher solubility in water **(Haynes, 2015)**, which facilitates O_3 flux into plant tissue. After penetrating through stomata, it immediately dissolves in the apoplastic fluid and generates primary reactive oxygen species (ROS) **(Iriti and Faoro, 2007)**. If not detoxified, these O_3-originated ROS react directly in the apoplastic fluid, the cell wall and/or plasma membrane, inducing a self-propagating oxidative burst of biogenic-ROS that spreads to the surrounding tissue, similar to the hypersensitive response (HR) resulting from incompatible host/pathogen interactions **(Pellinen et al., 1999; Vahisalu et al., 2010)**. These O_3 induced oxidative bursts might interfere with host/pathogen induced HR if they co-occur. Successful HR-mediated resistance requires appropriate timing and magnitude of ROS initiation and containment, which might be disrupted by O_3-induced ROS.

Ozone effects on wheat are dose dependent **(Liu et al., 2015b)**. Current O_3 concentrations are estimated to reduce grain yield by 10% with additional 20% losses estimated under future concentration **(Feng and Kobayashi, 2009; Van Dingenen et al., 2009)**. In addition to the biomass and yield losses, chlorotic and necrotic symptoms could affect wheat interactions with epiphytic and endophytic microbiome. Trends in O_3 concentrations suggest that O_3 effects on wheat plants are taking place at a global scale, however, the effects are more pronounced in developing countries, where food security is already endangered and greater food production is needed **(Shiferaw et al., 2013; Tester and Langridge, 2010)**. O_3 transport ranges from local to hemispheric scales, which increases baseline O_3 worldwide, including areas where emission of precursors have been controlled and reduced. The increasing baseline O_3 indicate that E-O_3 continues to be a problem at a global scale **(Cooper et al., 2014; Lee et al., 2014; Monks et al., 2015; Parrish et al., 2013)**.

Under low to moderate external ozone concentrations, the influx of ozone and associated ROS might be scavenged by the apoplastic antioxidants capacity, or cause some temporary effects, such as localized plasma membrane impairment and electrolyte leakage that do not cause cell death **(Vaultier and Jolivet, 2015)**. Although such temporary effects might not be visually observed, they consume some of the plant's energy to cope with, and might

interfere with critical physiological mechanisms, such as the initiation and containment of HR under pathogen attack.

However, under high O_3 concentrations currently encountered, and high stomatal conductance and low mesophyll resistance to gas diffusion (traits wheat breeders are indirectly selecting for), the influx of O_3 during the early phase of O_3 stress exceeds the apoplastic antioxidant capacity to detoxify ROS formed from O_3 degradation **(Pellinen et al., 1999; Vahisalu et al., 2010)**. The plant's antioxidant capacity could also fail to detoxify the high levels of ROS generated when O_3 is deposited to wet leaf surfaces, as the effective O_3 flux into the leaf will be increased causing acute symptoms, at O_3 levels expected only to cause chronic effects **(Altimir et al., 2006)**. If the plant over-reacts to O_3 by extreme stomatal closure that significantly decreases stomatal conductance, then photosynthesis rate, biomass production and seed yield will be negatively affected, despite the absence of visible injury **(Biswas et al., 2013; Meyer et al., 2000; Wattal and Siddiqui, 2015)**.

If the antioxidant machinery is not activated to balance O_3 flux, or the plant fail to restrict O_3 flux by adequate stomatal closure **(Biswas et al., 2008a)**, a self-propagated oxidative response will be initiated and will lead to localized or spreading program cell death (PCD), that will be observed as a visible chlorotic or necrotic symptom **(Vaultier and Jolivet, 2015)**. These symptoms will result in reduced photosynthesis, as the effective photosynthetic area will decrease **(Feng et al., 2012; Hassan, 2004; Pleijel et al., 2006)**, and accelerated leaf senescence will occur **(Burkart et al., 2013; Gelang et al., 2000; Ojanpera et al., 1998)**. Visible foliar symptoms might or might not be correlated to biomass and/or yield reductions, depending on the degree of injury, the injured tissue, and the timing of O_3 stress relative to the plant development **(Amundson et al., 1987; Feng et al., 2012; Hassan, 2004; Heagle et al., 2000; Mills et al., 2011; Ojanpera et al., 1998; Singh and Agrawal, 2010; Van Dingenen et al., 2009)**. For example, O_3 injury to leaves in the lower canopy at flowering or later will have less impact on yield than injuries on upper leaves, as lower leaves at the early growth stages contribute less to yield than flag leaf (F) or the second-top leaf (F-1). However, injured

leaves in lower canopy at early stages might have greater impact on plant establishment than later in the season.

Visible symptoms of ozone damage are not always correlated to yield reductions, as some varieties may respond to E-O_3 by significantly reducing stomatal conductance, and consequently, reducing photosynthesis. Therefore, some varieties might be negatively affected by O_3 stress in terms of biomass and yield, despite the absence of visible foliar injury. Therefore, identification of yield responses to O_3 is an important step in selecting parents to breed for high yield under O_3 stress. Visible injury responses are also expected to affect plant's interactions with epiphytic and endophytic microbiome, as O_3 has been found to affects wheat diseases, such as stem rust **(Heagle and Key, 1972)**, leaf rust **(Dohmen, 1987; Heagle, 1975; Pfleeger et al., 1999; Tiedemann, 1992a; Tiedemann et al., 1991; Tiedemann and Firsching, 2000)**, and powdery mildew **(Tiedemann, 1992a)**.

Therefore, visible O_3 injury responses should be taken into account when selecting host plants to be used in studying wheat/rust interactions, and parents selected for breeding future cultivars should be tested under E-O_3. This approach is critical for breeding resilient wheat varieties to be used in developing and heavily populated countries, such as China and India, where O_3 levels are expected to further increase **(Feng et al., 2012; Singh and Agrawal, 2010; Van Dingenen et al., 2009)**. In addition, this approach is also needed in wheat growing areas in the United States, as O_3 concentrations during the wheat growing season are yet to decrease, and remain high enough to impact yield **(Cooper et al., 2014; Lee et al., 2014; Monks et al., 2015; Parrish et al., 2013)**.

In general, wheat is known to be more sensitive to O_3 than other cereals **(Selldén and Pleijel, 1995)**. Oat (*Arena sativa* L) is considered relatively insensitive **(Pleijel et al., 1994)**, However, two spring oat varieties were found to have similar O_3 responses as two spring wheat varieties, and all of them did not appear to be O_3 sensitive **(Hartikainen et al., 2012)**. On the other hand, barley (*Hordeum vulgare* L) is considered an O_3-insensitive crop **(Mills et al., 2007; Selldén and Pleijel, 1995)**. Spring barely were found to be more tolerant than spring

wheat, and the differential response were attributed to the differences in stomatal responses to O_3 treatment, rather than the apoplastic ascorbate **(Kollist et al., 2000)**.

The differential O_3 responses are documented among wheat varieties **(Biswas et al., 2008b)**, indicating the importance of identifying the relative O_3 responses of varieties to be used in breeding programs and/or studying O_3 effects on wheat performance and interactions with other phytobiome components. A meta-analysis of 53 peer-reviewed studies published between 1980 and 2007, concluded that there was no significant response difference between spring wheat and winter wheat **(Feng et al., 2008)**. Another meta-analysis of 42 studies conducted in soil showed no difference between spring and winter wheat in total grain yield response to O_3, however, O_3 injury responses were not considered **(Broberg et al., 2015)**. There are reports from different parts of the world showing increased O_3-sensitivity in modern varieties of both spring **(Barnes et al., 1990; Pleijel et al., 2006; Velissariou et al., 1992)**, and winter wheat **(Biswas et al., 2008a; Biswas et al., 2008b; Biswas et al., 2013)**. However, there is limited information on the difference in O_3 responses between soft and hard red winter wheat cultivars, and the trends of their O_3 response when plotted against the date of their release.

Wheat is a very diverse crop; varieties are visually classified according to the endosperm texture or hardness (soft or hard) and color (white or red), as well as, growth habit (winter or spring) **(McFall and Fowler, 2009)**. Hardness is determined by the strength of binding between the starch granules and the protein network surrounding them. Soft wheat has loose binding between starch and protein matrix, due to lower protein percentage, usually less than 10%, which makes it easy to grind, and gives soft-texture flour, suitable for high dense products baked to low moisture content, such as cookies and crackers. Hard wheat has stronger binding between starch and protein matrix, due to higher protein content (about 12%) which makes it harder to grind, and gives coarse-texture flour, with more broken starch granules with high water uptake and provide more fermentable sugars for yeast, therefore, it is suitable for yeast-leavened bread, such as pan bread **(Thomason et al., 2009)**. In the United States, there

are five main market classes of bread wheat varieties typically grown in the eastern U. S. are soft red winter types.

This study was designed with the following objectives:

- Analyze the ozone monitoring data collected during March to May), from 2006 to 2015, at the Inwood Road field site of the USDA-ARS Plant Research Unit in Raleigh, NC.

- Investigate the relative ozone O_3 response of seedlings of 23 winter wheat genotypes, three varieties of spring wheat, as well as selected winter varieties of oat, barley, and triticale.

- Investigate O_3 sensitivity responses of hard and soft winter wheats, and the relationships between their year of release and O_3 sensitivity.

- Identify varieties having ozone tolerance that could be used in simultaneous breeding for O_3 tolerance and rust resistance.

- Identify O_3-sensitive varieties with different rust reaction types to be used in investigating the effects of O_3 stress on different types of rust resistance in wheat.

4.3. Materials and Methods

4.3.1. O_3-monitoring data

Hourly averages of ambient O_3 concentrations measured during wheat active growing season (March-May) over eight of the last ten years (2006-2008, 2010-2011, and 2013-2015) were provided by Samuel Ray & Walt Pursley, USDA-ARS Plant Research Unite in Raleigh, NC. Ozone hourly averages were summarized as daily daytime 12hr averages.

4.3.2. Experiment (1): Investigation of relative O_3 responses of some genotypes of spring and winter wheat, oat, barley, and triticale at the seedling stage.

Seedlings of 31 genotypes of winter and spring wheat, and winter oat, barley, and triticale genotypes as listed in Table (2), were phenotyped for O_3 symptom response. Seeds of genotypes with winter growth habit were vernalized in darkness for 6 weeks at 4°C by placing seeds between two layers of wet filtration paper in petri dishes. Two spring wheat varieties were vernalized using the same procedure for 10 days. After vernalization, seedlings were transplanted in 1 L pots filled with Fafard #2 Pro Mix (Fafard, Anderson, SC, USA), mixed with 5 g slow release fertilizer (Osmocote Plus, Scotts-Sierra Horticultural Products, Marysville, OH, USA), and grown in greenhouse with charcoal filtered (CF) air. At 21 days after transplanting, uniform plants at growth stage of 21-23 Zadoks were selected and fourth leaf on main stem was tagged.

Plants were moved to continuous stirred-tank reactors (CSTRs) and acclimated in CF-air for three days. After the acclimation, plants were exposed to one of four O_3 treatments (CF, 50, 75, 100 ppb, 8hr d^{-1}), under 60 % RH. After seven days of exposure, percentage of O_3 visible symptoms on the fourth leaf on the main stem were visually estimated using a 0-100% scale, where 0% was no visible damage and 100% was complete chlorosis.

The experimental design was split-plot in randomized complete block, with three blocks, with O_3 in the main plot, and genotype in the sub-plot. Three plants of each genotype were used per chamber, and data were averaged for the statistical analysis, using Proc. Glimmix, in SAS 9.4 (SAS Inc., Cary, NC, USA). Averages were separated using Tukey-Kramer adjustment.

Table (2): Different genotypes of winter and spring wheat, winter barley, oat and triticale and their market classes, accession ID, and date of cultivars release or variety and breeding line selection.

No.	Crop	Growth Habit	Color	Hardness	**Genotype**	Accession ID	Release or Selection Date
1	Wheat	Winter	Red	Hard	Turkey	CItr5757	1873
2					Kharkof	PI05641	1900
3					Scout 66	CItr13996	1967
4					TAM 107	PI495594	1985
5					Wesley	PI605742	1998
6					TAM 303	NIC	2003
7					Overland	PI647959	2007
8					ARS07-1214	NIC	2007
9					Everest	PI659807	2009
10					NuEast	PI657997	2009
11					Vision 30	PI661153	2010
12					Vision 45	PI667642	2012
13				Soft	Mediterranean	CItr3332	1819
14					Pioneer 26R15	PI633874	2003
15					NC-Neuse	PI633037	2003
16					Branson	PI639227	2005
17					Coker 9553	PI643092	2006
18					Jamestown	PI653731	2008
19					Shirley	PI656753	2009
20					USG 3120	NIC	2011
21					MD01W28-08-11	NIC	2011
22					NC-Yadkin	PI663206	2012
23			White	Hard	Appalachian White	PI657998	2009
24		Spring	Red	Hard	Red Fife	PI348919	1842
25			White		PBW 343	NIC	1995
26					Kingbird	NIC	2012
27	Barley	Winter	-	-	Charles	PI 634933	2003
28					Thoroughbred	PI 637845	2005
29	Oat	Winter	-	-	Horizon 201	PI 658000	2007
30					TAMO 411	NIC	2012
31	Triticale	Winter	-	-	Arcia	NIC	2001

4.3.3. Experiment (2): Investigation of relative O_3-yield responses of two winter wheat genotypes with differential visible injury responses during 2015.

Coker-9553 (O_3 moderately-sensitive) and MD01W28-08-11 (O_3 very-tolerant) were selected for validating their differential O_3 responses observed at seedling stage, by investigating their yield responses to post-heading O_3 stress. These two genotypes are parents of a doubled haploid (DH) population currently being phenotyped for novel adult-plant resistance to stem rust including races in the Ug99 lineage. If found to be differentially responding to O_3 in term of yield responses, these two genotypes and their DH population could be used to breed for stem rust and O_3 tolerance simultaneously. Vernalized seedlings were transplanted in 1 L pots filled with Fafard #2 Pro Mix (Fafard, Anderson, SC, USA), mixed with 5 g slow release fertilizer (Osmocote Plus, Scotts-Sierra Horticultural Products, Marysville, OH, USA), and grown in CF-air in greenhouse until heading stage. Plants of the two genotypes were moved to 2.4 m tall × 3 m diameter Open-top field Chambers (OTCs), where they were acclimated for 3 days then grown until harvest, under four O_3 treatments (CF, 50, 75 and 100 ppb, 12 hr. average). At maturity, heads from individual plants were manually harvested and kernels were separated using single-head thrasher.

The experimental design was split-plot in randomized complete block, with three blocks, and O_3 levels as the main plot, and genotype in the sub-plot. Three plants of each genotype were used per chamber, and data were averaged for the statistical analysis that was performed using Proc. Glimmix, in SAS 9.4 (SAS Inc., Cary, NC, USA). Yield averages were separated using Tukey-Kramer adjustment.

4.4. Results

4.4.1. O_3-monitoring data

Ambient O_3 concentrations measured during wheat active growing season (March-May) over eight of the last 10 years (2006-2008, 2010-2011, and 2013-2015) at a typical sub-

urban location in Raleigh NC, USA, indicated that the 12hr average of hourly-average O_3 concentrations were mostly above the threshold of 40 ppb for sensitive plants, as illustrated in Figure (24). This indicated the importance of considering O_3 responses of breeding materials used in this region. The general 12 hr. average over the entire aforementioned period was 43.82 ppb.

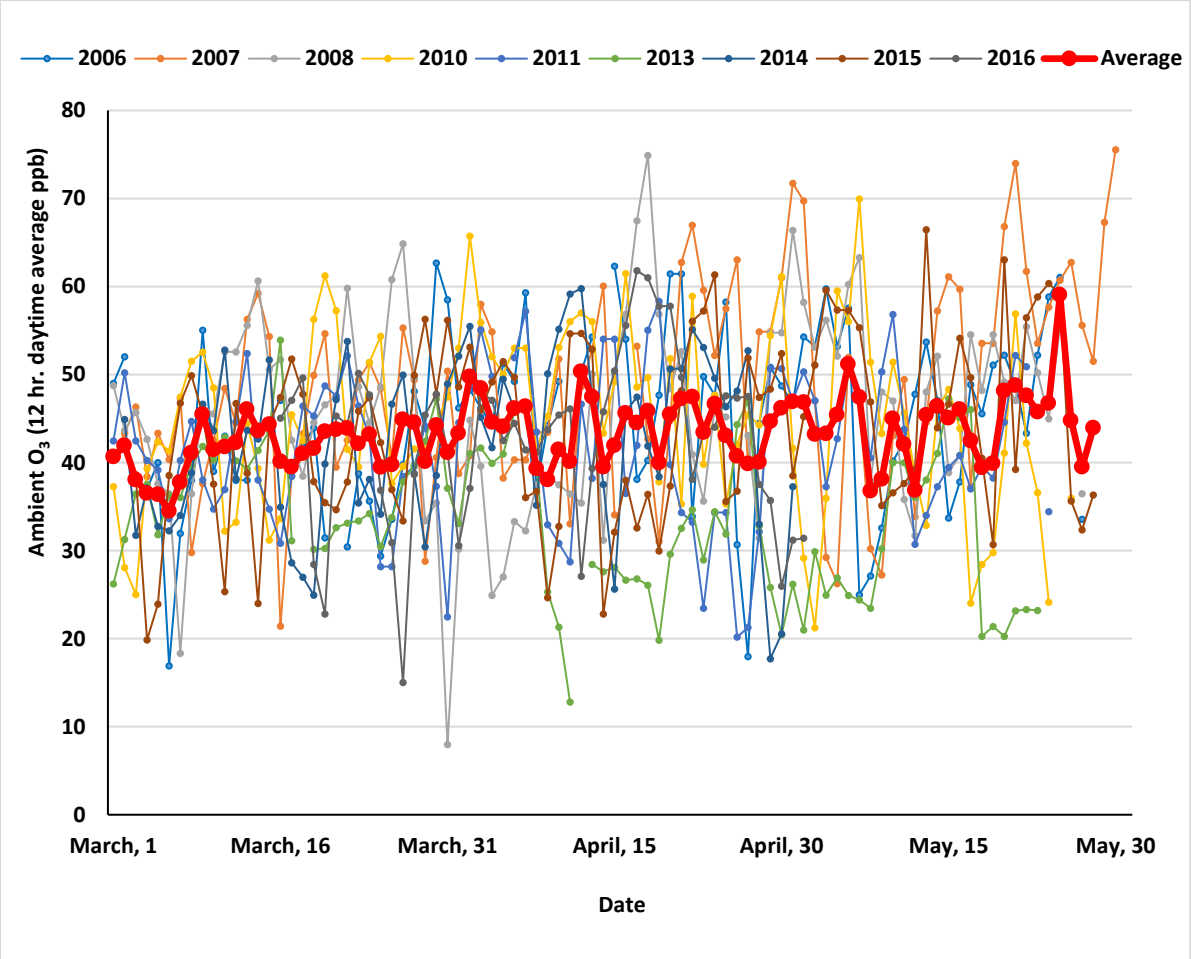


Figure (24): Ozone monitoring data during wheat active growing season (March-May), summarized as 12hr daily average, at wheat open field plots in a sub-urban location, at the USDA-ARS Plant Science Unit field site, 5 km south of Raleigh, NC, U.S.A. Elevation was 110 m above sea level, and provided by Samuel Ray & Walt Pursley.

Monthly and active growing season average O_3 concentrations showed general trends of decline over years that increase in magnitude toward the end of the season as illustrated in Figure (25). For example, the rate of decline in average O_3 concentrations during May is higher than the decline rate during April, which in turn is higher than the decline rate in March.

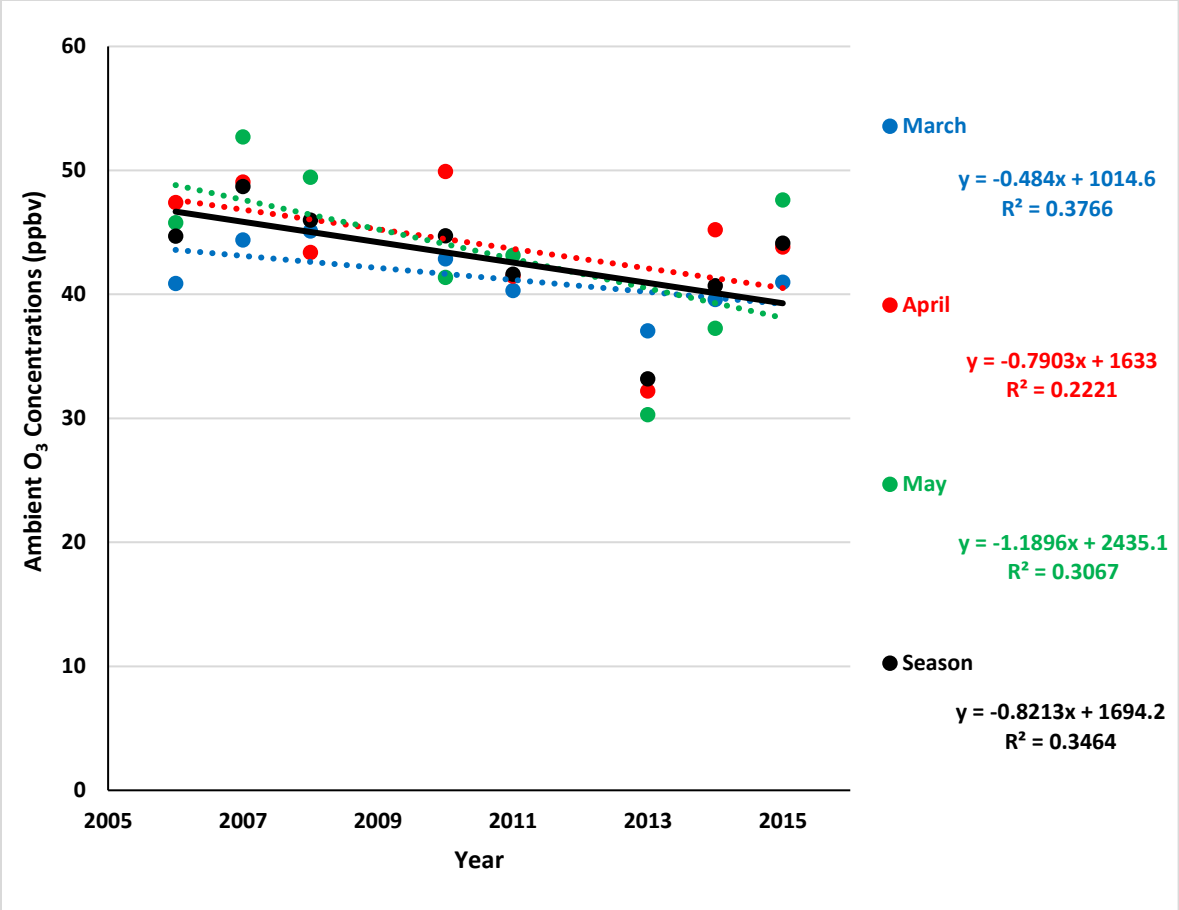


Figure (25): Trends in monthly and active growing average O_3 concentrations monitored at a typical sub-urban location, in Raleigh NC, during wheat active growing season (March-May). Initial data were summarized as 12hr daily average. Data provided by Samuel Ray & Walt Pursley, USDA-ARS, Plant Research Unit, Raleigh, NC, USA.

As illustrated in Figure (26), hourly average O_3 concentrations during the 2015 season when yield tests were performed mostly showed the temporal stochasticity of O_3 concentrations at a given location at low altitude. Cycles fluctuating between different peak and bottom magnitudes were observed as a result of different factors controlling local O_3 concentrations. Overnight high O_3 concentrations for several days and low daily peaks were common, as shown in Figure (27).

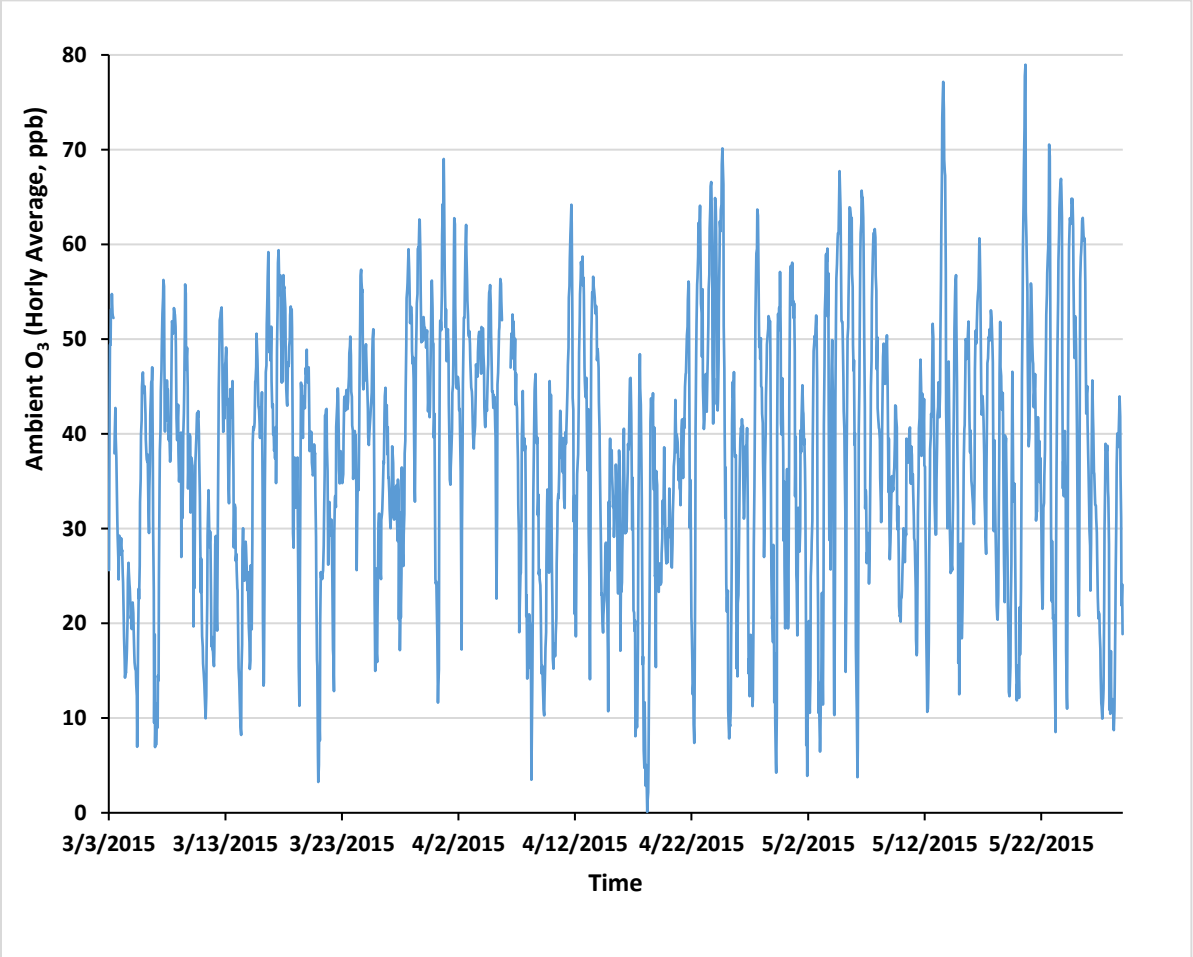


Figure (26): Hourly average O_3 monitoring data during the wheat active growing season (March-May 2015), at the USDA-ARS Plant Science Unit field site, 5 km south of Raleigh, NC, U.S.A. Elevation was 110 m above sea level, and provided by Samuel Ray & Walt Pursley.

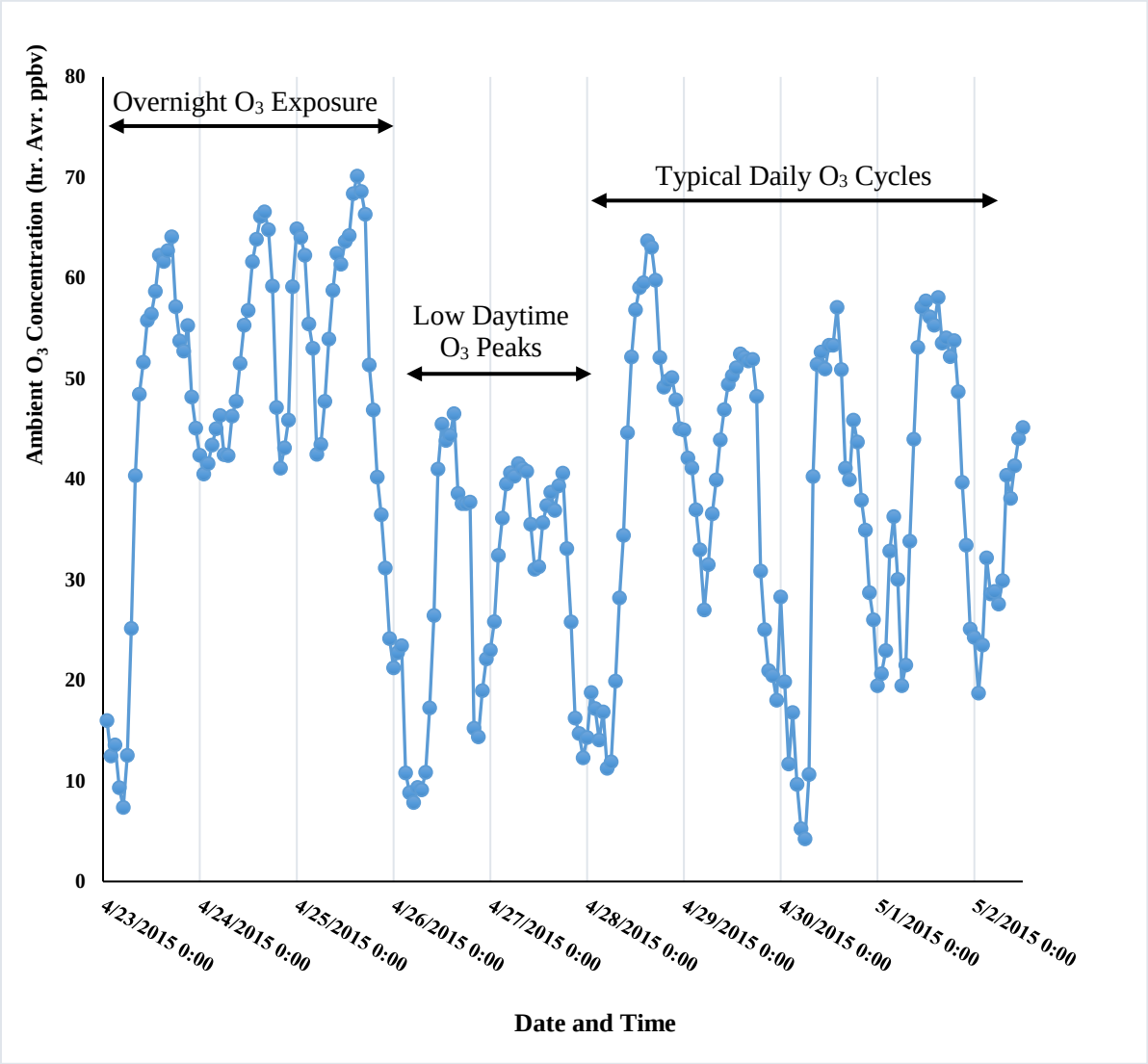


Figure (27): Hourly-average O_3 concentrations at the USDA-ARS OTC field site in a sub-urban location, in Raleigh NC, during wheat grain filling stage (April 23 - May 3, 2015). Data provided by Samuel Ray & Walt Pursley, USDA-ARS, Plant Research Unit, Raleigh, NC, USA.

4.4.2. Results of Experiment (1):

Seedling screening in CSTRs using visible symptoms as response measure indicated that the tested winter wheat varieties were on average less sensitive than the average response of the three tested spring wheat genotypes at 50 and 75 ppb O_3, but all had similar responses at 100 ppb as illustrated in Figure (28). The tested winter wheat and oat varieties had very similar average responses at all O_3 concentrations. The tested variety of triticale was more tolerant than wheat at the higher concentrations. In contrast, barley was shown to be O_3 insensitive relative to the other tested species, especially under high O_3 concentrations. These results confirm that wheat on average is more sensitive than most of the other cereals.

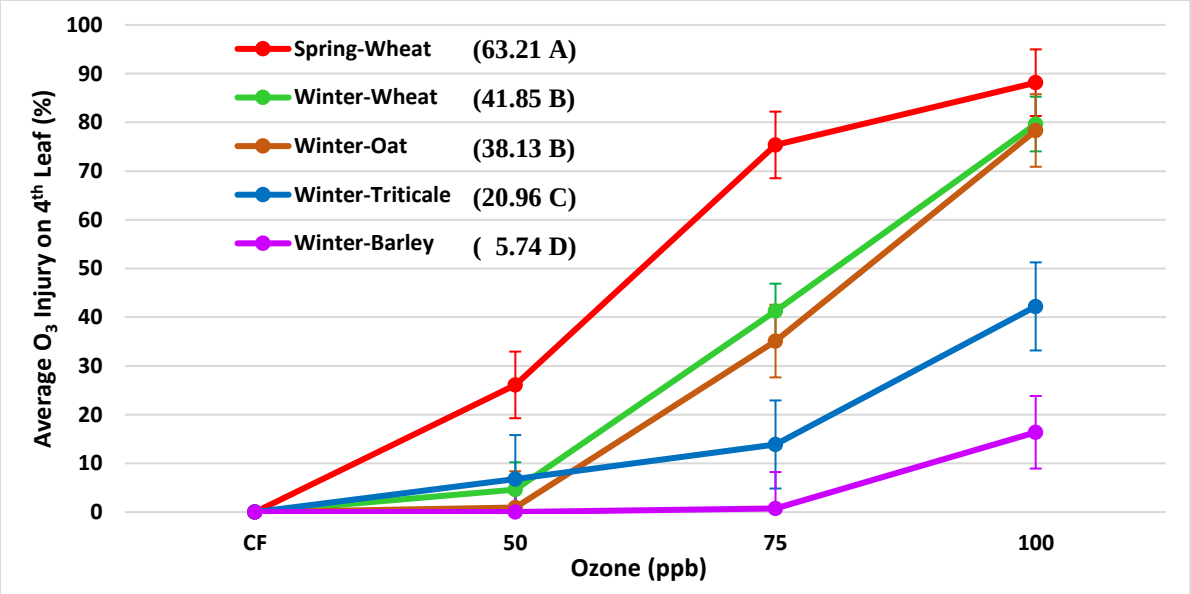


Figure (28): Relative O_3 injury response on the fourth leaf on the main stem of 24 genotypes winter wheat, comparing to tow genotypes of winter wheat oat and barley, and one winter genotype of triticale. Data are averages of visual injury estimated after seven days of exposure to 50, 75, and 100 ppb O_3, for 7hr d^{-1}, in continuous stirred tank reactors (CSTRs), at the seedling stage. (Error bar = SE., $\alpha = 0.05$).

To investigate the differences in average O_3 responses between the hard and soft red classes of winter wheat and their relative response to other cereals tested, the data were reanalyzed with wheat classes included in the group factor, and the results are shown in Figure (29). The main finding is that the tested HRWW varieties were in average more sensitive to O_3 visible symptoms than SRWW. However, HRWW varieties were not as sensitive as the spring wheat varieties.

On the other hand, the tested SRWW were intermediate in their average response between oat and triticale. There were significant differences between varieties within each of the two classes of RWW, and even larger differences between the most sensitive varieties (mainly HRWW) and the most tolerant ones (mainly SRWW). The three spring wheat varieties tested also exhibited significant different, whereas the oat and barley verities did not show significant differences in response within species.

Another interesting finding is that the SRWW breeding line MD01W28-08-11 was O_3 insensitive under the O_3 treatments used in this study. This line also has adult-plant resistance to stem rust races in including the Ug99-lineage. Kingbird is also resistant to rust diseases at adult-plant stage, but was one of the most sensitive genotypes.

To investigate the effects of breeding on O_3 responses in red winter wheat, the average O_3 responses were plotted against the genotype selection or release date, as illustrated in Figure (30). Considering the entire set of genotypes tested, surprisingly, the trend in visible injury is negative, which means that newer varieties tends to show less injury than older ones. This trend seems to be derived only by HRWW. This is because SRWW almost showed no trend; however, this lack of trend might have been greatly affected by the low sensitivity of the only old variety (Mediterranean) released in 1819.

Because of the higher number of genotypes selected or released after the year 2000, we considered the trend in this period separately, as illustrated in Figure (31). Both classes of red wheat showed stronger negative correlation. The SRWW had larger slope than HRWW. This might also has been affected by the tolerance of the breeding line MD01W28-08-11.

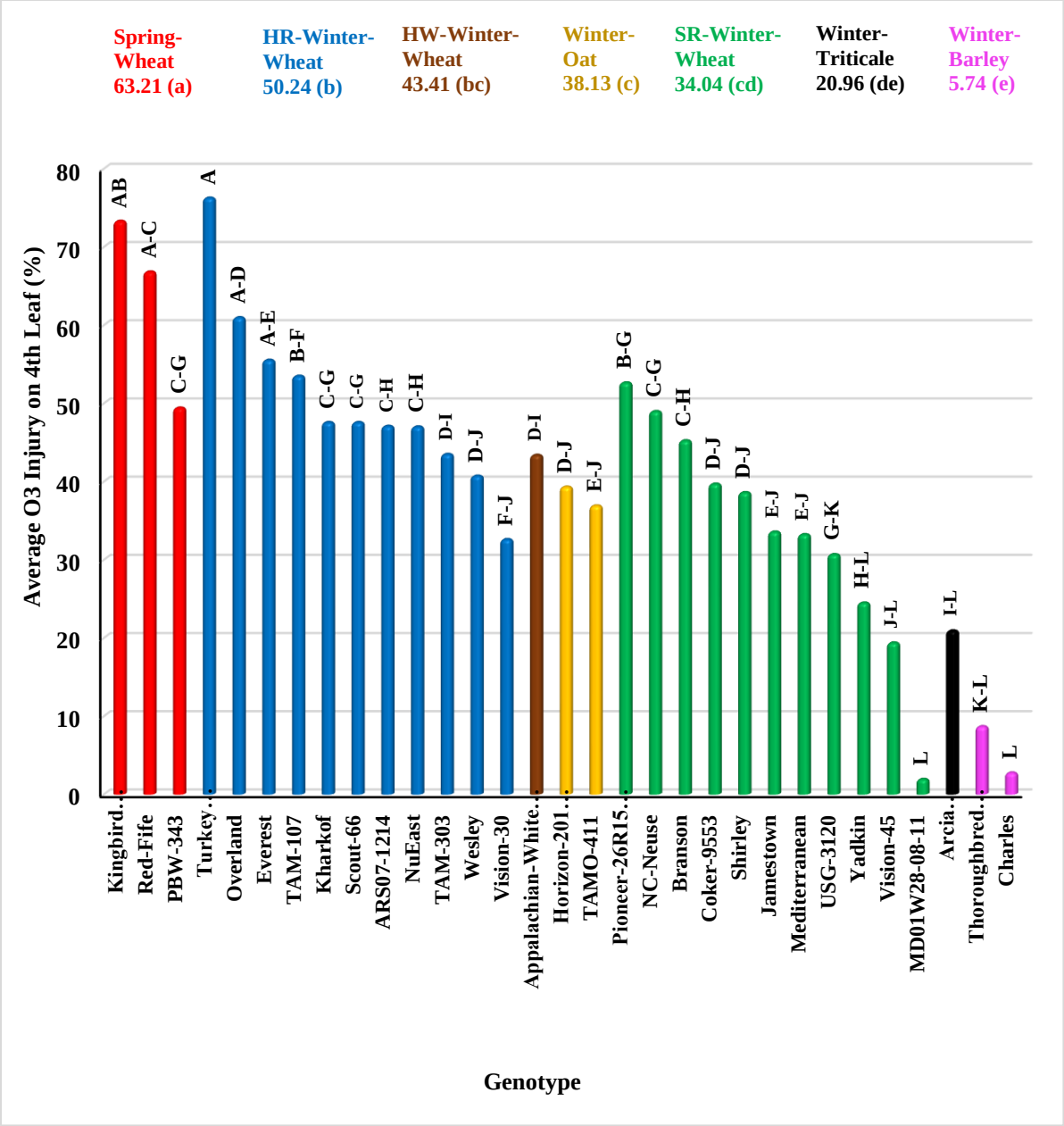


Figure (29): O_3 injury response on the fourth leaf on the main stem of 23 genotypes of winter wheat, comparing to three genotypes of spring wheat, two genotypes of winter oat and barley, and one winter genotype of triticale. Data are averages of visual injury estimated after seven days of exposure across three O_3 concentrations (50, 75, and 100 ppb, for 8hr d^{-1}), in continuous stirred tank reactors (CSTRs), at seedling stage. (Different letters indicate significant differences according to Tukey's adjustment at α=0.05).

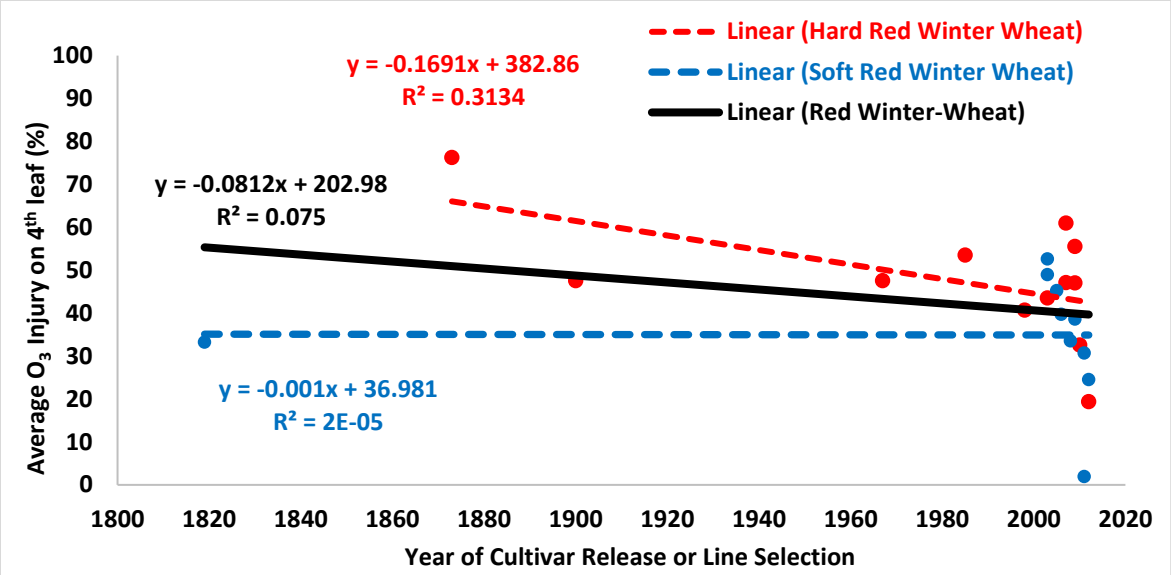


Figure (30): Trends in average O_3 visible responses visually estimated on the fourth leaf on the main stem of some soft and hard red winter wheat varieties, released or selected in the United States over the last two centuries. The black line represents the slope of all data points.

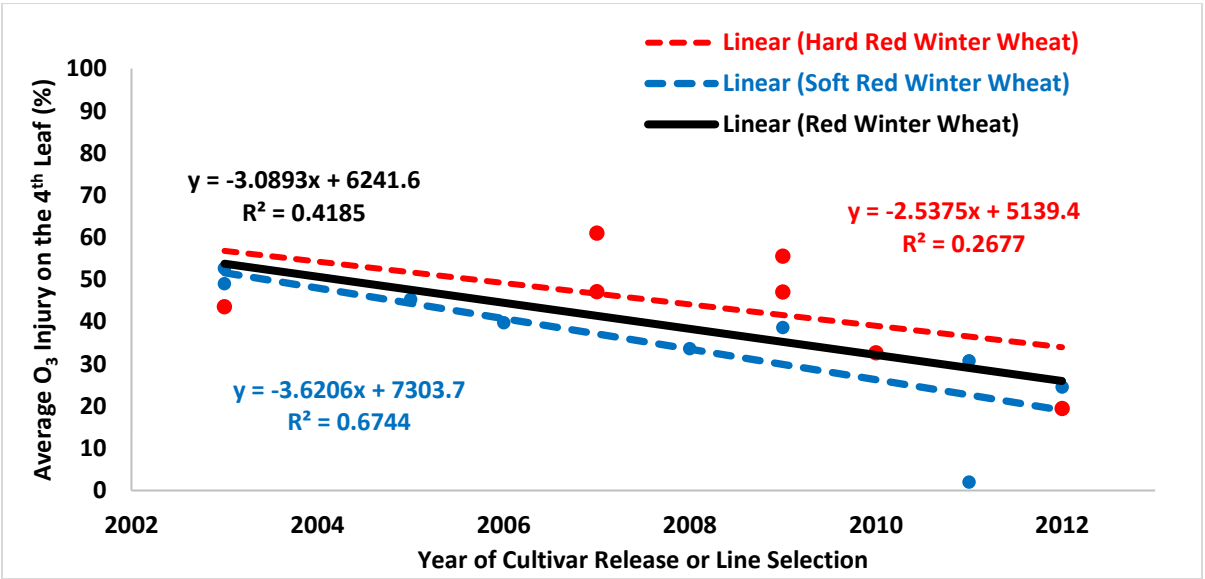


Figure (31): Trends in average O_3 visible responses visually estimated on the fourth leaf on the main stem of some soft and hard red winter wheat varieties, released or selected in the United States since the year 2000. The black line represents the slope of all data points.

4.4.3. Results of Experiment (2):

Based upon the visible injury results, two parents of a Doubled Haploid (DH) population were found to be significantly different. These two genotypes are Coker-9553 (O_3-moderately sensitive, stem rust-susceptible) and MD01W28-08-11 (O_3-toleant, adult-plant resistant to stem rust). As illustrated in Figure (32), the total seed yield results confirmed the differential responses between the two genotypes. Surprisingly, MD01W28-08-11 showed significant yield increase under near ambient (50 ppb) and moderately high (75 ppb) O_3 concentrations relative to the CF control, without any yield loss under the highest O_3 concentration tested. On the other hand, Coker-9553 showed gradual yield reductions that become significant in the 100 ppb O_3 treatment.

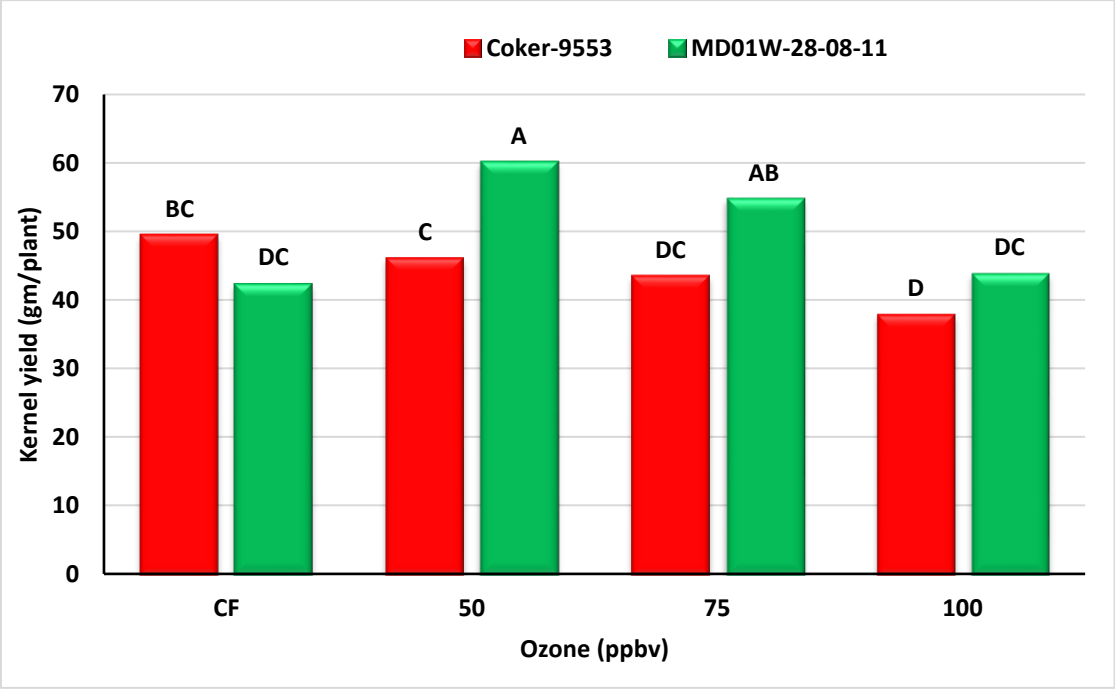


Figure (32): O_3-yield responses of two soft red winter wheat genotypes, exposed from heading to harvest, to different O_3 treatments (CF, 50, 75 and 100 ppb, 12hr average O_3, diurnal profile), in Open-top field Chambers (OTCs).

4.5. Discussion

Breeding hard red winter wheat varieties that are adapted to the humid rain-fed climate in the eastern United States is a major objective to wheat-breeders in this area, as it will benefit wheat growers, millers, and consumers **(Sethi, 2015)**. Breeders are also incorporating stem rust resistance that still effective against the rapidly evolving races in the Ug99-lineage, especially adult-plant resistance, as it is more durable than single genes **(Singh et al., 2011)**. O_3 concentration trends over the entire region indicated that O_3 levels are increasing during winter and are not declining during spring **(Cooper et al., 2014; Lee et al., 2014; Monks et al., 2015; Parrish et al., 2013)**, this will likely result in more O_3 stress during future wheat growing seasons. In addition, incorporation of O_3 tolerance to breeding programs is essential for avoiding the inadvertent selection for O_3 sensitivity that lead to O_3-sensitive modern wheat varieties in many parts of the world **(Biswas et al., 2008b; Biswas et al., 2013; Pleijel et al., 2006)**.

One of the objectives of this study was to validate the reported regional trends **(Cooper et al., 2014; Lee et al., 2014; Monks et al., 2015; Parrish et al., 2013)**, using local O_3 monitoring data collected in a sub-urban location in Raleigh NC. The results showed that spring 12hr daytime O_3 concentrations at the monitored site are declining, and the magnitude of decline rate increases from March to May. However, the general average concentration over the entire period is still above the threshold of 40 ppb. In addition, daytime peaks were significantly above this threshold, all throughout the season, and lasted in some cases for several consecutive days. Therefore, it is very likely that wheat plants were experiencing negative O_3 effects, especially during the grain filling stage late in the season. Consequently, we might conclude that it is important for breeding programs in the eastern US to consider the O_3 responses of the breeding material being used.

Another objective of this study was to investigate the relative O_3 responses of red winter wheat in relation to some other wheat classes and cereal crops. The results suggested that winter wheat might be - on average - more tolerant to O_3 than spring wheat in terms of O_3

visible symptoms. However, a meta-analysis of the yield responses reported in 53 studies (1980 and 2007) concluded that yield responses of the two categories of growth habit in response to O_3 were similar **(Feng et al., 2008)**. These authors came into this conclusion despite the fact that winter wheat showed these similar responses at O_3 concentrations that were 20% higher comparing to the O_3 concentrations causing similar responses by spring wheat. This difference in the concentrations used, and the fact that winter wheat did not exhibit higher yield reductions are clear indications that winter wheat is more tolerant to O_3 than spring wheat. Meanwhile, spring wheat was more sensitive to O_3 effects on chlorophyll content, which might be an indicator for higher visible symptoms, which was not reported in the comparison in the meta-analysis. The significant O_3-induced stomatal closure and chlorophyll content of spring wheat are indicators of an over-reaction to O_3 stress by restricting gas exchange restricting CO_2, which might have also resulted in lower photosynthesis and reduced chlorophyll maintenance.

The development of visible O_3 symptoms on plant leaves is important in determining plant interactions with the epiphytic and endophytic microbiome and were found to affect wheat interactions with plant pathogens such as wheat rusts, and powdery mildew. On the other hand, the absence of visible symptoms might result from significant reduction in stomatal conductance that restricts photosynthesis and consequently, reduce biomass and seed yields. Therefore, O_3 tolerance in terms of foliar injury should be validated using yield data, and this step is a necessary to avoid any inadvertent selection for varieties with O_3-sensitive yield. The observed differences between and within SRWW and HRWW, suggests the suitability of simultaneous breeding for hardness and O_3-tolerance. The SRWW could be used as a source of O_3 tolerance when crossed with HRWW.

The diversity in rust resistance and O_3 tolerance within the genotypes tested provide suitable hosts for rust/O_3 studies. The host's response to O_3 determines the magnitude of rust/O_3 interactions within the host's tissue. Conclusions drawn from studies using O_3 tolerant varieties may lead to conclusions that do not apply to rust/O_3 interactions on sensitive hosts and vice versa. Host plants with unknown O_3 responses are not suitable for studying rust/O_3 interactions for several reasons: First, it difficult to select O_3 treatments to be tested without

knowing the relative ozone response of the host, especially if the objective of the study is to investigate the effects of sub-symptomatic O_3 treatment. This might explain the lack of studies investigating the impact of near-ambient, near-threshold, sub-symptomatic, and sub-symptomatic O_3 stress on diseases. Second, the interpretation of any effects on the disease must take into account the environmental effects on the host. For example, conclusions drown using symptomatic reactions (high O_3 stress and sensitive host) do not apply on sub-symptomatic reactions (low O_3 stress and a tolerant host).

A major difference between spring and winter wheat is their vernalization requirement, which could be considered as a cold stress. Effective ROS control is essential for plant's ability to cope with different sorts of stresses including biotic and a biotic stress. It might be postulated that vernalization might have a priming effect on winter wheat plants against O_3 visible symptoms. However, there is limited information to verify such hypothesis. Results presented here indicate that triticale seems to be more tolerant to O_3 than wheat, and it is was found also to be more winter hardy and freezing tolerant than wheat **(Rapacz et al., 2015)**. Studying the effects of vernalization on winter wheat responses to O_3 might be a suitable approach to test this hypothesis.

The observed negative trends in O_3 injury responses of red winter wheat might be not apply to all red winter wheat, as the number of genotypes used in this study in not large, as well as the range of release date. However, this negative trend might a sign of an inadvertent selection for non-symptomatic plants in general, which might have resulted in selecting against sensitive varieties in terms of visible symptom responses. These negative trends in foliar injury contradicts several reports showing increasing trends of yield loss of modern varieties of both spring **(Barnes et al., 1990; Pleijel et al., 2006; Velissariou et al., 1992)**, and winter wheat **(Biswas et al., 2008a; Biswas et al., 2008b; Biswas et al., 2013)**.

Differential yield responses are essential for yield genetic gain under O_3 stress. The contrasting yield and visible symptoms responses between Coker-9553 and MD01W28-08-11, make them a suitable parent-pair for breeding O_3 tolerant SRWW. Observing yield differences

between and MD01W28-08-11 and a variety in the in the lower half of the sensitivity range like Coker-9553 indicates a high likelihood for finding stronger differential yield responses with genotypes in the upper half of the sensitivity range. Consequently, we could conclude that MD01W28-08-11 could be used as a source of resistance in simultaneous breeding for high kernel hardness and O_3 tolerance in eastern US. Screening the DH population from the cross between Coker-9553 and MD01W28-08-11 for both rust resistance and O_3 tolerance are first steps towards identifying the quantitative trait loci QTL(s), and genetic markers associated with both traits. However, the population genotypic data is required for such analysis.

The significant yield increase of MD01W28-08-11 at 50 and 70 ppb O_3 is an indication of O_3 hormesis on tolerant wheat varieties. The elucidation of the mechanisms of such yield increase could advance our understanding of the effects of low O_3 concentrations.

This study investigated a subset of each of the tested cereal crops and wheat classes. Therefore, it provides additional information on the relative responses of the tested material, which may not apply to the entire species or wheat classes. Screening wheat-breeding materials used for a specific objective is the best practice to ensure a high potential for adding O_3-tolerance genetic gain.

5. Dissecting the Effects of O_3 and CO_2 Concentration, Exposure Timing and Duration on the Disease Components of Stem and Leaf Rusts on Winter Wheat

5.1. Abstract

The resurgence of rust diseases and the elevated O_3 levels are major limiting factors to global wheat production and food security. This research is addressing the lack of information on the effects of current elevated levels of O_3 and/or CO_2 on disease components of leaf and stem rust diseases. The results of four different experiments presented here contradict the current consensus that elevated O_3 is suppressive to wheat rusts, and provide evidence that sub-symptomatic O_3 treatments are conducive to some biotrophic pathogens.

The first experiment aimed at determining the effects of four combinations of CO_2 (400, 570 ppm) and O_3 (10, 50 ppb) on leaf rust on four winter wheat varieties ('Coker 9553', 'NC Neuse', 'Jamestown' and 'NuEast') known to differ in their response to leaf rust (race MBTNB). Plants were exposed in OPECs, inoculated at GS 39-40 Zadoks, and disease severity and components were estimated on the flag leaf and F-1. Results showed that elevated O_3 (singly or combined with elevated CO_2) increased disease severity and pustule size, and the increase was more pronounced on susceptible varieties. CO_2 treatment had no significant effect on disease severity. The second experiment investigated the effects of four combinations of CO_2 (400, 570 ppm) and O_3 (0, 50 ppb) on stem rust (race QFCSC) on Coker 9553 and NC Neuse. The results of percent sporulation area assessed at 21DAI, using image analysis software Assess 2.2 showed that O_3 (singly and in combination) increased disease severity of stem rust on susceptible wheat variety 'Coker 9553', and gas treatments did not alter the disease response of the resistant variety 'NC Neuse'.

The third experiment aimed at confirming the differential effects of different O_3 concentrations (CF, 50, 70, and 90 ppb) on stem rust on Coker 9553. Seedlings at the 21-22 Zadoks stage were exposed in OPECs for six weeks. Plants were inoculated after the third week and disease was assessed using Assess 2.2 at 7, 14, and 21 DAI. Treatment with 50 ppb significantly increased percent sporulation on the two uppermost leaves, whereas the higher

concentrations showed no significant difference from CF, however, there were significant difference between the two leaf positions within each of the two treatments, with more disease on the younger leaves. The fourth experiment investigated the effects of four different combinations of CO_2 (400, 570 ppm) and O_3 (0, 50 ppb), and seven combinations of exposure timing and duration on the disease severity of stem rust on Coker 9553. Plants were exposed at 21-22 Zadoks for six weeks, inoculated after the third week of exposure, and present sporulation was assessed 21 DAI. Results showed that only continuous O_3 treatment for six weeks and O_3 exposure for three weeks prior to the inoculation (long predisposition), significantly increased disease relative to the CF control, which suggested a predisposition effect, rather than a post-inoculation disease enhancement.

In conclusion, a consistent disease increase of both leaf and stem rust of wheat were evident under near-ambient but not under relatively high O_3 concentration. The O_3-induced disease increase was more pronounced with the increased susceptibility to the pathogen, as reflected in larger pustule size, and accelerated pustule formation on susceptible varieties only, which are likely to enhance rust epidemics.

5.2. Introduction

Global wheat production and food security are threatened by elevated ground level ozone (O_3) concentrations and the resurgence of rust diseases. Wheat is the most sensitive major agricultural crop to O_3 concentrations resulting from manmade air pollution **(Booker et al., 2009)**. Current O_3 concentrations are estimated to cause approximately 10% global average yield loss, which is expected to increase by additional 20% under future concentrations **(Feng and Kobayashi, 2009; Van Dingenen et al., 2009)**.

O_3 is one of nature's most powerful oxidizers; upon diffusion through stomata, the extremely unstable O_3 molecules immediately dissolve into the apoplastic sap, generating reactive oxygen species (ROS) burst, attacking molecules in the apoplastic sap, cell wall and membranes **(Vaultier and Jolivet, 2015)**. Type and magnitude of resulting O_3 effects depend on the dose, the antioxidant capacity of the tissue, as well as the timely activation of O_3 tolerance mechanisms at proper magnitude **(Liu et al., 2015b)**. Consequently, O_3 effects on plants are very diverse, and may be expressed differently on different parts of the plant canopy. This is expected to cause dose dependent effects on wheat plants' performance, and accordingly affect their interactions with their phytobiome, including pathogens.

O_3 effects on host/pathogen interactions vary depending on the timing of O_3 exposure and the pathogen infection, the O_3 concentration, the growth stage of host plant, other priming or predisposing environmental factors, such as elevated CO_2, temperature, drought and water vapor pressure deficit **(Fuhrer, 2003)**. Because of O_3 dose-dependent phytotoxicity, and the difference in sensitivity between and within plant species, four O_3 treatment ranges could be proposed, which would defer from one species to another, and from one genotype to another, and even from one tissue to another or one canopy part to another or one growth stage to another within the same genotype:

(1) Non-detrimental O_3 treatment, which is the exposure to low O_3 concentrations that could be scavenged by the host's antioxidant machinery without causing significant negative effects. In fact, some tolerant wheat genotypes (e.g. MD01W28-08-11) showed yield

increase in response to low O_3 treatments **(Mashaheet et al., 2016a)**. This phenomenon is also known as Hormesis, which refers to the beneficial effects (such as improved performance, stress tolerance, growth or yield) as a result of the exposure to low doses of a stressor that is otherwise detrimental or lethal when applied at higher doses.

(2) Sub-symptomatic O_3 treatment, which is the exposure to a moderately low O_3 stress (right below the visible foliar injury threshold), and would result in significant increase in antioxidant activities that successfully detoxify O_3 and prevent visible symptom development, at the cost of some metabolic energy. The plant would suffer some temporarily impairment in function and structure, that will be repaired without leaving visible symptoms. This might also require some stomatal conductance adaptation, and might result in reduced photosynthesis.

(3) Chronic symptomatic O_3 treatments, which is the exposure to moderately high O_3 stress (at or right above the visible injury threshold), for extended periods, resulting in gradual exhaustion to the antioxidant machinery and plant resources depletion. As a result, the plant gradually develop O_3 visible symptoms, such as leaf chlorosis and accelerated senescence.

(4) Acute symptomatic O_3 treatments, which result from significantly high O_3 stress (significantly higher than the visible injury threshold). The high O_3 flux under this type of exposure exceeds the capacity of the antioxidant scavenging mechanisms, and results in significant non-biogenetic ROS burst; followed by a biogenic ROS burst and programed cell death. The results of this type of stress may initially appear as water soaked areas from cellular collapse that dry out and turn into necrotic symptoms.

Several reviews summarized the effects of climate change on plant diseases **(Chakraborty et al., 2008; Chappelka and Grulke, 2016; Eastburn et al., 2011; Fuhrer, 2003; Garrett et al., 2006; Ghini et al., 2008; Heagle, 1982; Luck et al., 2011; Manning and v. Tiedemann, 1995; Newton et al., 2011; Pangga et al., 2011; Pautasso et al., 2012; Scherm and Coakley, 2003; West et al., 2012; Yanez-Lopez et al., 2012)**. One review

focused on wheat diseases **(Juroszek and von Tiedemann, 2013)**, and one report focused on obligate parasites in cereals **(Heagle, 1975)**. Others reports highlighted the climate change effects on rust diseases **(Chakraborty et al., 2011; Helfer, 2014)**. The consensus is that obligate (biotrophic) parasites are expected to be generally inhibited by elevated O_3, as they need a healthy host to grow. On the other hand, infection by facultative (necrotrophic) pathogens are expected to increase, decrease or remain unchanged depending on the dose, as necrotrophs kill plant tissue to feed on the dead material **(Pfleeger et al., 1999)**.

Wheat rusts are major wheat diseases caused by three fungal pathogens: Stem (Black) rust (Sr) is caused *Puccinia graminis* f. sp. *tritici* (*Pgt*); Leaf (Brown) rust (Lr) is caused by *Puccinia triticina* Eriks. (*Pt*); Stripe (Yellow) rust (Yr) is caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*) **(Morgounov et al., 2012)**. These fungi are obligate parasites; hence, they are biotrophic and require living host to grow on. It was assumed that O_3-induced negative effects on the host would negatively affect rust diseases **(Manning and v. Tiedemann, 1995; Pfleeger et al., 1999)**. In contrast, necrotrophic parasites feeding on dead tissue are favored by injured host tissue **(Manning and v. Tiedemann, 1995)**. This hypothesis might be valid only under symptomatic O_3 stress and acute exposure but not under lesser O_3 effects, where the weakened non-injured host might be more suitable source of nutrients and exhibits less efficient resistance, which would be easier for the pathogen to overcome. In addition, O_3 effects on a particular disease depend on the timing of exposure in relation to: the host's growth stage and physiological status of the infected tissue, the pathogen's infection, and/or the particular host/pathogen combination **(Pfleeger et al., 1999)**. The complex interactions between these factors might explain the discrepancy of O_3/rust interaction outcomes reported, summarized in ***Table (***Table (3).

Table (3): Reports on the interactions of elevated O_3, CO_2 and rust pathogens on wheat plants.

Disease	Host Growth Stage & Sampled Tissue	Host's O_3 response & Visible Symptoms	Host/rust reaction type	Disease Stage	O_3 and CO_2 Treatments, Exposure system	Effects of Gas Treatments on Rust Diseases	Suggested Mechanism	Reference
Stem rust	7 days old seedlings Attached leaves	n/a Presumably Sensitive both injured & non-injured tissue	Presumably Susceptible	For 6h (0, 24, or 48 h) before inoculation	O_3 (60, 120, 240 ppb) In Closed Chambers	- Pre-inoculation exposure had no effect on spore germination. - Immediate exposure before inoculation had no effect penetrations or infection. - Exposure 24 and 48h before inoculation to 240 ppb reduced penetration and infection.	- - - Indirectly via injured tissue	(Heagle and Key, 1973a)
				For 4 days right after inoculation	(60, 120, 240 ppb, 6h d^{-1}) In Closed Chambers	- Post-inoculation exposure had no effect on germination and penetration and invasion for 4 days post inoculation with non-stressed spores on healthy tissue. - Negative effects on rust fungal growth and spore production on injured tissue.	- -	
				For 18 days after inoculation	(60, 120, 180 ppb, 6h d^{-1}) In Closed Chambers	- Reduced spore production. -No O_3 effect on germination and infections by spores formed under O_3-stress.	- Indirectly, via injured host tissue	
Stem rust	7 days old seedlings Attached leaves	Both injured and non-injured	Presumably Susceptible	17h after inoculation (during infection)	O_3 (240 ppb, 6h) In Closed Chambers	- O_3 had no effect on germination penetration in any treatment used. - Stem rust germination and appressoria protected host tissue from O_3 injury at site of infection.	- Rust produces diffusible element protecting tissue from O_3 injury.	(Heagle and Key, 1973b)
				After 1-4h during spore germination	(300 ppb, 5h) In Closed Chambers	- Rust-protection against O_3 injury starts 1h after incubation, even without appressoria.	- Protection is not because of restricted O_3 flux, as it does not require appressoria.	
				For 5h during spore germination	(300 ppb, 5h) In Closed Chambers	- Rust-protection from O_3 injury is confined to infection site only, and does not spread to surrounding tissue.		

Table (4): Continued.

Disease	Host Growth Stage & Sampled Tissue	Host's O_3 response & Visible Symptoms	Host/rust reaction type	Disease Stage	O_3 and CO_2 Treatments, Exposure system	Effects of Gas Treatments on Rust Diseases	Suggested Mechanism	Reference
Leaf rust	n/a (Young plants, 15-20 cm)	No visible symptoms	Presumably Susceptible	Pre-inoculation	O_3 (85 ppb 3.5d, 7h d^{-1}) & (105 ppb 7d, 7h d^{-1})* In glass chambers in a closed room	Significant reduction of infection frequency (receptivity)	Several speculations include structural, metabolic changes.	(Dohmen, 1987)
Leaf rust	20-29, 30-39, 50-59 and 60-65 Detached segments of the two upper leaves	n/a Presumably sensitive (Spring wheat cv. Tubro), No injury (Sub-symptomatic tissue)	Presumably Susceptible	For 5 days (7 h d^{-1}) before inoculation	O_3 (about 40, 80, 120 ppb)* In Climate Chambers	40 ppb increased disease severity, with increased growth stages 80 and 120 ppb reduced disease severity on younger plants (GS 29 and 51), and increased disease severity on older plants -Disease severity, pustule density (receptivity), and spore production effects all were in agreement - O_3 treatment had no effect on latent period	No disease-enhancement mechanism was discussed. Senescence-like disease inhibition under high O_3	(Tiedeman n, 1992a)
Leaf rust	Started at one month after planting till maturity	n/a, (suspected tolerant and sensitive, but they were found to be similar in sensitivity) Yes, visible injury	Presumably Susceptible	Before inoculation and after penetration Inoculation started at flag leaf stage, repeated weekly	O_3 (0, 90 ppb) In OTCs	Little evidence that ozone exposure reduced the severity of the diseased as measured by area under disease progress curve. Smaller pustule size under O_3 treatment Lack of synergism between O_3 and leaf rust on all host parameters.	-The absence of exposure during germination and penetration. -The lack of direct interaction between O_3 and the pathogen. - Injured tissue might explain reduced severity.	(Pfleeger et al., 1999)

Table (4): Continued.

Disease	Host Growth Stage & Sampled Tissue	Host's O_3 response & Visible Symptoms	Host/rust reaction type	Disease Stage	O_3 and CO_2 Treatments, Exposure system	Effects of Gas Treatments on Rust Diseases	Suggested Mechanism	Reference
Leaf rust	From planting till maturity	n/a Presumably sensitive (Spring wheat cv. Tubro) Yes, visible injury	Presumably Susceptible	Thought the entire season (both gases), accept for 2 days after inoculation Inoculation at tillering (GS 21-25), and repeated at GS 37-39 and GS 51	O_3 (20, 80 ppb) CO_2 (370-400, 620-650 ppm) O_3 + CO_2 In closed chambers	-Rust accelerated O_3 injury development by 14 days -O_3 had no effect on germination, penetration and infection structures - reduced disease severity, and spore production - Increased latent period - CO_2 slightly increased disease severity only on lower canopy -When combined, O_3 effects on disease were more pronounced than CO_2	-O_3 injury and early senescence restricted the host tissue available for the pathogen -CO_2 increased carbohydrates -CO_2 delayed O_3 symptoms by 14 days, but did not prevent them	(Tiedemann and Firsching, 1998; Tiedemann and Firsching, 2000)
Stem rust Leaf rust Stripe rust	n/a	n/a	Presumably Susceptible	From inoculation through Latent period	CO_2 (+0.03, 0.15, 0.3, 0.75, 1.5, 4.5 and 6 times ambient, In Glass cuvettes in the greenhouse	No effect on germination, penetration, or infection Accelerated sporulation (0.15-0.75 %)	Increased carbohydrates	Gassner and Straib (1930), in German, reported by (Manning and v. Tiedemann, 1995)
Stem rust Leaf rust	Presumably adult-plants	n/a	Susceptible Resistant	Before and throughout the Entire cycle	CO_2 (750, 390 ppm) In phytotron	Increased disease severity of both diseases on susceptibles, and no effect on resistant	Increased carbohydrates	(Bencze et al., 2013)

*, assuming 1 ppb=2 µg m^{-3}

Previous studies investigating O_3 effects on wheat rusts were conducted mainly under relatively high O_3 concentrations. Studies focusing on near-ambient or near-threshold concentrations are lacking. Yearly average baseline O_3 at rural or remote sites of the northern hemisphere ranges from 29 to 49 ppb **(Cooper et al., 2014)**. O_3 concentrations increase from this baseline at sea level with the increased elevation to range from 37 to 59 ppb **(Cooper et al., 2014)**. Although higher concentrations might be experienced in heavily polluted areas, O_3 concentrations in the range of 29-59 ppb might be more relevant to wheat growing areas and season.

The most recent reported trends of O_3 concentration indicate significant improvement in peak concentrations, stabilization or slight increase in background over large areas of the USA and Europe, and significant increase in China **(Cooper et al., 2014; Lee et al., 2014; Monks et al., 2015; Parrish et al., 2013)**. Therefore, O_3 continues to be a significant stress during wheat growing season. However, most of the O_3 treatments experimentally investigated (60-300 ppb) are relatively high for the wheat growing season and are expected to be in the range of chronically symptomatic to acute symptomatic treatments for such a sensitive crop, as confirmed by the symptomatic effects reported on the host and provide as an explanation for disease decrease Table (3).

Studies in the range of 29-59 ppb are lacking. Only one study investigated the effect of 40 ppb treatment **(Tiedemann, 1992a)**. In this study, five days of exposure to 40 ppb O_3 for seven hours a day, prior to the inoculation did not cause O_3-induced foliar injury and increased disease severity of leaf rust at all growth stages. Even the higher concentrations (80 and 120 ppb, 5h d^{-1}, for 5 days) increased disease severity in the absence of host injury. However, this study was conducted using detached leaf segments on Benzimidazole-agar. These results were not confirmed by other studies using attached leaves. Studies investigating the effects of O_3 treatments in the sub-symptomatic range (46.4-55.5 ppb) showed 3-5 fold increase in disease severity of poplar leaf rust **(Karnosky et al., 1999; Percy et al., 2002)**. This was attributed to increased leaf witting and, and decreased phenolic glycoside concentrations under elevated O_3, which were not observed under enhanced CO_2. The results of these three studies are the basis

for the hypothesis that continuous treatment with sub-symptomatic O_3 treatments using near ambient or near threshold concentrations (e.g. 50 ppb) would weaken the host plant and enhance rust diseases, whereas relatively-high O_3 treatment would restrict the pathogen by reducing the viable host tissue available to the pathogen. The mechanism of such effect might be either pre-inoculation predisposition and/or post-inoculation disease enhancement.

Elevated CO_2 was found to increase leaf and stem rusts only on susceptible hosts, by increasing the carbohydrate content in the host tissue **(Bencze et al., 2013)**. By the same mechanism, CO_2 reduced the effects of relatively high O_3 concentrations on leaf rust, when the two elevated concentrations of the two gases were combined **(Tiedemann and Firsching, 1998; Tiedemann and Firsching, 2000)**. Based up on these two studies, we could postulate that under combination of near-threshold O_3 treatment (50 ppb) and elevated CO_2, the increased carbohydrates in weakened tissues would increase rust diseases. We could also postulate that this increase will be contingent on the host resistance. The exposure concentration, timing, duration and the corresponding disease stage might determine the magnitude of these effects.

Research Objectives:

a. Investigation of the effects of entire season treatment with near-ambient O_3 and CO_2 on leaf rust, on winter wheat varieties with different rust responses, at adult-plant stage.

b. Investigation of the effects of near-ambient O_3 and CO_2 exposure on stem rust on winter wheat at adult-plant stage.

c. Investigation of the effects of different O_3 concentrations on stem rust on winter wheat.

d. Investigation of the effects of near-ambient O_3 and CO_2 exposure timing and duration on stem rust on winter wheat at seedling stage.

To achieve the objectives of this study, four different experiments were conducted, each of which focused on one of the objectives above.

5.3. Experiment (1): Effects of Near-Ambient O_3 and CO_2 on Disease Components of Leaf Rust, on O_3-sensitive Winter Wheat Varieties, with Different Reaction Types to Rust

5.3.1. Material and Methods of Experiment (1)

5.3.1.1. Plant Materials

Four winter wheat genotypes ('Coker 9553', 'NC Neuse', 'Jamestown' and 'NuEast') known to differ in their response to leaf rust (susceptible, moderately susceptible, moderately resistant and resistant, respectively) were selected for this experiment. These varieties are moderately sensitive to O_3 **(Mashaheet et al., 2014)**. The seeds were vernalized in darkness in the refrigerator for 6 weeks at 4°C by placing seeds between two layers of wet filter paper in petri dishes. Vernalized seedling were planted in 1 L pots filled with Fafard #2 Pro Mix (Fafard, Anderson, SC, USA), mixed with 5 g slow release fertilizer (Osmocote Plus, Scotts-Sierra Horticultural Products, Marysville, OH, USA). Plants were grown in Charcoal Filtered air (CF) in greenhouse. At four weeks after planting, uniform plants were selected, and the main stem, the first tiller and second tiller were tagged.

5.3.1.2. Gas Exposure

Plants were acclimated for seven days in Outdoor-Plant Environment chambers (OPECs) **(Flowers et al., 2007)**. After acclimation, plants were grown from growth stage 29-31 Zadoks until maturity under one of four combinations of CO_2 (400, 570 ppm, steady state) and O_3 (10, 50 ppb, 12hr average of 24hr diurnal profile), at 60% relative humidity, and 25/ 16°C day/night temperature cycle. The four different gas treatments were CF (10 ppb O_3, 400 ppm CO_2), O_3 (50 ppb O_3, 400 ppm CO_2), O_3+CO_2 (50 ppb O_3, 570 ppm CO_2), and CO_2 (10 ppb O_3, 570 ppm CO_2). Two replicates of each gas treatment were used, with three plants per genotype in each.

5.3.1.3. Disease Inoculation

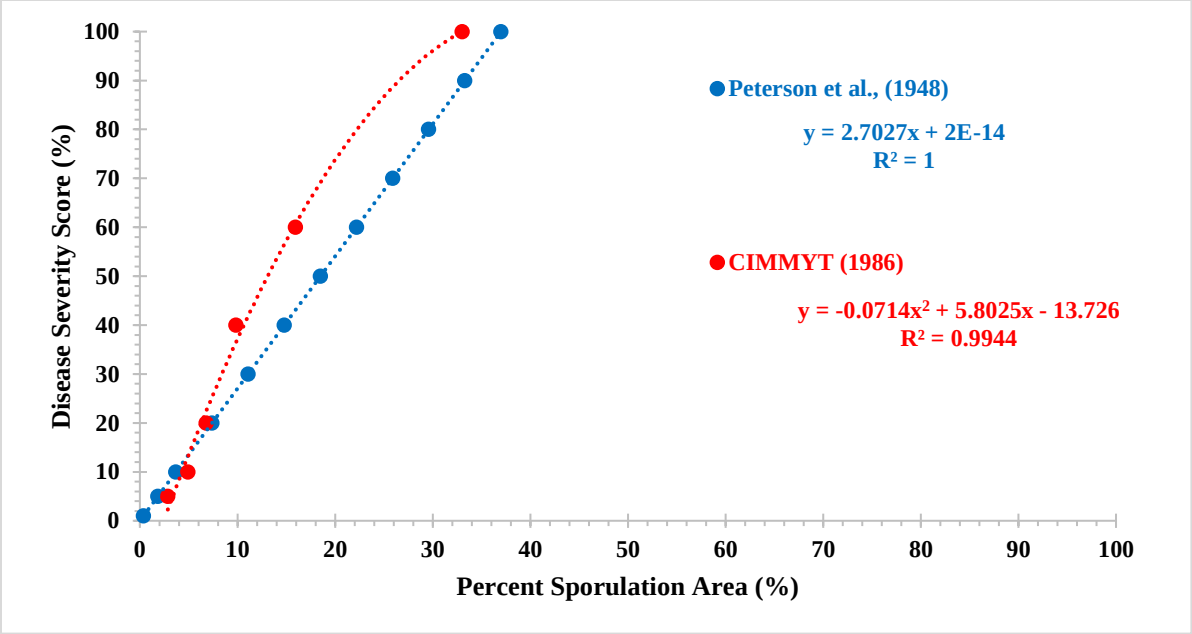
Plants were inoculated with dry urediniospores spores (0.04 gm spores per chamber) of leaf rust race MBTNB **(Tiedemann and Firsching, 1998; Tiedemann and Firsching, 2000)** right after flag leaf emergence (Zadoks growth stage of 39-40). Spores were uniformly spread over plants by reversed air current pumped with air pump through spore collection block. Due formation were only allowed for one night at inoculation at gas exposure was disrupted. Plants were left wet for three hours after sunrise, and then were allowed to dry out for two hours before the gas exposure was resumed, to avoid wet exposure. At inoculation, no O_3 visible symptoms were observed on the flag leaf or F-1.

5.3.1.4. Disease Estimation

Disease severity were visually estimated on flag leaf (F) and the second top leaf (F-1) on the main stem and the first tiller according to the CIMMYT Rust Scoring Guide illustrated in Figure (33). Disease severity of the four scored laves were averaged per plant for analysis. When uredinia were first observed, 5 cm length in the middle of the flag leaf blade was marked **(Singh and Huerta-Espino, 2003)**. For the latent period, pictures of marked area were taken at 7-11 and 14-15 days after inoculation (DAI). Numbers of ruptured pustules were counted and the proportion of ruptured pustules to the final number of pustules at 15 DAI was calculated. The area under the sporulation curve (AUSC) was calculated. Images of the same area were taken using a Dino-Lite AM4113ZT Polarizing Digital Microscope (AnMo Electronics Corporation, Hsinchu 300, Taiwan), and the provided Dino-Lite software were used to measure pustule length and width. Pustule size was calculated according to the following formula **(Lee and Shaner, 1985)**: Pustule size = length (mm) × width (mm) × π/4.

5.3.1.5. Statistical Analysis

Data were analyzed using mixed procedure in SAS 9.2 (SAS Inc., Cary, NC, USA). Averages were separated using Tukey-Kramer adjustment.



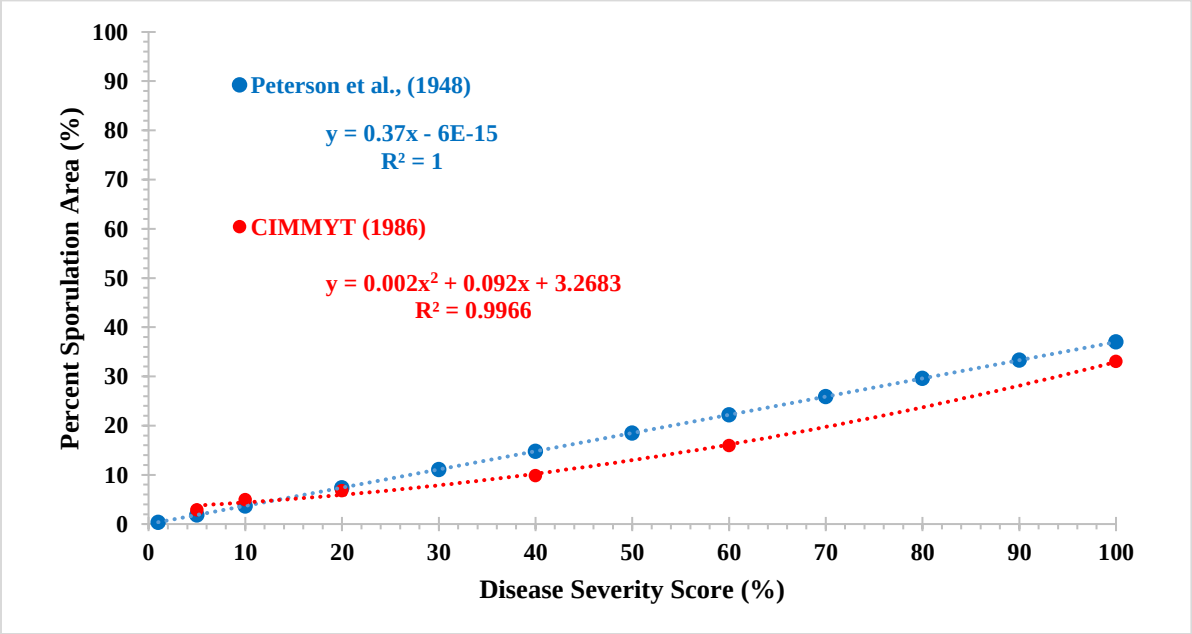


Figure (33): The relationships between the assessed percent sporulation area using APS Assess 2.2 and the corresponding disease severity scores illustrated in the CIMMYT Rust Scoring Guide scale **(CIMMYT, 1986)**, and the published modified Cobb scale **(Pask et al., 2012; Peterson et al., 1948; Roelfs et al., 1992)**.

5.3.2. Results and Discussion of Experiment (1)

5.3.2.1. Disease Severity of Leaf Rust

Singly, elevated O_3 increased disease severity of leaf rust on the susceptible variety Coker 9553 and moderately susceptible variety NC Neuse. This finding confirms previous results obtained from detached leaf segments on Benzimidazole-agar after exposure to sub-symptomatic O_3 treatments **(Tiedemann and Firsching, 1998; Tiedemann and Firsching, 2000)**, which suggests that biotrophic pathogens could be enhanced by sub-symptomatic O_3 stress at near ambient concentrations, on susceptible hosts, as illustrated in Figure (34).

CO_2 treatment of 570 ppm had no significant effect on disease severity on any of the tested varieties. Slightly higher CO_2 concentration (620-650 ppm) caused a slight increase in disease severity **(Tiedemann and Firsching, 1998; Tiedemann and Firsching, 2000)**, whereas a higher concentration (750 ppm) significantly increased disease severity only on the susceptible variety **(Bencze et al., 2013)**. Together, these results suggest a slight trend of increasing disease enhancement with increased CO_2 concentration.

Combined treatment with both elevated O_3 and CO_2 also resulted in an increased disease severity on Coker 9553, an intermediate response on NC Neuse, and no effect on Jamestown and NuEast, another gas treatment by host resistance trend. In previous studies, elevated CO_2 was found to delay O_3 symptom development by 14 days, but it did not completely prevent it, and eventually, CO_2 slightly ameliorated the O_3-enhancement of leaf rust disease severity **(Tiedemann and Firsching, 1998; Tiedemann and Firsching, 2000)**.

Over all, due to the absence of gas treatment effects on disease severity on the moderately resistant and resistant varieties, along with the increasing effect of gas treatment on the susceptible varieties, it was concluded that there is a trend of increased protection against O_3-induced disease severity with the increased level of rust resistance in the varieties tested. These two aforementioned trends indicate the importance of further investigation of the effects of current O_3 concentration on biotrophic pathogens, as supposed to the relatively high

concentrations anticipated after decades. In addition, the importance of simultaneous breeding for O_3 tolerance and rust resistance, as it would result in triple benefit. It would reduce the direct effects of both stressors; in addition to avoiding the synergism between the two stresses coincident on vulnerable varieties (sensitive-susceptible).

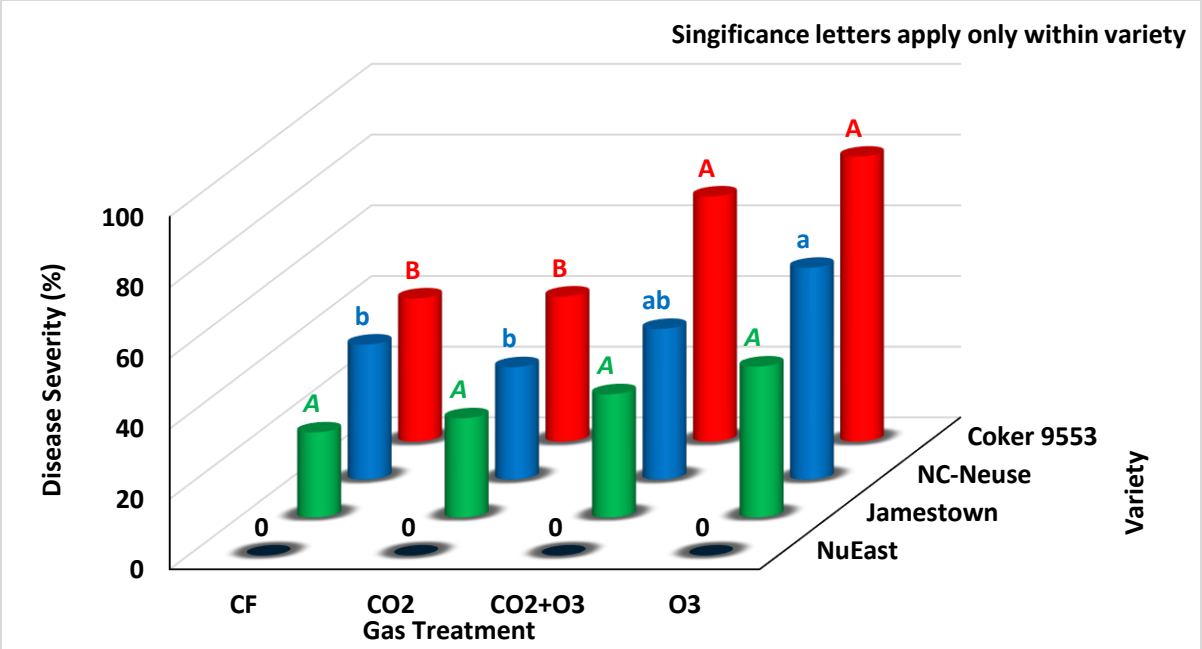


Figure (34): Effects of gas treatments on average disease severity of leaf rust race MMTNB, on four winter wheat varieties with different reaction types [Coker 9553 (susceptible), NC Neuse (moderately susceptible) Jamestown (moderately resistant) and NuEast (resistant)]. Four treatments [CF (10 ppb O_3, 400 ppm CO_2), O_3 (50 ppb O_3, 400 ppm CO_2), O_3+CO_2 (50 ppb O_3, 570 ppm CO_2), and CO_2 (10 ppb O_3, 570 ppm CO_2)] were applied in OPECs, at 60% relative humidity, and 25, 16°C day/night temperature cycle. Data is the average of two replicates of each gas treatment, with three plant per genotype in each. Disease severity was visually estimated, on flag and second top leaf, on the main stem and the first tiller, and scores were averaged per plant for analysis. Significant differences in means were determined by Tukey's tests. Letters apply only within variety.

5.3.2.2. Pustule Size on Flag Leaf

Singly and in combination with elevated CO_2, O_3 significantly increased pustule sizes on Coker 9553 (susceptible). There were no gas treatment effects on pustule size of leaf rust on any of the other varieties tested as illustrated in Figure (35). This finding identified one of the mechanisms by which O_3 increased disease severity of leaf rust. Hence, it is important for wheat breeders to selecting against O_3 sensitivity and rust susceptibility simultaneously. Gas treatment effects on parameters such as pustule size, which might be not captured by visual disease estimation, indicate the importance of precise methods for disease quantification and investigation of the effects on different disease components.

5.3.2.3. Area under sporulation curve (AUSC) on Flag Leaf

Latent period of leaf rust showed differential response to gas treatments on different varieties as shown in Figure (36). On Coker 9552 (susceptible), O_3 hastened sporulation. CO_2 retarded sporulation on NC Neuse when used singly or in combination with O_3. Gas treatment had no significant effect on latent period of leaf rust on NuEast.

In conclusion, results from experiment (1) is in favors of the hypothesis that leaf rust is enhanced by sub-symptomatic O_3 treatments. This hypothesis needs to be tested on stem rust, as there is no study that looked at sub-symptomatic O_3 treatment.

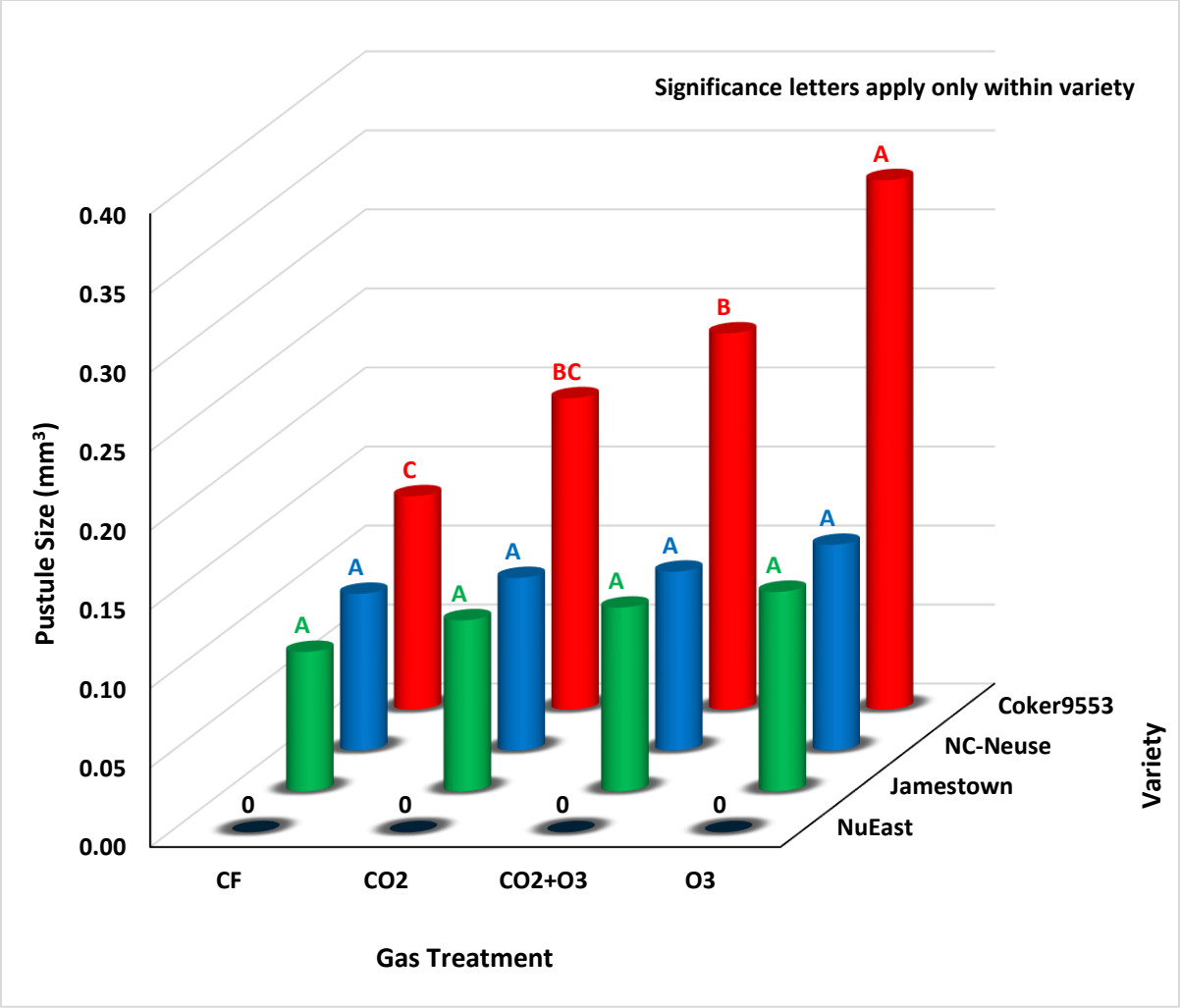


Figure (35): Effect of Gas treatment on pustule size (mm^3) of leaf rust race MMTNB, on four winter wheat varieties with different reaction types [Coker 9553 (susceptible), NC Neuse (moderately susceptible) Jamestown (moderately resistant) and NuEast (resistant)]. Treatments included four different combinations of O_3 and CO_2 [CF (10 ppb O_3, 400 ppm CO_2), O_3 (50 ppb O_3, 400 ppm CO_2), O_3+CO_2 (50 ppb O_3, 570 ppm CO_2), and CO_2 (10 ppb O_3, 570 ppm CO_2)], at 60% relative humidity, and 25, 16°C day/night temperature cycle. Data is the average of two replicates of each gas treatment, with three plants per genotype in each. Pustule length and width were measured on middle of flag of the main stem, and were used for pustule size calculation. Significant differences in means were determined by Tukey's tests. Letters apply only within variety.

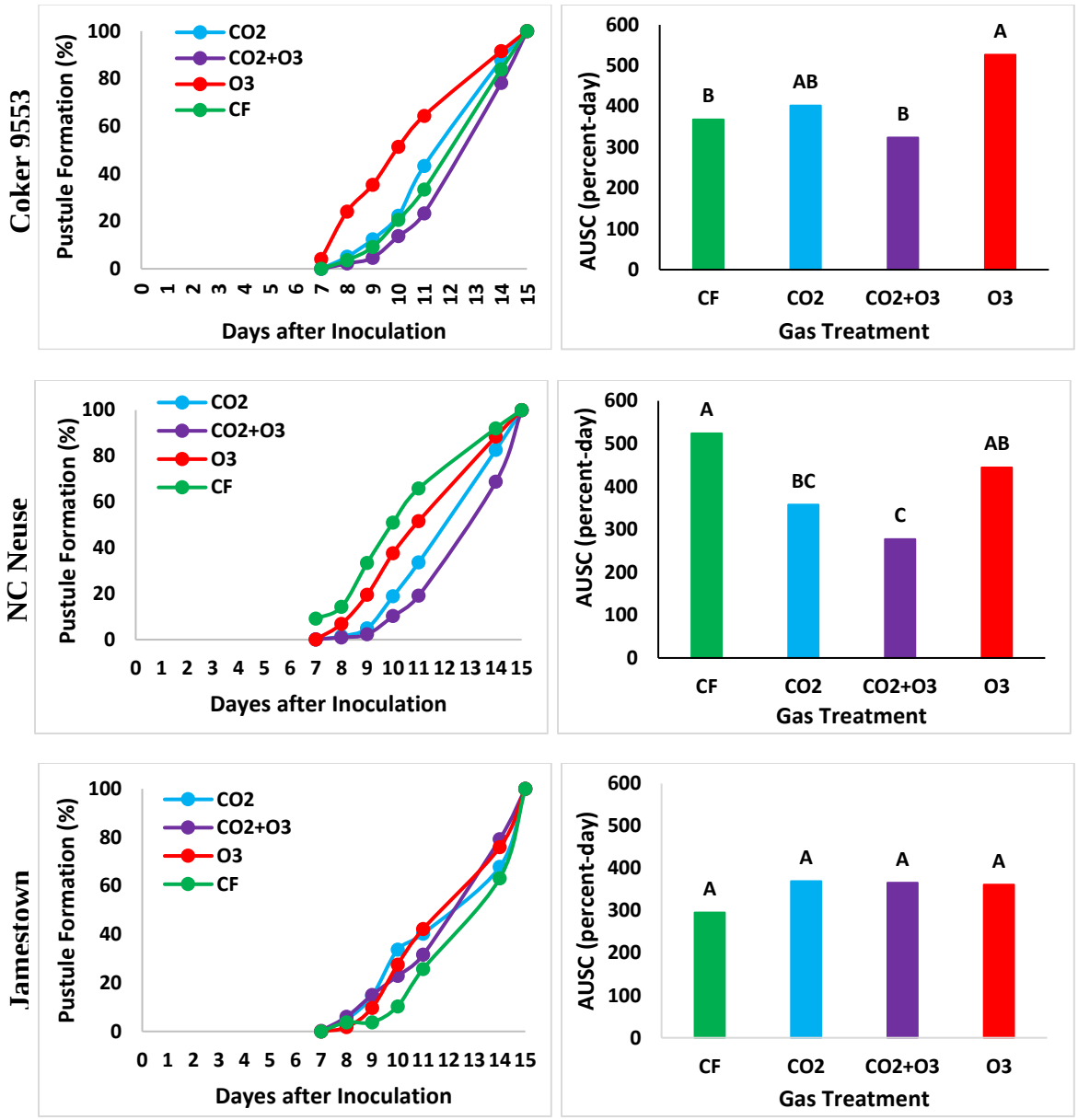


Figure (36): Effect of four combinations of CO_2 (400, 570 ppm) and O_3 (10, 50 ppb) on latent period [percent pustule formation and area under sporulation curve (AUSC)] of leaf rust race MMTNB, on three winter wheat varieties: Coker 9553 (susceptible); NC Neuse (m. susceptible); Jamestown (m. resistant)]. Treatments were applied in OPECs, at 60% relative humidity, and 25/16°C day/night temperatures. Data is the average of two replicates of each gas treatment, with three plants per variety in each. Significant differences in mean were determined by Tukey's tests. Letters apply only within variety.

5.4. Experiment (2): Effects of Near-Ambient O_3 and CO_2 on Disease Severity of Stem Rust, on O_3-sensitive Winter Wheat Varieties, with Different Reaction Types

5.4.1. Material and Methods of Experiment (2)

Two winter wheat genotypes ('Coker 9553' and 'NC Neuse') known to differ in their response to stem rust (susceptible and resistant, respectively) were selected for this experiment. These varieties are moderately sensitive to O_3 **(Mashaheet et al., 2014)**. Plants were vernalized, planted, grown in the greenhouse, selected for uniformity, tagged, acclimated and exposed using the same materials and methods as experiment (1).

Plants were inoculated with urediniospores spores of stem rust race QFCSC suspended in Tween 20 solution. Each variety was represented in each gas treatment by four plants, which were grouped and quantitatively sprayed with 10 ml suspension containing 0.01 gm spore using reversed air current pumped with air pump through spore collection block. The incubation process was conducted as experiment (1).

At 21 days after inoculation, the leaf sheath of the flag leaf of the main stem and the first tiller were sampled and photographed. The images were analyzed using the image analysis software Assess 2.2 (APS, Saint Paul, Minnesota, USA), and percent sporulation area were assessed. The relationships between the assessed percent sporulation area and the disease severity scores assigned according to the most commonly used scales [modified Cobb scale **(Pask et al., 2012; Peterson et al., 1948; Roelfs et al., 1992)**, and the CIMMYT Rust Scoring Guide scale **(CIMMYT, 1986)]** are shown in Figure (33).

5.4.2. Results and Discussion of Experiment (2)

5.4.2.1. Percent sporulation area of Stem rust

Statistical analysis indicated that there was no difference in response between the flag leaf sheath from the main stem and the flag leaf from the first tiller, however, the gas treatment has a significant effects. Singly and in combination with elevated CO_2, O_3 increased disease

severity of stem rust on the susceptible wheat variety 'Coker 9553'. Gas treatments did not alter the disease response of the resistant variety 'NC Neuse'. This is the first report on O_3-induced disease enhancement, and the first report on the effects of sub-symptomatic O_3 treatment on stem rust. These results confirms our previous result obtained using leaf rust. These results suggests that current ambient O_3 concentrations might have a double impact on wheat production; the first is the direct O_3 inhibition on wheat yield, and the second is indirect by increasing effects of rust. It is clear that a study that includes both injurious and sub-symptomatic O_3 treatment is critical in order to confirm the differential effects of different O_3 concentrations.

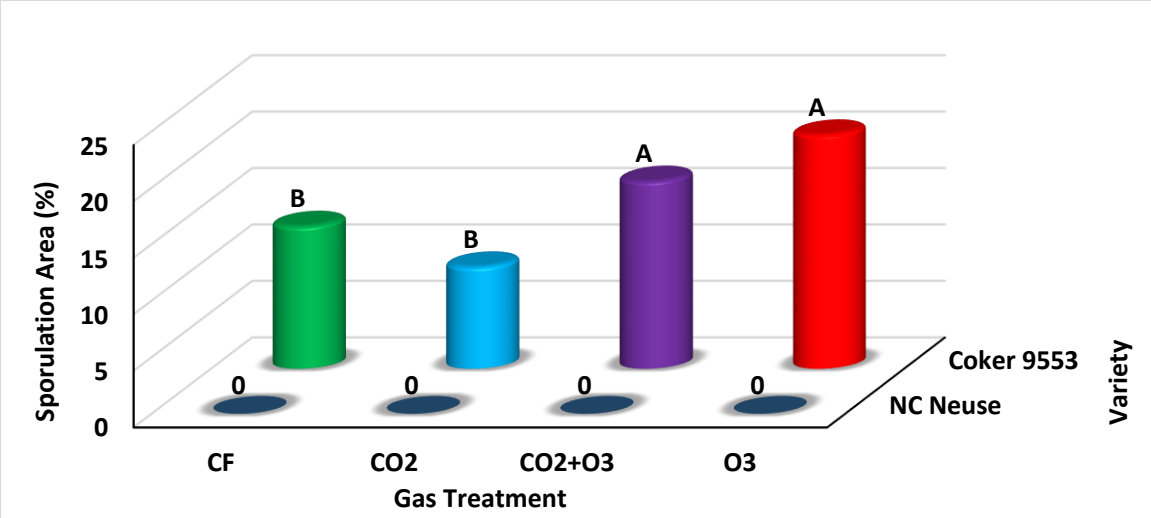


Figure (37): Effect of Gas treatment on percent sporulation are of stem rust race QFCSC, on winter wheat varieties Coker 9553 (susceptible) and NC Neuse (resistant). Four different gas treatments were conducted in OPECs [CF (0 ppb O_3, 400 ppm CO_2), O_3 (50 ppb O_3, 400 ppm CO_2), O_3+CO_2 (50 ppb O_3, 570 ppm CO_2), and CO_2 (0 ppb O_3, 570 ppm CO_2)], at 60% relative humidity, and 25, 16°C day/night temperature cycle. Data is the average of two replicates of each gas treatment, with three plants per genotype in each. Significant differences in mean were determined by Tukey's tests. Letters apply only within variety.

5.5. Experiment (3): Effects of Different O_3 Concentrations on Stem Rust at Seedling Stage

5.5.1. Material and Methods of Experiment (3)

Seeds of winter wheat genotypes Coker-9553 (rust-susceptible, O_3-moderately sensitive) were planted and grown plastic containers under charcoal-filtered air in a greenhouse for four weeks, then seedling with uniform growth stage of 21-22 Zadoks **(Tottman, 1987; Zadoks et al., 1974)** were selected and the main stem were tagged.

Then plants were moved to Out-door Plant Environment Chambers (OPECs) for three days of acclimation, followed by three weeks of exposure to four different O_3 concentrations (CF, 50, 70, and 90 ppb), applied as 12hrs averages of a 24hrs predefined diurnal profile shown in Figure (38). Temperatures were maintained at 25/16 day/night cycle, at near-ambient CO_2 concentration (400 ppm), and 50% RH. The experiment included eight OPECs divided into two blocks, each treatment was assigned to one chamber per block.

After three weeks of exposure, plants were inoculated with stem rust race QFCSC. Fresh spores maintained on susceptible plants in the greenhouse were collected and cleaned of plant debris. Each nine plants were sprayed with spore suspension in distilled deionized water solution of Tween 20. Plants were placed on a rotating carousel, sprayed with a hand sprayer, and returned to the assigned OPEC. At sunset, the OPEC exposure systems were turned off and the relative humidity were increased to 100% and maintained above 95% to allow dew formation overnight. In the morning, the plants were left in still air for three hours and then the air circulation was restored in each OPEC until the plants were completely dry (to avoid wet exposure to O_3. Then the O_3 treatments were restored.

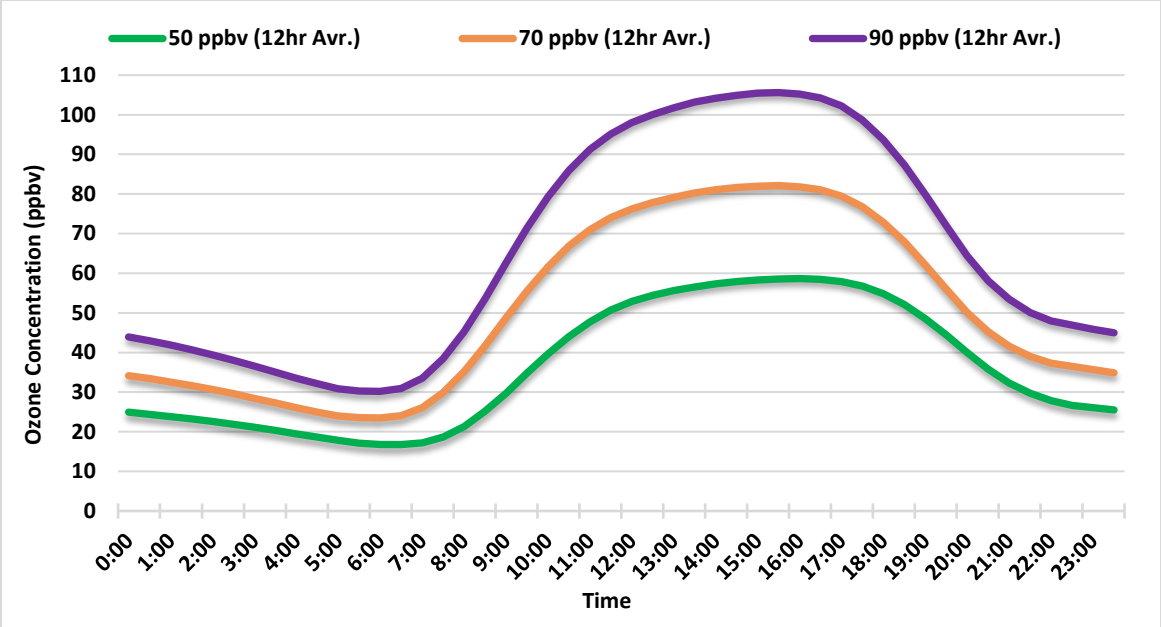


Figure (38): Target 24hr diurnal profiles for the three O_3 treatments with 12hr average of 50, 70 and 90 ppb, applied in Outdoor-plant Environment Chambers (OPECs), to study the effects of different O_3 concentration on stem rust at seedling stage.

The top two leaves on the main stem were tagged at inoculation and three plants per treatment were harvested 7, 14, and 21 days after inoculation (DAI) as illustrated in Figure (39). Leaves were scanned using a flat bed scanner scanner and images were analyzed using the APS Assess 2.2, image analysis software (APS, Saint Paul, Minnesota, USA). The observed present sporulation area estimated using the software was statistically analyzed using SAS 9.4 (SAS Inc., Cary, NC, USA) as shown in Figure (40), and averages were separated using Tukey's adjusted p-values.

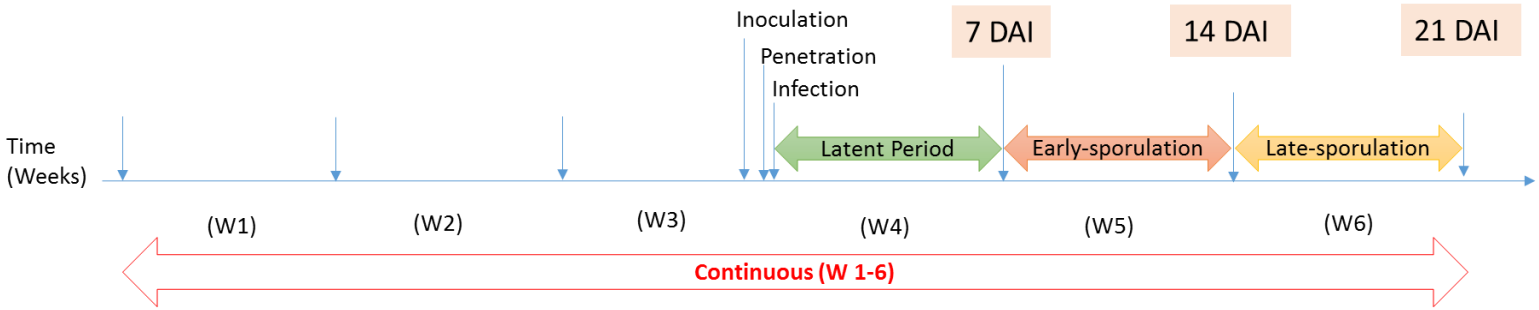


Figure (39): Timeline for O_3 treatment, stem rust inoculation, and disease sampling, used to study the effects of different O_3 concentrations on stem rust at the seedling stage in experiment (3).

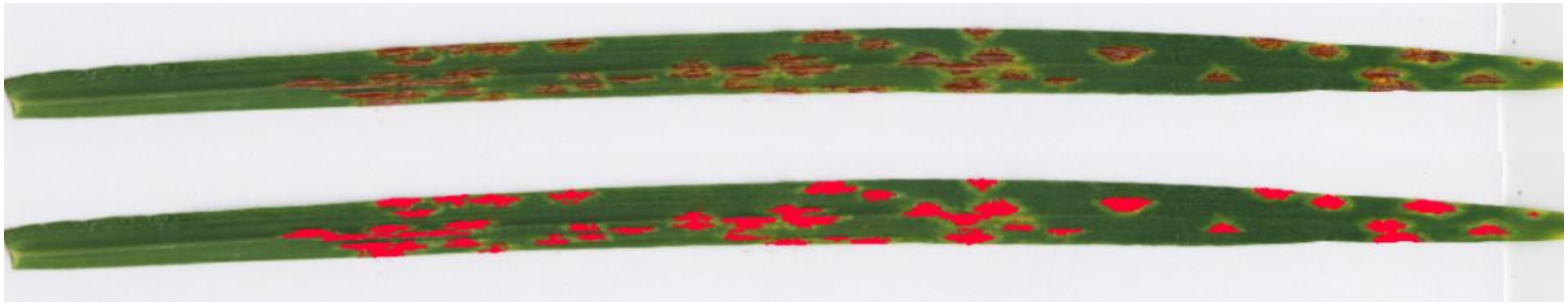


Figure (40): Assessment of percent sporulation area using scanning laves and image analysis using APS Assess 2.2. Top leaf shows the actual stem rust sporulation area, and the bottom leaf shows the sporulation area identified by the APS Assess 2.2.

5.5.2. Results and discussion of Experiment (3)

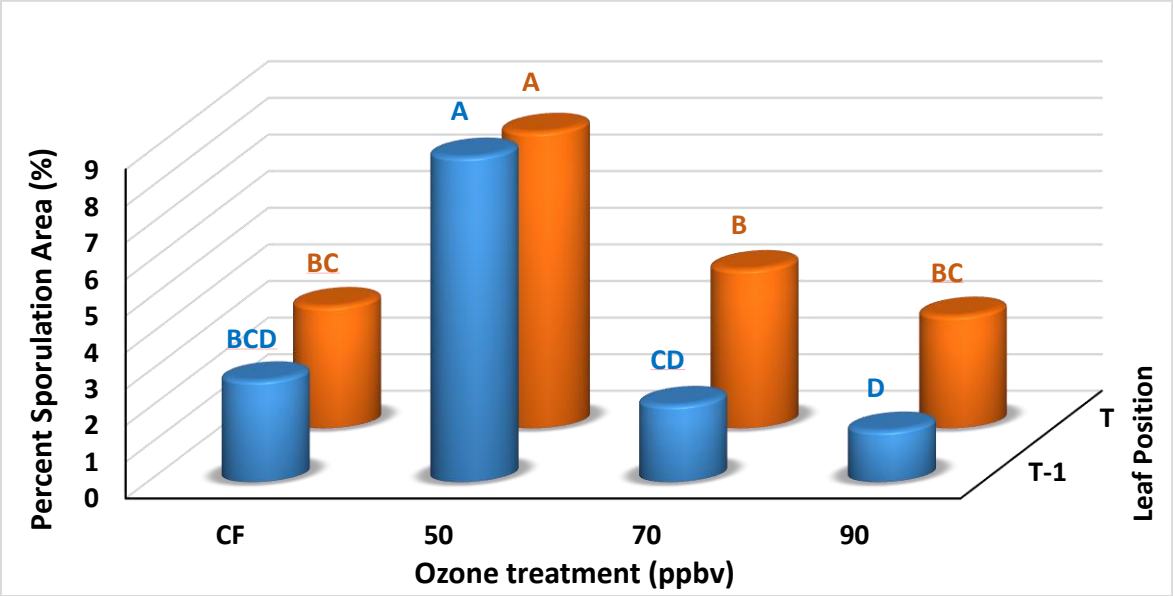
Statistical analysis of percent sporulation area showed no significant second order interaction between Concentration, exposure timing and duration, and leaf position; however, two of the first order interactions were significant, Concentration x leaf position and concentration x sampling time (DAI).

Both leaf positions showed significantly high sporulation at 50 ppb O_3, with no significant differences between the two, which indicates that near-threshold O_3 stress is conducive to stem rust, on more than one leaf position and order, within the canopy as illustrated in Figure (41). There were no significant differences between leaf positions at CF and 50 ppb. However, percent sporulation area at both of the high O_3 concentrations (70, 90 ppb) showed differential response between leaf positions, where the older leaves (T-1) showed significantly less disease than younger ones (T). However, only the older leaves at 90 ppb showed significantly less disease than the younger leaf position on the control plants. These findings indicate that O_3 effects on stem rust, and rust diseases in general are not linearly correlated with concentration. The sub-symptomatic concentrations are conducive to the disease whereas the relatively high concentrations are suppressive.

The change in sporulation area over time in CF, 50 and 70 ppb showed a typical increase until 14DAI, followed by a slight decrease at 21DAI. On the other hand, 90 ppb showed a decreased sporulation area after 7DAI. However, these changes over time were only significant at 50 ppb as illustrated in Figure (41).

In conclusion, these results support the hypothesis that sub-symptomatic O_3 treatments have different effects on rust disease, which might apply to other biotrophic pathogens, such as powdery mildew. However, further studies are needed to validate this generalization. These O_3 effects could be a result of O_3-induced predisposition and/or O_3-induced disease enhancement. In addition, there is no information on the timing and dose requirements for such effects to take place, as well as the possible protection from current and future elevated CO_2 levels.

(a)



(b)

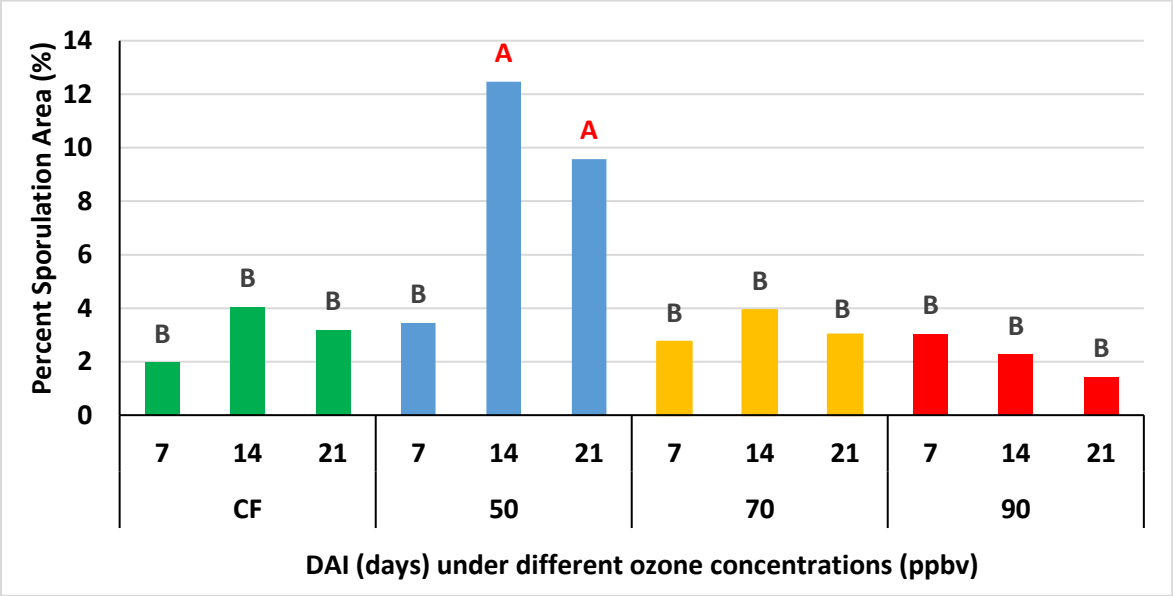


Figure (41): Effect of continuous treatment with different O_3 concentrations (50, 70, and 90 ppb 12hr average of 24hr diurnal profile on percent sporulation area of stem rust (race QFCSC) at two leaf positions [top leaf (T) and second top leaf (T-1)]) on the main stem (a), and at 3 different times after inoculation (7, 14, 21 DAI) (b). Winter wheat cultivar Coker-9553 used was grown in OPECs, at 50% RH, 25/16 °C (day/night), and 400 ppm CO_2.

5.6. Experiment (4): Effects of O_3 and CO_2 concentration, exposure timing and duration on stem rust on winter wheat at seedling stage

5.6.1. Material and Methods of Experiment (4)

In this study, Coker-9553 was used to examine the effects of both the timing and the duration of gas exposure on stem rust disease severity. Seeds were vernalized, planted, grown in the greenhouse, selected for uniformity, tagged, acclimated and exposed using the same materials and methods as experiment (3).

Then plants were moved for three days of acclimation in Out-door Plant Environment Chambers (OPECs) maintaining 50% relative humidity, and 25, 16°C day/night temperature cycle. After acclimation, plants were exposed for six weeks to one of three gas treatments [O_3 (50 ppb O_3, 400 ppm CO_2), O_3+CO_2 (50 ppb O_3, 570 ppm CO_2), and CO_2 (0 ppb O_3, 570 ppm CO_2)], comparing to a CF control (0 ppb O_3, 400 ppm CO_2). For each of these three gas treatments (O_3, O_3+CO_2 and CO_2), seven different combinations of timing and duration of exposure were applied, each of which targeted a specific stage of the host/pathogen interaction with inoculation occurring after the initial 3 weeks of gas treatment. The combinations of timing and duration were as follows: 1^{st}-6^{th} weeks (continuous); 1^{st}-3^{rd} weeks (long predisposition); 3^{rd} week (short predisposition); 4^{th}-6^{th} weeks (whole disease cycle); 4^{th} week (latent period); 5^{th} week (sporulation); and 6^{th} week (post-sporulation). Plants were kept in CF in the days in which they are not assigned for elevated gas treatment, as shown in Figure (42).

Disease inoculation was conducted after the 3^{rd} week of exposure using the same inoculation procedure as experiment (3), however, disease data were collected only once at 21 DAI. The statistical analysis and mean difference identification were conducted using the same procedure used in experiment (3).

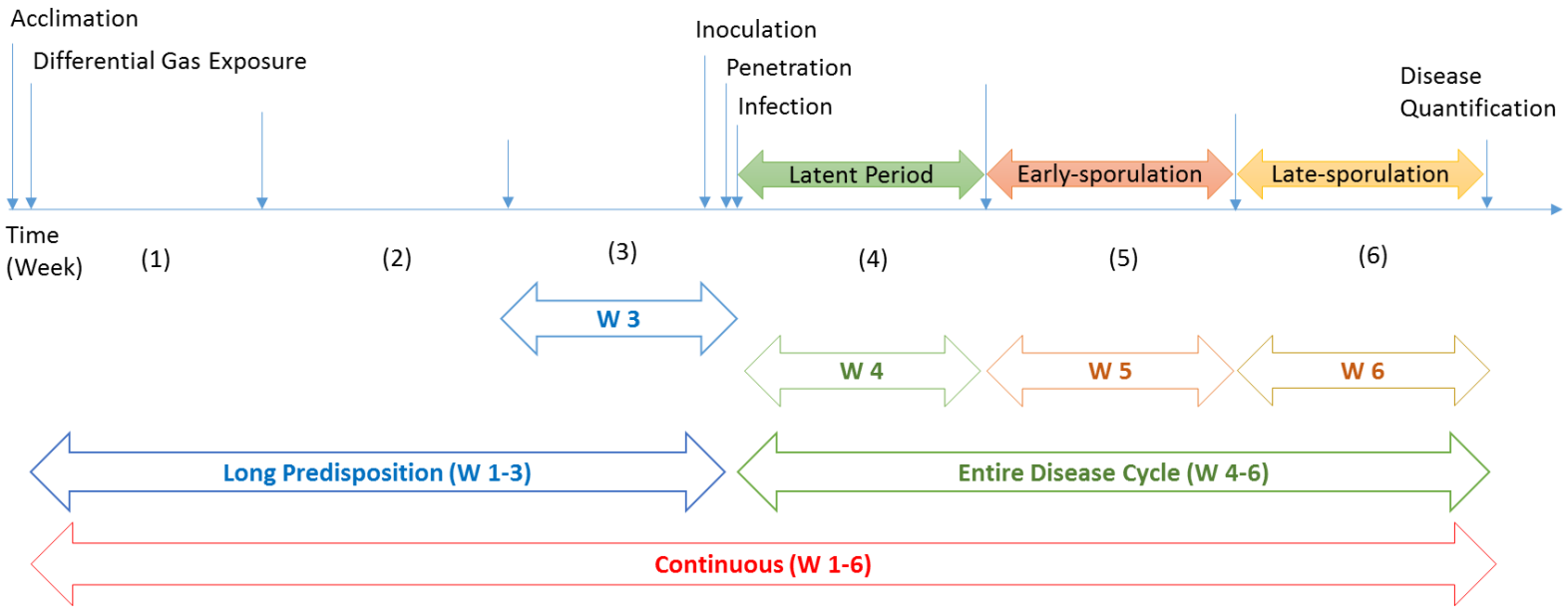


Figure (42): Timeline for stem rust inoculation and assessment, and different timing and duration of gas exposure, and their relation to disease stage, used to study the effects of different O_3 and CO_2 concentration, exposure timing and duration on stem rust on winter wheat at seedling stage in experiment (4).

5.6.2. Results and Discussion of Experiment (4)

Results showed that only continuous O_3 treatment and O_3 exposure for three weeks prior to the inoculation (long predisposition), significantly increased disease relative to the control (CF). No other treatments were significantly different from the control. This is strong evidence that current ambient O_3 effects (mimicked by 50 ppb in this study) on wheat interactions with rust diseases are not currently recognized and thus under estimated, and that more research efforts are needed to quantify these effects under fully open field conditions. Because there was no significant difference between the continuous treatment and the long predisposition before inoculation, we could conclude that the rust increase under 50 ppb O_3 was due to a predisposition effect, rather than a post-inoculation disease enhancement.

Continuous treatment with CO_2 and the combinations of O_3+CO_2 showed intermediate response, confirming previously observed results. However, because the CO_2 concentration used is the anticipated level after three decades, the CO_2 amelioration to O_3 effects might not be a big factor in mediating the presumable current O_3-induced disease increase. Rust is known to be insensitive to O_3 **(Heagle and Key, 1973a; Heagle and Key, 1973b)**, and this experiment provide more evidence for such insensitivity in host tissue, as O_3 treatments with 50 ppb during latent or sporulation periods did not alter the disease.

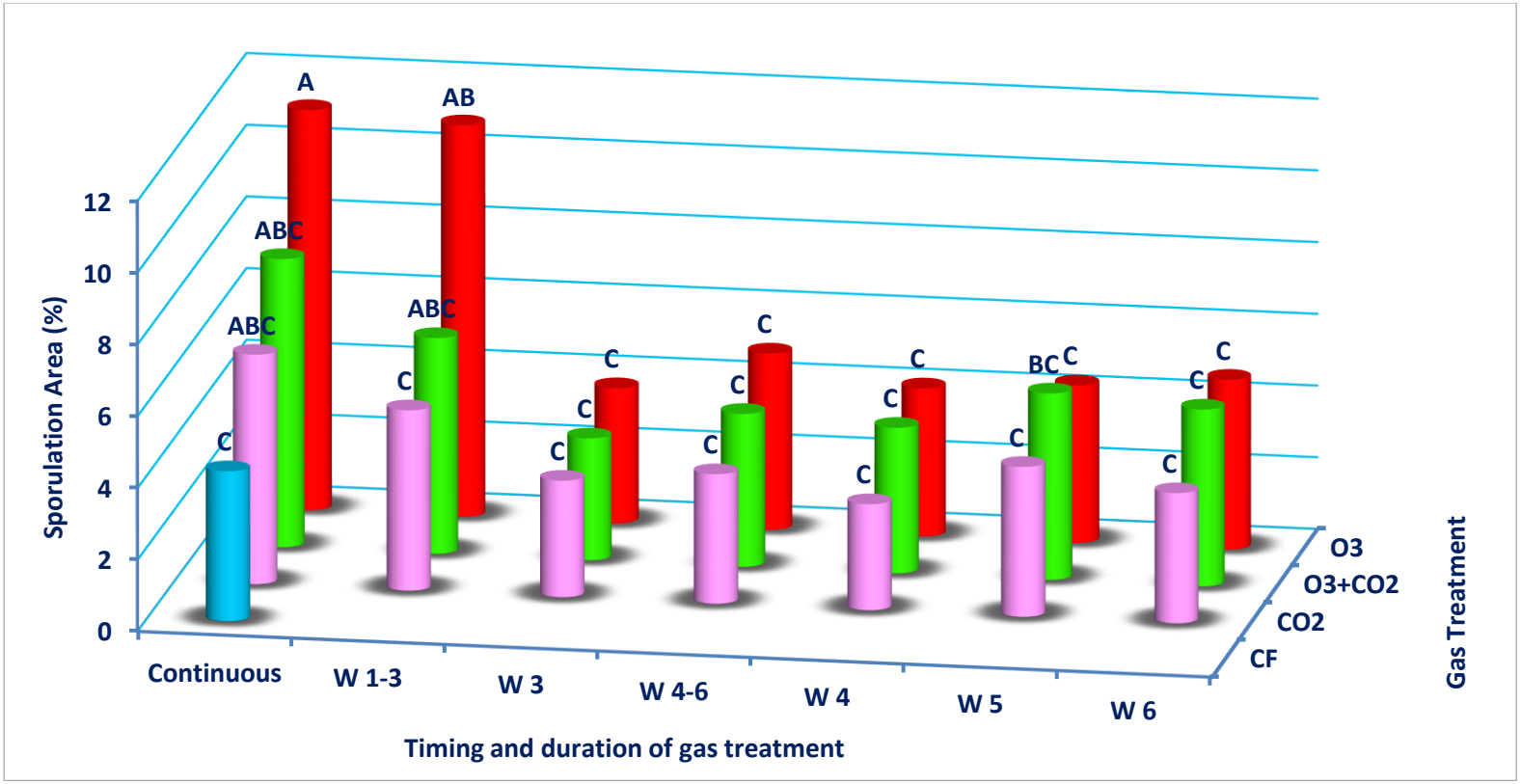


Figure (43): Effect of gas treatments [CF control (0 ppb O_3, 400 ppm CO_2), O_3 (50 ppb O_3, 400 ppm CO_2), O_3+CO_2 (50 ppb O_3, 570 ppm CO_2), and CO_2 (0 ppb O_3, 570 ppm CO_2)], exposure timing (relative to the disease stage) and duration (1-6 weeks) on stem rust (race QFCSC) on percent sporulation area at two leaf position [top leaf (T) and second top leaf (T-1)]) on the main stem, at 3 different times after inoculation (7, 14, 21 DAI) on winter wheat cultivar Coker-9553, grown in OPECs, at 50% RH, 25/16 °C (day/night), and 400 ppm CO_2.

5.7. General Discussion and Conclusion

Relatively high O_3 concentration limit obligate parasites (biotrophic pathogens) by limiting the viable host tissue required for all pathogen stages. The extrapolation of the suppressive effects of high O_3 concentration led to the current consensus that elevated O_3 is suppressive to biotrophic pathogens including wheat rust **(Manning and v. Tiedemann, 1995; Pfleeger et al., 1999)**. O_3 concentrations that do not exert this relatively high degree of stress on the host were shown to cause opposite effects on detached wheat leaf segments **(Tiedemann, 1992a)**, as well as in other rust systems, but not on attached wheat leaves. Therefore, this study was attempted to fill in this knowledge gap by investigating the effects of near-ambient (which are also near threshold) O_3 concentrations on leaf and stem rusts of wheat.

This study showed consistent disease increase of both leaf and stem rust of wheat, under near-ambient but not under relatively high O_3 concentration. The O_3-induced disease increase was more pronounced with increased susceptibility to the pathogen, as reflected in larger pustule size (increased inoculum sources), and accelerated pustule formation (shorter generations), which are likely to increase rust epidemics. The decreased O_3 effects with increased rust resistance night indicate some cross connections between rust resistance and O_3 tolerance, at least when the two stressors are present sat the same time. This trend also suggests the importance of simultaneous breeding for both stressors, which could result in a double slow rusting effect.

O_3-induced rust disease enhancement was observed on different canopy layers (the top and second from the top leaves), indicating that O_3 effects apply at a whole canopy scale. These effects also seem to be due to O_3-induced predisposition, rather than post-infection disease enhancement, which means that the negative synergetic effect could take place even if the two stressors did not coincide. Elevated CO_2 concentrations did not seem to provide enough protection against O_3 effects on disease, and mainly caused disease increase in the absence of O_3 stress.

There are several mechanisms, by which sub-symptomatic O_3 stress could be conducive to biotrophic pathogens, and especially rust pathogens:

a- O_3-enhanced leaf wettability and spore germination

O_3 is known to affects cuticle production **(Kontunen-Soppela et al., 2007)**, chemistry and physical properties **(Percy et al., 2002)**, as well as topology **(Karnosky et al., 1999)**. These effects were found to affect the wettability and free-water film formation on leaves, which is a key requirement for spore germination **(Percy et al., 2002)**. Near-ambient O_3 (around 50 ppb) treatments increased the disease severity of poplar leaf rust by four folds compared to ambient concentrations (36.0-38.8 ppb), and the combination of O_3 and CO_2 showed threefold increase, whereas CO_2 alone did not cause changes **(Percy et al., 2002)**. These authors attributed this increase partially to the changes in leaf wettability, because of changes in cuticular wax structure, as O_3 altered the ratio of the most hydrophobic (hydrocarbons) to least (fatty acids) hydrophobic cuticular, which is expected to increase leave wettability **(Holloway, 1969)**. Elevated concentrations of either O_3 or CO_2, as well as their combination were found to increase the synthesis of fatty acids, which stimulate host recognition by the pathogens **(Percy et al., 2002)**. In addition, changes in cuticle topology were observed under near-ambient O_3 treatment that caused fivefold increase in aspen leaf rust **(Karnosky et al., 1999)**.

b- Rust protection to the host cells in the infection locus against O_3

Stem rust was found to protect the sub-stomatal mesophyll cells from O_3 damage, when plants are exposed 2-4 hours after inoculation, which was attributed to fungal diffusible factors produced during germination **(Heagle and Key, 1973b)**. This protection could result from the direct effects of a factor from the pathogen, or from plant shifts in some aspect of the host's metabolism in response to the pathogen factor. The biotrophs are more likely to exhibit this ability after penetrations, which would lead to differential O_3 tolerance within the host tissue, especially under sub-symptomatic O_3 treatments.

The effect of stem rust infection on the O_3 response of the host infected tissue was also investigated **(Heagle and Key, 1973b)**. Stem rust was found to produce diffusible elements that resulted in significant protection against O_3 injury at the infection site. Some evidence for post-infection rust protection to the host tissue against O_3 damage was observed in this study as the green islands surrounding rust pustules even in totally injured tissue as shown in Figure (44). We could also see in Figure (44-f), some infection loci that failed to sporulate due to the severe O_3 stress.

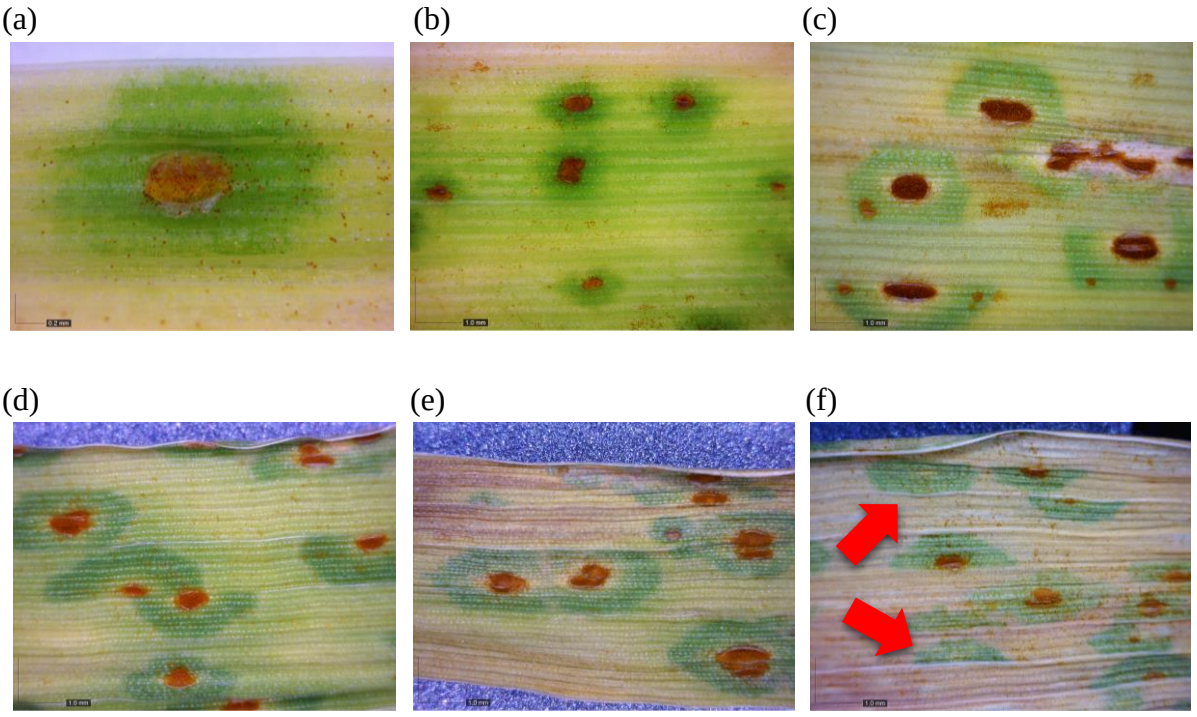


Figure (44): Green islands surrounding stem rust pustules on O_3-stressed leaves of winter wheat variety 'Coker 9553', indicating post-infection rust protection to the leaf tissue against O_3 damage.

c- O_3-induced reduction of resistance compounds

Resistance and antioxidant compounds production are part of plat's secondary metabolism and compete for available plant resources. Attenuated non-injured plants exposed to near-ambient O_3 treatments would have fewer resources available for resistance compounds, which makes it easier for the pathogen to overcome the weakened plant resistance. The fourfold increase in polar leaf rust under near-ambient O_3 treatment was associated with decreased phenolic glycoside concentrations (important defense compounds of poplar), whereas they were increased under elevated CO_2 **(Percy et al., 2002)**.

d- O_3-induced electrolyte leakage

Low to moderate O_3 stress could cause sub-symptomatic effects, such as membrane disintegration, leading to electrolyte leakage into the apoplast **(Zheng et al., 2011)**. This type of effects were found to alter wheat interactions with necrotrophic pathogens by increasing disease severity **(Tiedemann and Pfähler, 1994)**, which could have similar effects on biotrophic pathogens, such as wheat rusts. Induction of electrolyte leakage into the apoplast is known to be a pathogenicity factor in some diseases.

Disintegration of cell membranes and electrolyte leakage would allow the pathogen to timely acquire host-originated nutrients needed after the exhaustion of the spore's reserve. The spore supply is enough for sending very few haustoria to adjacent host cells after penetration through stomata **(Leonard and Szabo, 2005)**. If these haustoria failed to establish the nutrient acquisition from the host, the infection fails to progress **(Leonard and Szabo, 2005)**. Easy nutrient acquisition or availability in the apoplast for the rust fungus to utilize once penetrated, could result in higher infection success rate that could be observed as more pustules per area (also known as Receptivity).

Leaf rust pathogens with different infection type were found to cause differential and correspondent electrolyte leakage shortly after infection, and the differences in electrolyte leakage between races gradually vanished over time. These changes in electrolyte leakage were

correlated with peroxidase activity **(Saini et al., 1988)**. Extreme rust resistance of some wheat varieties is attributed to their ability to significantly reduce electrolyte leakage into the apoplast and extremely susceptible reactions were associated with the opposite response **(Thatcher, 1943)**. Stresses that increases electrolyte leakage such as cold were found increase rust and reduce host resistance **(Thatcher, 1943)**. This indicates the importance of the electrolyte leakage shortly after infection, and suggests that O_3-induced electrolyte leakage might have similar effects on rust. The host cells are much more sensitive to such effects on plasma membrane permeability, which explained why host cells are killed before the pathogen cells start to be negatively affected **(Thatcher, 1943)**.

e- Disruption of receptors and resistance proteins

Many of the rust resistance genes are coding for nucleotide-binding site leucine-rich repeats (NBS-LRR) which are receptor protein domains located on the apoplastic side of the plasma membrane Many of the rust resistance genes encoding NBS-LRR protein domains are usually resistance proteins (RP) located on the apoplastic side of the plasma membrane **(Kolmer, 2005)**. These domains are known as signal receptors especially for diseases signals. Because of their locations, the NBS-LRR proteins are vulnerable to O_3-induced ROS in the apoplast. This might result in protein denaturation and function loss.

f- Resources relocation from injured into healthy tissue

Chronic O_3 exposure causes accelerated senescence. Enzymes involved in regulated cellular disassembly, and nutrient translocation, such as RNases and nucleases, showed increased activities in wheat flag leaf tissues from plants chronically exposed to O_3 **(Booker, 2004)**. Hence, this increased activity would allow more nutrients for the fungus once it penetrate the host tissue, and continue to provide the pathogen as long as the surrounding tissue is still viable.

g- Interference of ROS scavenging physiology with timely activation of HR

Activated ROS scavenging mechanisms might interfere with critical and time-sensitive physiological mechanisms for resistance, such as the initiation and containment of HR under pathogen attack. The HR is an indicative of pathogen attack **(Wrzaczek et al., 2010)** usually leading to resistance responses **(Overmyer et al., 2003)**. The up-regulation of resistance genes in response to a chronic stressor like O_3 would be unsuitable and cannot be maintained for an extended period, as the continuous upregulation of resistance genes consumes from plant resources. Hence, O_3 stressed plants might uncouple the HR and the up-regulation of resistance genes, when HR is not induced by pathogen, which might result in lessened resistance responses when plant is attacked by the pathogen while under O_3 stress.

h- Increased carbon metabolism and sugar availability

Plant pathogens such as rust fungi are sugar dependent **(Bolton et al., 2008)**. O_3 stress increases carbon metabolism and results in increased sugar catabolism as a plant adaptations mechanism to impaired photosynthesis **(Li et al., 2015)**. The resulting sugars will be also available to the pathogen colonizing the host tissue.

i- Ozone induced lipoxygenase pathway

The plant lipoxygenase (LOX) pathway is known to be induced in plants by both pathogen attack **(Leonard and Szabo, 2005)**, and O_3 stress **(Vaultier and Jolivet, 2015)**. The formation of appressoria, vesicles and haustorium mother cells are induced by 'trans-2-hexen-1-ol', a six-carbon derivative, one of several six-carbon derivatives of lipoxygenase (LOX) pathway **(Leonard and Szabo, 2005)**.

j- Ozone interference with interplay of plant hormones

O_3 tolerance to oxidative stress requires precise control of the interplay of plant hormones, such as Jasmonic acid (JA) and methyl jasmonate (MeJA), which protect tissues from ROS-induced cell death and thus counteract the effects of SA and ethylene, two hormones

are known to increase in response to both O_3 stress and pathogen attack **(Wrzaczek et al., 2010)**. Jasmonate treatment reduced the amount of SA produced in response to ozone **(Rao et al., 2000)**. JA also antagonizes ethylene signaling, and ethylene inhibited JA-induced gene expression **(Tuominen et al., 2004)**. Ethylene production that interferes with stomatal closure **(Wilkinson et al., 2011)** might enhance rust penetration.

These crossroads between rust and O_3 stress might explain the increased rust disease severity, under sub-symptomatic O_3 treatments. Understanding the effects of gas treatments on disease components is critical for estimating rust epidemics and modelling future disease levels. Research on these essential parameters is needed for increasing wheat yield and feeding the growing populations worldwide in a changing climate and shifting pathogen.

Breeding wheat for O_3 stress tolerance could increase potential, attainable and actual yield of wheat. Wheat varieties with oxidative stress tolerance would be expected to have higher yield potential than less tolerant varieties, as oxidative stress is part of several normal plant physiological processes (e.g. light interception and photosynthesis). However, oxidative stress tolerant varieties would have much higher attainable yield under oxidative stress resulting from different stressors, especially elevated O_3. Furthermore, varieties with oxidative stress tolerance are less vulnerable to rust diseases if they are attacked by virulent rust strains.

References

Afzal, F., S.K. Chaudhari, A. Gul, A. Farooq, H. Ali, S. Nisar, B. Sarfraz, K.J. Shehzadi and A. Mujeeb-Kazi. 2015. Bread wheat (*Triticum aestivum* L.) under biotic and abiotic stresses: An overview. p. 293-317. *In* K.R. Hakeem (ed.) Crop production and global environmental issues. Springer International Publishing.

Agrios, G.N. 2005. Plant pathology. Elsevier, Burlington.

Ainsworth, E.A., A. Rogers, and A.D.B. Leakey. 2008. Targets for crop biotechnology in a future high-CO_2 and high-O_3 world. Plant Physiol. 147:13-19.

Ali, I., A. P. Roelfs, and J. Huerta-Espino. 1994. Inheritance of leaf rust resistance in wheat cultivars Morocco and Little Club. Plant Disease 78:383-384.

Altimir, N., P. Kolari, J.P. Tuovinen, T. Vesala, J. Back, T. Suni, M. Kulmala, and P. Hari. 2006. Foliage surface ozone deposition: A role for surface moisture? Biogeosciences 3:209-228.

American Phytopathological Society. 2016. Phytobiomes: A roadmap for research and translation. American Phytopathological Society, St. Paul, MN. USA. http://www.phytobiomes.org/roadmap/Pages/default.aspx

Amundson, R.G., R.J. Kohut, A.W. Schoettle, R.M. Raba, and P.B. Reich. 1987. Correlative reductions in whole-plant photosynthesis and yield of winter wheat caused by ozone. Phytopathology 77:75-79.

Archibald, A.T., J.G. Levine, N.L. Abraham, M.C. Cooke, P.M. Edwards, D.E. Heard, M.E. Jenkin, A. Karunaharan, R.C. Pike, P.S. Monks, D.E. Shallcross, P.J. Telford, L.K. Whalley, and J.A. Pyle. 2011. Impacts of HO_x regeneration and recycling in the oxidation of isoprene: Consequences for the composition of past, present and future atmospheres. Geophysical Research Letters 38:1-6.

Avnery, S., D.L. Mauzerall, J. Liu, and L.W. Horowitz. 2011a. Global crop yield reductions due to surface ozone exposure: 1. Year 2000 crop production losses and economic damage. Atmospheric Environment 45:2284-2296.

Avnery, S., D.L. Mauzerall, J. Liu, and L.W. Horowitz. 2011b. Global crop yield reductions due to surface ozone exposure: 2. Year 2030 potential crop production losses and

economic damage under two scenarios of O3 pollution. Atmospheric Environment 45:2297-2309.

Barnes, J.D., D. Velissariou, A.W. Davison, and C.D. Holevas. 1990. Comparative ozone sensitivity of old and modern Greek cultivars of spring wheat. The New Phytologist 116:707-714.

Barnes, J., J. Bender, T. Lyons, and A. Borland. 1999. Natural and man-made selection for air pollution resistance. Journal of Experimental Botany 50:1423-1435.

Bencze, S., G. Vida, K. Balla, E. Varga-László, and O. Veisz. 2013. Response of wheat fungal diseases to elevated atmospheric CO_2 level. Cereal Research Communications 41:409-419.

Biswas, D.K., and G.M. Jiang. 2011. Differential drought-induced modulation of ozone tolerance in winter wheat species. Journal of Experimental Botany 62:4153-4162.

Biswas, D.K., H. Xu, Y.G. Li, B.L. Ma, and G.M. Jiang. 2013. Modification of photosynthesis and growth responses to elevated CO_2 by ozone in two cultivars of winter wheat with different years of release. Journal of Experimental Botany 64:1485-1496.

Biswas, D.K., H. Xu, Y.G. Li, M.Z. Liu, Y.H. Chen, J.Z. Sun, and G.M. Jiang. 2008a. Assessing the genetic relatedness of higher ozone sensitivity of modern wheat to its wild and cultivated progenitors/relatives. Journal of Experimental Botany 59:951-963.

Biswas, D.K., H. Xu, Y.G. Li, J.Z. Sun, X.Z. Wang, X.G. Han, and G.M. Jiang. 2008b. Genotypic differences in leaf biochemical, physiological and growth responses to ozone in 20 winter wheat cultivars released over the past 60 years. Global Change Biology 14:46-59.

Bolton, M.D., J.A. Kolmer, and D.F. Garvin. 2008. Wheat leaf rust caused by *Puccinia triticina*. Molecular Plant Pathology 9:563-575.

Booker, F., R. Muntifering, M. McGrath, K. Burkey, D. Decoteau, E. Fiscus, W. Manning, S. Krupa, A. Chappelka, and D. Grantz. 2009. The ozone component of global change: Potential effects on agricultural and horticultural plant yield, product quality and interactions with invasive species. Journal of Integrative Plant Biology 51:337-351.

Booker, F.L. 2004. Influence of ozone on ribonuclease activity in wheat (*Triticum aestivum*) leaves. Physiologia Plantarum 120:249-255.

Booker, F.L., K.O. Burkey, K. Overmyer, and A.M. Jones. 2004. Differential responses of G-protein *Arabidopsis thaliana* mutants to ozone. New Phytologist 162:633-641.

Braun, H., G. Atlin and T. Payne. 2010. Multi-location testing as a tool to identify plant response to global climate change. p. 115-138. *In* M.P. Reynolds (ed.) Climate change and crop production. CABI, London, UK.

Broberg, M.C., Z. Feng, Y. Xin, and H. Pleijel. 2015. Ozone effects on wheat grain quality – A summary. Environmental Pollution 197:203-213.

Brosché, M., E. Merilo, F. Mayer, P. Pechter, I. Puzõrjova, G. Brader, J. Kangasjärvi, and H. Kollist. 2010. Natural variation in ozone sensitivity among *Arabidopsis thaliana* accessions and its relation to stomatal conductance. Plant, Cell & Environment 33:914.

Burkart, S., J. Bender, B. Tarkotta, S. Faust, A. Castagna, A. Ranieri, and H.J. Weigel. 2013. Effects of ozone on leaf senescence, photochemical efficiency and grain yield in two winter wheat cultivars. Journal of Agronomy & Crop Science 199:275-285.

Burkey, K.O., S.J. Ray, W.A. Pursley and R.W. Zobel. 2016. Updates on studies of ozone-temperature interactions in the field. *In* Updates on studies of ozone-temperature interactions in the field. 29th task force meeting, UNECE ICP vegetation   February 29 – March 4, 2016 2016. ICP Vegetation Programme Coordination Centre, Dubna, Russia.

Burton, A., K. Burkey, T. Carter Jr, J. Orf, and P. Cregan. 2016. Phenotypic variation and identification of quantitative trait loci for ozone tolerance in a fiskeby III × Mandarin (ottawa) soybean population. Theoretical & Applied Genetics 129:1113-1125.

Cabane, M., D. Afif, and S. Hawkins. 2012. Chapter 7 - Lignins and abiotic stresses. Adv. Bot. Res. 61:219-262.

Cape, J.N., R. Hamilton, and M.R. Heal. 2009. Reactive uptake of ozone at simulated leaf surfaces: Implications for 'non-stomatal' ozone flux. Atmospheric Environment 43:1116-1123.

Chakraborty, S., J. Luck, G. Hollaway, G. Fitzgerald, and N. White. 2011. Rust-proofing wheat for a changing climate. Euphytica 179:19-32.

Chakraborty, S., J. Luck, G. Hollaway, A. Freeman, R. Norton, K.A. Garrett, K. Percy, A. Hopkins, C. Davis, and D.F. Karnosky. 2008. Impacts of global change on diseases of agricultural crops and forest trees. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 3:1-15.

Chang, C., E. Faust, X. Hou, P. Lee, H.C. Kim, B.C. Hedquist, and K. Liao. 2016. Investigating ambient ozone formation regimes in neighboring cities of shale plays in the northeast united states using photochemical modeling and satellite retrievals. Atmospheric Environment 142:152-170.

Chappelka, A.H., and N.E. Grulke. 2016. Disruption of the 'disease triangle' by chemical and physical environmental change. Plant Biology Journal 18:5-12.

Chen, W., C. Wellings, X. Chen, Z. Kang, and T. Liu. 2014. Wheat stripe (yellow) rust caused by *Puccinia striiformis* f. sp. *tritici*. Molecular Plant Pathology 15:433-446.

Chen, W., L. Zhang, X. Y. Li, R. Ye, Q. Li, J. Zhu, N. Fang, L. Wang, Z. Wu, and W.R. Horvath. 2015. Elevated ozone increases nitrifying and denitrifying enzyme activities in the rhizosphere of wheat after 5 years of fumigation. Plant Soil 392:279-288.

CIMMYT. 1986. Rust scoring guide. CIMMYT., Mexico, DF.

Colette, A., W. Aas, L. Banin, and et al. 2016. Air pollution trends in the EMEP region between 1990 and 2012. Norwegian Institute for Air Research, P.O. Box 100, NO-2027 Kjeller, Norway.

Cooper, O.R., D.D. Parrish, J. Ziemke, N.V. Balashov, M. Cupeiro, I.E. Galbally, S. Gilge, L. Horowitz, N.R. Jensen, J.F. Lamarque, V. Naik, S.J. Oltmans, J. Schwab, D.T. Shindell, A.M. Thompson, V. Thouret, Y. Wang, and R.M. Zbinden. 2014. Global distribution and trends of tropospheric ozone: An observation-based review. Elementa: Science of the Anthropocene 2:29.

D'haese, D., K. Vandermeiren, H. Asard, and N. Horemans. 2005. Other factors than apoplastic ascorbate contribute to the differential ozone tolerance of two clones of trifolium repens L. Plant, Cell & Environment 28:623-632.

Darino, M.A., M.J. Dieguez, D. Singh, L.R. Ingala, M.F. Pergolesi, R.F. Park, R.A. McIntosh, and F. Sacco. 2015. Detection and location of *Lr11* and other leaf rust resistance genes in the durably resistant wheat cultivar Buck Poncho. Euphytica 206:135-147.

Davydov, V.A. 1999. Characterization of the stomatal apparatus in monosomic lines of Chinese Spring wheat. Russian Journal of Genetics 35:458-461.

De Kok, L.J., D.A. Grantz, and K.O. Burkey. 2016. Plants and the changing environment. Plant Biology Journal 18:3-4.

Dicke, M. 2016. Plant phenotypic plasticity in the phytobiome: a volatile issue. Current Opinion in Plant Biology 32:17-23.

Dizengremel, P., D.L. Thiec, M.P. Hasenfratz-Sauder, M. Vaultier, M. Bagard, and Y. Jolivet. 2009. Metabolic-dependent changes in plant cell redox power after ozone exposure. Plant Biology (Stuttgart, Germany) 11 Suppl. 1:35-42.

Dohmen, G.P. 1987. Secondary effects of air pollution: Ozone decreases brown rust disease potential in wheat. Environmental Pollution 43:189-194.

Eastburn, D.M., A.J. McElrone, and D.D. Bilgin. 2011. Influence of atmospheric and climatic change on plant pathogen interactions. Plant Pathology 60:54-69.

Eversole, K., C. Feuillet, K.F.X. Mayer, and J. Rogers. 2014. Slicing the wheat genome. Science 345:285-287.

FAO. 2016. Faostat 2016: Visited November 11, 2016. http://faostat.fao.org/beta/en/#data/TP

Fares, S., R. Weber, J. H. Park, D. Getner, J. Karlik, and A.H. Goldstein. 2012. Ozone deposition to an orange orchard: Partitioning between stomatal and non-stomatal sinks. Environmental Pollution 169:258-266.

Feng, Z., K. Kobayashi, and E. A. Ainsworth. 2008. Impact of elevated ozone concentration on growth, physiology, and yield of wheat (*Triticum aestivum* L.): A meta-analysis. Global Change Biology 14: 2696-2708.

Feng, Z., H. Tang, J. Uddling, H. Pleijel, K. Kobayashi, J. Zhu, H. Oue, and W. Guo. 2012. A stomatal ozone flux–response relationship to assess ozone-induced yield loss of winter wheat in subtropical China. Environmental Pollution 164:16-23.

Feng, Z., and K. Kobayashi. 2009. Assessing the impacts of current and future concentrations of surface ozone on crop yield with meta-analysis. Atmospheric Environment 43:1510-1519.

Fetch, T., T. Zegeye, R.F. Park, D. Hodson, and R. Wanyera. 2016. Detection of wheat stem rust races TTHSK and PTKTK in the Ug99 race group in Kenya in 2014. Plant Disease 100:1495-1495.

Finkelstein, P.L., A.W. Davison, H.S. Neufeld, T.P. Meyers, and A.H. Chappelka. 2004. Sub-canopy deposition of ozone in a stand of Cutleaf Coneflower. Environmental Pollution 131:295-303.

Fiore, A.M., F.J. Dentener, O. Wild, C. Cuvelier, M.G. Schultz, P. Hess, C. Textor, M. Schulz, R.M. Doherty, L.W. Horowitz, I.A. MacKenzie, M.G. Sanderson, D.T. Shindell, D.S. Stevenson, S. Szopa, R. Van Dingenen, G. Zeng, C. Atherton, D. Bergmann, I. Bey, G. Carmichael, W.J. Collins, B.N. Duncan, G. Faluvegi, G. Folberth, M. Gauss, S. Gong, D. Hauglustaine, T. Holloway, I.S.A. Isaksen, D.J. Jacob, J.E. Jonson, J.W. Kaminski, T.J. Keating, A. Lupu, E. Marmer, V. Montanaro, R.J. Park, G. Pitari, K.J. Pringle, J.A. Pyle, S. Schroeder, M.G. Vivanco, P. Wind, G. Wojcik, S. Wu, and A. Zuber. 2009. Multimodel estimates of intercontinental source-receptor relationships for ozone pollution. Journal of Geophyical Research 114:D04301.

Fiscus, E.L., F.L. Booker, and K.O. Burkey. 2005. Crop responses to ozone: Uptake, modes of action, carbon assimilation and partitioning. Plant, Cell & Environment 28:997-1011.

Flowers, M.D., E.L. Fiscus, K.O. Burkey, F.L. Booker, and J.B. Dubois. 2007. Photosynthesis, chlorophyll fluorescence, and yield of snap bean (*Phaseolus vulgaris* L.) genotypes differing in sensitivity to ozone. Environmental & Experimental Botany 61:190-198.

Francl, L.J. 2001. The disease triangle: A plant pathological paradigm revisited. The Plant Health Instructor [Online]. DOI: 10.1094/PHI-T-2001-0517-01, http://www.apsnet.org/edcenter/instcomm/TeachingArticles/Pages/DiseaseTriangle.aspx

Frei, M. 2015. Breeding of ozone resistant rice: Relevance, approaches and challenges. Environmental Pollution 197:144-155.

Fuhrer, J. 2003. Agroecosystem responses to combinations of elevated CO_2, ozone, and global climate change. Agriculture, Ecosystems & Environment 97:1-20.

Garrett, K.A., S.P. Dendy, E.E. Frank, M.N. Rouse, and S.E. Travers. 2006. Climate change effects on plant disease: Genomes to ecosystems. Annual Review of Phytopathology 44:489-509.

Gelang, J., H. Pleijel, E. Sild, H. Danielsson, S. Younis, and G. Selldén. 2000. Rate and duration of grain filling in relation to flag leaf senescence and grain yield in spring wheat (*Triticum aestivum*) exposed to different concentrations of ozone. Physiologia Plantarum 110:366-375.

Ghini, R., E. Hamada, and W. Bettiol. 2008. Climate change and plant diseases. Scientia Agricola 65:98-107.

Haider, N. 2013. The origin of the B-genome of bread wheat (*Triticum aestivum* L.). Genetika 49:303-314.

Hartikainen, K., M. Kivimäenpää, A. Nerg, and T. Holopainen. 2012. Significance of leaf structure and emission of volatile organic compounds in ozone tolerance of oat and wheat. Botany 90:121-135.

Hassan, I.A. 2004. Interactive effects of salinity and ozone pollution on photosynthesis, stomatal conductance, growth, and assimilate partitioning of wheat (*Triticum aestivum* L.). Photosynthetica 42:111-116.

Haynes, W.M. 2015. CRC handbook of chemistry and physics, 95th edition. CRC Press, Hoboken.

Heagle, A.S., J.E. Miller, and W.A. Pursley. 2000. Growth and yield responses of winter wheat to mixtures of ozone and carbon dioxide. Crop Sci. 40:1656-1664.

Heagle, A.S. 1982. Interactions between air pollutants and parasitic plant diseases. p. 333-348. *In* D.P. Ormrod (ed.) Effects of gaseous air pollution in agriculture and horticulture. Butterworth-Heinemann.

Heagle, A.S., and L.W. Key. 1973a. Effect of ozone on the wheat stem rust fungus. Phytopathology 63:400.

Heagle, A.S., and L.W. Key. 1973b. Effect of *Puccinia graminis* f. sp. *tritici* on ozone injury in wheat. Phytopathology 63:609.

Heagle, A.S., and L.W. Key. 1972. Effect of ozone on the wheat stem rust fungus. Phytopathology 63:397-397-400.

Heagle, A.S. 1975. Response of three obligate parasites to ozone. Environmental Pollution (1970) 9:91-95.

Heath, R.L., and P.E. Frederick. 1979. Ozone alteration of membrane permeability in Chlorella. Plant Physiology 64:455-459.

Heck, W.W., J.A. Dunning, and R.B. Philbeck. 1978. A continuous stirred tank reactor (CSTR) system for exposing plants to gaseous air contaminants: Principles, specifications, construction, and operation. Department of Agriculture, Agricultural Research Service, Southern Region, New Orleans, LA. USA.

Heggestad, H.E., and J.T. Middleton. 1959. Ozone in high concentrations as cause of tobacco leaf injury. Science 129:208-210.

Helfer, S. 2014. Rust fungi and global change. New Phytologist 201:770-780.

Herbinger, K., M. Tausz, A. Wonisch, G. Soja, A. Sorger, and D. Grill. 2002. Complex interactive effects of drought and ozone stress on the antioxidant defence systems of two wheat cultivars. Plant Physiology and Biochemistry 40:691-696.

Heun, M., R. Schäfer-Pregl, D. Klawan, R. Castagna, M. Accerbi, B. Borghi, and F. Salamini. 1997. Site of einkorn wheat domestication identified by DNA fingerprinting. Science 278:1312-1314.

Hiebert, C.W., J.A. Kolmer, C.A. McCartney, J. Briggs, T. Fetch, H. Bariana, F. Choulet, M. N. Rouse, and W. Spielmeyer. 2016. Major gene for field stem rust resistance co-locates with resistance gene *Sr12* in 'Thatcher' wheat. PLoS One 11:e0157029.

Hill, A.C., and N. Littlefield. 1969. Ozone effect on apparent photosynthesis, rate of transpiration, and stomatal closure in plants. Environmental Science & Technology 3:52-56.

Holloway, P.J. 1969. Chemistry of leaf waxes in relation to wetting. Journal of the Science of Food & Agriculture 20:124-128.

Hoshika, Y., M. Watanabe, N. Inada, and T. Koike. 2013. Model-based analysis of avoidance of ozone stress by stomatal closure in siebold's beech (*Fagus crenata*). Annals of Botany 112:1149-1158.

ICP Vegetation. 2011. Ozone pollution: A hidden threat to food security. ICP Vegetation Programme Coordination Centre, Centre for Ecology and Hydrology, Environment Centre Wales, Deiniol Road, Bangor, Gwynedd, LL57 2UW, UK.

IPCC. 2014a. IPCC fifth assessment synthesis report; contribution of working groups I, II and III to the fifth assessment report of the intergovernmental panel on climate change; [core writing team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland.

IPCC. 2014b. Climate change 2013: The physical science basis: Working group I contribution to the fifth assessment report of the intergovernmental panel on climate change. Cambridge University Press, New York.

Iriti, M., and F. Faoro. 2009. Ozone-induced changes in plant secondary metabolism. p. 245-268. *In* S.N. Singh (ed.) Climate change and crops. Springer Berlin Heidelberg.

Iriti, M., and F. Faoro. 2008. Oxidative stress, the paradigm of ozone toxicity in plants and animals. Water Air Soil Pollution 187:285 301.

Jacob, D.J. 1999. Introduction to atmospheric chemistry. Princeton University Press.

Joo, J.H., S. Wang, J.G. Chen, A.M. Jones, and N.V. Fedoroff. 2005. Different signaling and cell death roles of heterotrimeric G protein α and β subunits in the Arabidopsis oxidative stress response to ozone. The Plant Cell 17:957-970.

Joshi, A.K., M. Azab, M. Mosaad, M. Moselhy, M. Osmanzai, S. Gelalcha, G. Bedada, M.R. Bhatta, A. Hakim, P.K. Malaker, M.E. Haque, T.P. Tiwari, A. Majid, M.R.J. Kamali, Z. Bishaw, R.P. Singh, T. Payne, and H.J. Braun. 2011. Delivering rust resistant wheat to farmers: A step towards increased food security. Euphytica 179:187-196.

Juroszek, P., and A. von Tiedemann. 2013. Climate change and potential future risks through wheat diseases: A review. European Journal of Plant Pathology. 136:21 33.

Kadono, T., D. Tran, R. Errakhi, T. Hiramatsu, P. Meimoun, J. Briand, M. Iwaya-Inoue, T. Kawano, and F. Bouteau. 2010. Increased anion channel activity is an unavoidable event in ozone-induced programmed cell death. PloS One 5:e13373.

Kangasjärvi, J., P. Jaspers, and H. Kollist. 2005. Signalling and cell death in ozone-exposed plants. Plant, Cell & Environment 28:1021-1036.

Kanna, M., M. Tamaoki, A. Kubo, N. Nakajima, R. Rakwal, G.K. Agrawal, S. Tamogami, M. Ioki, D. Ogawa, H. Saji, and M. Aono. 2003. Isolation of an ozone-sensitive and jasmonate-semi-insensitive Arabidopsis mutant (oji1). Plant Cell Physiology 44:1301-1310.

Karnosky, D.F., B. Mankovska, K. Percy, R.E. Dickson, G.K. Podila, J. Sober, D.F. Karnosky, G. Hendrey, M.D. Coleman, M. Kubiske, K.S. Pregitzer, J.G. Isebrands, A. Noormets, K. Percy, R.E. Dickson, G.K. Podila, J. Sober, A. Noormets, G. Hendrey, M.D. Coleman, M. Kubiske, K.S. Pregitzer, and J.G. Isebrands. 1999. Effects of tropospheric O_3 on trembling aspen and interaction with CO_2: Results from an O_3-gradient and a face experiment. Water, Air, & Soil Pollution 116:311-322.

Kerstiens, G., and K.J. Lendzian. 1989. Interactions between ozone and plant cuticles. I. ozone deposition and permeability. The New Phytologist 112:13-19.

Khoder, M.I. 2009. Diurnal, seasonal and weekdays–weekends variations of ground level ozone concentrations in an urban area in greater Cairo. Environmental Monitoring and Assessment 149:349-362.

Kim, K., Y. Kwon, J. Lee, M. Eun, and J. Sohn. 2004. QTL mapping and molecular marker analysis for the resistance of rice to ozone. Molecules and Cells 17:151.

Knott, D.R. 1993. Agronomic and quality characters of near-isogenic lines of wheat carrying genes for stem rust resistance. Euphytica 68:33-41.

Kollist, H., H. Moldau, L. Mortensen, S.K. Rasmussen, and L.B. Jørgensen. 2000. Ozone flux to plasmalemma in barley and wheat is controlled by stomata rather than by direct reaction of ozone with cell wall ascorbate. Journal of Plant Physiology 156:645-651.

Kolmer, J.A. 2015. Leaf rust resistance in wheat line RL6062 is an allele at the *Lr3* locus. Crop Science 55:2186-2190.

Kolmer, J.A. 2005. Tracking wheat rust on a continental scale. Current Opinion in Plant Biology 8:441-449.

Kontunen-Soppela, S., V. Ossipov, S. Ossipova, and E. Oksanen. 2007. Shift in birch leaf metabolome and carbon allocation during long-term open-field ozone exposure. Global Change Biology 13:1053-1067.

Kumar, N., P.L. Kulwal, A. Gaur, A.K. Tyagi, J.P. Khurana, P. Khurana, H.S. Balyan, A.K. Tyagi, N. Kumar, P.L. Kulwal, A. Gaur, P.K. Gupta, J.P. Khurana, P. Khurana, and P.K. Gupta. 2006. QTL analysis for grain weight in common wheat. Euphytica 151:135-144.

Ledford, H. 2015. Plant dwellers take the limelight. Nature 523:137-138.

Lee, H.J., S.W. Kim, J. Brioude, O.R. Cooper, G.J. Frost, C.H. Kim, R.J. Park, M. Trainer, and J.H. Woo. 2014. Transport of NO_x in East Asia identified by satellite and in situ measurements and lagrangian particle dispersion model simulations. Journal of Geophysical Research: Atmospheres 119:2574-2596.

Lee, T.S., and G. Shaner. 1985. Oligogenic inheritance of length of latent period in six slow leaf-rusting wheat cultivars. Phytopathology 75:636.

Leonard, K.J., and L.J. Szabo. 2005. Stem rust of small grains and grasses caused by *Puccinia graminis*. Molecular Plant Pathology 6:99-111.

Li, Q., X. Bao, C. Lu, X. Zhang, J. Zhu, Y. Jiang, and W. Liang. 2012. Soil microbial food web responses to free-air ozone enrichment can depend on the ozone-tolerance of wheat cultivars. Soil Biology and Biochemistry 47:27-35.

Li, X., Y. Deng, Q. Li, C. Lu, J. Wang, H. Zhang, J. Zhu, J. Zhou, and Z. He. 2013. Shifts of functional gene representation in wheat rhizosphere microbial communities under elevated ozone. ISME Journal 7:660-671.

Li, Y., M. Muthuramalingam and R. Mahalingam. 2015. Plant responses to tropospheric ozone. p. 1-14. *In* P.K. Jaiwal, R.P. Singh, and O.P. Dhankher (eds.) Genetic manipulation in plants for mitigation of climate change. Springer India.

Ling, H., S. Zhao, D. Liu, J. Wang, H. Sun, C. Zhang, H. Fan, D. Li, L. Dong, Y. Tao, C. Gao, H. Wu, Y. Li, Y. Cui, X. Guo, S. Zheng, B. Wang, K. Yu, Q. Liang, W. Yang, X. Lou, J. Chen, M. Feng, J. Jian, X. Zhang, G. Luo, Y. Jiang, J. Liu, Z. Wang, Y. Sha, B. Zhang, H. Wu, D. Tang, Q. Shen, P. Xue, S. Zou, X. Wang, X. Liu, F. Wang, Y. Yang, X. An, Z. Dong, K. Zhang, X. Zhang, M. Luo, J. Dvorak, Y. Tong, J. Wang, H. Yang, Z. Li, D.

Wang, A. Zhang, and J. Wang. 2013. Draft genome of the wheat A-genome progenitor *Triticum urartu*. Nature 496:87-90.

Liu, C., X. Yang, H. Zhang, X. Wang, Z. Zhang, Y. Bian, B. Zhu, Y. Dong, and B. Liu. 2015a. Genetic and epigenetic modifications to the BBAA component of common wheat during its evolutionary history at the hexaploid level. Plant Molecular Biology 88:53-64.

Liu, X., L. Sui, Y. Huang, C. Geng, and B. Yin. 2015b. Physiological and visible injury responses in different growth stages of winter wheat to ozone stress and the protection of spermidine. Atmospheric Pollution Research 6:596-604.

Long, S.P., E.A. Ainsworth, A.D.B. Leakey, and P.B. Morgan. 2005. Global food insecurity. treatment of major food crops with elevated carbon dioxide or ozone under large-scale fully open-air conditions suggests recent models may have overestimated future yields. Philosophical Transactions of the Royal Society B: Biological Sciences 360:2011-2020.

Lu, C., Y. Cao, C. He, X. Bao, R. Fang, Y. Wang, X. Chen, Y. Shi, and Q. Li. 2016. Effects of elevated O_3 and CO_2 on the relative contribution of carbohydrates to soil organic matter in an agricultural soil. Soil Tillage Research 159:47-55.

Luck, J., M. Spackman, A. Freeman, P. Tre¸bicki, W. Griffiths, K. Finlay, and S. Chakraborty. 2011. Climate change and diseases of food crops. Plant Pathology 60:113-121.

Luig, N.H. 1983. A survey of virulence genes in wheat stem rust, *Puccinia graminis* f. sp. *tritici*. Verlag Paul Parey., Berlin, Hamburg.

Madkour, S.A., and J.A. Laurence. 2002. Egyptian plant species as new ozone indicators. Environmental Pollution 120:339-353.

Manning, W.J., and A. v. Tiedemann. 1995. Climate change: Potential effects of increased atmospheric carbon dioxide (CO_2), ozone (O_3), and ultraviolet-B (UV-B) radiation on plant diseases. Environmental Pollution 88:219-245.

Marcussen, T., S.R. Sandve, L. Heier, M. Spannagl, M. Pfeifer, , K.S. Jakobsen, B.B.H. Wulff, B. Steuernagel, K.F.X. Mayer, and O. Olsen. 2014. Ancient hybridizations among the ancestral genomes of bread wheat. Science 345: 1250092, 1-6.

Mashaheet, A.M. 2016. Elevated surface ozone and phytobiomes: Challenges and opportunities. *In* Phytobiome Convening Meeting. Jul. 29, 2016. American Phytopathological Society, Tampa, FL, USA.

Mashaheet, A.M., D.S. Marshall and K.O. Burkey. 2016a. Simultaneous breeding for resistance to the emerging stem rust races of the Ug99 lineage and ozone tolerance in winter wheat. *In* The Emerging Plant Diseases and Global Food Security International Symposium. March 23-24, 2016. North Carolina State University, Raleigh, NC, USA.

Mashaheet, A.M., D.S. Marshall and K.O. Burkey. 2015. Effects of elevated O_3 and CO_2 on stem rust disease on winter wheat; *In* 2015-Eastern Wheat and Southern Small Grain Workers Conference; April 28-30, 2015. Richmond, VA, USA.

Mashaheet, A.M., D.S. Marshall and K.O. Burkey. 2014. Effects of climate change on the components of wheat leaf rust disease on winter wheat. *In* 2014 APS-CPS Joint Meeting. Aug. 9-13, 2014. American Phytopathological Society, Minneapolis, MN, USA.

Mashaheet, A.M., D.S. Marshall, K.O. Burkey, A. Abdelrhim and R. Ullah. 2016b. Screening the monosomic lines of the Chinese Spring wheat variety for ozone tolerance. *In* 2016 APS Annual Meeting. Jul. 30 - Aug. 3, 2016. American Phytopathological Society, Tampa, FL, USA.

Mashaheet, A.M., D.S. Marshall, K.O. Burkey, A. Abdelrhim and R. Ullah. 2016c. Screening some rust universal-susceptible wheat varieties for ozone tolerance and some applications. *In* 2016 APS Annual Meeting. Jul. 30 - Aug. 3, 2016. American Phytopathological Society, Tampa, FL, USA.

Mayee, C.D., and V.V. Datar. 1989. Measuring plant disease. p. 485-496. *In* V.P. Agnihotri, N. Singh, H.S. Chaube, U.S. Singh, and T.S. Dwivedi (eds.) Perspectives in phytopathology. Today and tomorrow's Printers & Publishers, New Delhi, India.

Mayer, K., J. Rogers, J. el Dole, and et al. 2014. A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. Science 345: 1251788, 1-11.

McElroy, C.T., and P.F. Fogal. 2008. Ozone: From discovery to protection. Atmosphere-Ocean 46:1-13.

McFall, K.L., and M.E. Fowler. 2009. Overview of wheat classification and trade. p. 437-454. *In* Regentsessor, Brett F Carver Editor (ed.) Wheat science and trade. Wiley-Blackwell.

McIntosh, R.A., and Z.A. Pretorius. 2011. Borlaug global rust initiative provides momentum for wheat rust research. Euphytica 179:1-2.

McIntosh, R.A., C.R. Wellings, and R.F. Park. 1995. Wheat rusts: An atlas of resistance genes. CSIRO Publishing, Melbourne.

McLEOD, A.R. 1995. An open-air system for exposure of young forest trees to sulphur dioxide and ozone. Plant, Cell & Environment 18:215-225.

Meyer, U., B. Köllner, J. Willenbrink, and G.H.M. Krause. 2000. Effects of different ozone exposure regimes on photosynthesis, assimilates and thousand grain weight in spring wheat. Agriculture, Ecosystems and Environment 78:49-55.

Middleton, G.K., J.R. Bennett, and H.C. Newton. 1973. Registration of McNAIR 701 Wheat (reg. no. 529). 13:585.

Miller, G., N. Suzuki, S. Ciftci-Yilmaz, and R. Mittler. 2010. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant, Cell & Environment 33:453-467.

Mills, G., A. Buse, B. Gimeno, V. Bermejo, M. Holland, L. Emberson, and H. Pleijel. 2007. A synthesis of AOT40-based response functions and critical levels of ozone for agricultural and horticultural crops. Atmospheric Environment 41:2630-2643.

Mills, G., F. Hayes, D. Simpson, L. Emberson, D. Norris, H. Harmens, and P. Buker. 2011. Evidence of widespread effects of ozone on crops and (semi-)natural vegetation in europe (1990–2006) in relation to AOT40- and flux-based risk maps. Global Change Biol. 17:592-613.

Monks, P.S., A.T. Archibald, A. Colette, O. Cooper, M. Coyle, R. Derwent, D. Fowler, C. Granier, K.S. Law, G.E. Mills, D.S. Stevenson, O. Tarasova, V. Thouret, E. von Schneidemesser, R. Sommariva, O. Wild, and M.L. Williams. 2015. Tropospheric ozone and its precursors from the urban to the global scale from air quality to short-lived climate forcer. Atmos. Chem. Phys. 15:8889-8973.

Morgan, P.B., T.A. Mies, G.A. Bollero, R.L. Nelson, and S.P. Long. 2006. Season-long elevation of ozone concentration to projected 2050 levels under fully open-air conditions substantially decreases the growth and production of soybean. New Phytologist 170:333-343.

Morgounov, A., H.A. Tufan, R. Sharma, B. Akin, A. Bagci, H. Braun, Y. Kaya, M. Keser, T.S. Payne, K. Sonder, and R. McIntosh. 2012. Global incidence of wheat rusts and powdery mildew during 1969–2010 and durability of resistance of winter wheat variety bezostaya 1. European Journal of Plant Pathology 132:323-340.

Newton, A.C., S.N. Johnson, A.C. Newton, P.J. Gregory, and P.J. Gregory. 2011. Implications of climate change for diseases, crop yields and food security. Euphytica 179:3-18.

Ojanpera, K., E. Patsikka, and T. Ylaranta. 1998. Effects of low ozone exposure of spring wheat on net CO_2 uptake, Rubisco, leaf senescence and grain filling. New Phytologist 138:451-460.

Örvar, B.L., J. McPherson, and B.E. Ellis. 1997. Pre-activating wounding response in tobacco prior to high-level ozone exposure prevents necrotic injury. The Plant Journal: For Cell and Molecular Biology 11:203-212.

Overmyer, K., M. Brosché, and J. Kangasjärvi. 2003. Reactive oxygen species and hormonal control of cell death. Trends Plant Sci. 8:335-342.

Pangga, I.B., J. Hanan, and S. Chakraborty. 2011. Pathogen dynamics in a crop canopy and their evolution under changing climate. Plant Pathology 60:70-81.

Parrish, D.D., K.S. Law, J. Staehelin, R. Derwent, O.R. Cooper, H. Tanimoto, A. Volz-Thomas, S. Gilge, H.-. Scheel, M. Steinbacher, and E. Chan. 2013. Lower tropospheric ozone at northern midlatitudes: Changing seasonal cycle. Geophysical Research Letters 40:1631-1636.

Pask, A., J. Pietragalla, D.M. Mullan, and M.P. Reynolds. 2012. Physiological breeding II: A field guide to wheat phenotyping. CIMMYT., Mexico, D.F.

Pautasso, M., T.F. Döring, M. Garbelotto, L. Pellis, and M.J. Jeger. 2012. Impacts of climate change on plant diseases-opinions and trends. European Journal of Plant Pathology 133:295 313.

Pellinen, R., T. Palva, and J. Kangasjärvi. 1999. Subcellular localization of ozone-induced hydrogen peroxide production in birch (*Betula pendula*) leaf cells. The Plant Journal 20:349-356.

Percy, K.E., C.S. Awmack, R.L. Lindroth, M.E. Kubiske, B.J. Kopper, J.G. Isebrands, K.S. Pregitzer, G.R. Hendrey, R.E. Dickson, B.J. Kopper, K.E. Percy, D.F. Karnosky, R. Harrington, C.S. Awmack, J. Sober, E. Oksanen, D.R. Zak, R.L. Lindroth, G.R. Hendrey, K.S. Pregitzer, J.G. Isebrands, M.E. Kubiske, D.R. Zak, E. Oksanen, J. Sober, R. Harrington, and D.F. Karnosky. 2002. Altered performance of forest pests under atmospheres enriched by CO_2 and O_3. Nature 420:403-407.

Peterson, R.F., A.B. Campbell, and A.E. Hannah. 1948. A diagrammatic scale for estimating rust intensity on leaves and stems of cereals. Canadian Journal of Research 26c:496-500.

Pfleeger, T.G., M.A. da Luz, and C.C. Mundt. 1999. Lack of a synergistic interaction between ozone and wheat leaf rust in wheat swards. Environmental & Experimental Botany 41:195-207.

Pleijel, H., A.B. Eriksen, H. Danielsson, N. Bondesson, and G. Selldén. 2006. Differential ozone sensitivity in an old and a modern Swedish wheat cultivar-grain yield and quality, leaf chlorophyll and stomatal conductance. Environmental & Experimental Botany 56:63-71.

Pleijel, H., G. Wallin, P.E. Karlsson, L. Skarby, and G. Sellden. 1994. Ozone deposition to an oat crop (*Avena sativa* L.) grown in open-top chambers and in the ambient air. Atmospheric Environment 28:1971-1979.

Pretorius, Z.A., R.P. Singh, W.W. Wagoire, and T.S. Payne. 2000. Detection of virulence to wheat stem rust resistance gene *Sr31* in *Puccinia graminis*. f. sp. *tritici* in Uganda. Plant Disease 84:203-203.

Quarrie, S.A., A. Steed, C. Calestani, and et al. 2005a. A high-density genetic map of hexaploid wheat (*Triticum aestivum* L.) from the cross Chinese Spring × SQ1 and its use to compare QTLs for grain yield across a range of environments. Theoretical and Applied Genetics 110:865-880.

Quarrie, S.A., A. Kaminska, A. Dodmani, I. Gonzalez, C. Gillespie, P. Bilsborrow, and J. Barnes. 2007. QTLs governing ozone impacts on wheat yield. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 146:S261.

Quarrie, S.A., S.P. Quarrie, R. Radosevic, D. Rancic, A. Kaminska, J.D. Barnes, M. Leverington, C. Ceoloni, and D. Dodig. 2006. Dissecting a wheat QTL for yield present

in a range of environments: From the QTL to candidate genes. Journal of Experimental Botany 57:2627-2637.

Quarrie, S.A., M. Gulli, C. Calestani, A. Steed, N. Marmiroli, S.A. Quarrie, M. Gulli, C. Calestani, A. Steed, S.A. Quarrie, M. Gulli, C. Calestani, and A. Steed. 1994. Location of a gene regulating drought-induced abscisic acid production on the long arm of chromosome 5A of wheat. Theoret. Appl. Genetics 89:794-800.

Quarrie, S.A., A. Steed, C. Calestani, A. Semikhodskii, C. Lebreton, C. Chinoy, N. Steele, D. Pljevljakusić, E. Waterman, J. Weyen, J. Schondelmaier, D.Z. Habash, P. Farmer, L. Saker, D.T. Clarkson, A. Abugalieva, M. Yessimbekova, Y. Turuspekov, S. Abugalieva, R. Tuberosa, M.-. Sanguineti, P.A. Hollington, R. Aragués, A. Royo, D. Dodig, A. Royo, R. Aragués, S.A. Quarrie, P.A. Hollington, A. Steed, C. Calestani, A. Semikhodskii, C. Lebreton, C. Chinoy, N. Steele, D. Pljevljakusić, E. Waterman, J. Weyen, J. Schondelmaier, D.Z. Habash, P. Farmer, L. Saker, D.T. Clarkson, A. Abugalieva, M. Yessimbekova, Y. Turuspekov, S. Abugalieva, R. Tuberosa, and M.-. Sanguineti. 2005b. A high-density genetic map of hexaploid wheat (*Triticum aestivum* L.) from the cross chinese spring × SQ1 and its use to compare QTLs for grain yield across a range of environments. Theoretical and Applied Genetics 110:865-880.

Rao, M.V., H. Lee, R.A. Creelman, J.E. Mullet, and K.R. Davis. 2000. Jasmonic acid signaling modulates ozone-induced hypersensitive cell death. The Plant Cell 12:1633-1646.

Rapacz, M., M. Sasal, H.M. Kalaji, and J. Kościelniak. 2015. Is the OJIP test a reliable indicator of winter hardiness and freezing tolerance of common wheat and triticale under variable winter environments? Plos One 10:e0134820.

Roelfs, A.P., E.E. Saari, and R.P. Singh. 1992. Rust diseases of wheat: Concepts and methods of disease management. Maize and Wheat Improvement Center, Mexico, D.F.

Rogers, H.H., H.E. Jeffries, E.P. Stahel, W.W. Heck, L.A. Ripperton, and A.M. Witherspoon. 1977. Measuring air pollutant uptake by plants: A direct kinetic technique. Journal of the Air Pollution Control Association 27:1192.

Roll-Hansen, N. 1985. A new perspective on lysenko? Annals of Science 42:261-278.

Saini, R.S., H.K.L. Chawla, D.S. Wagle, R.S. Saini, and H.K.L. Chawla. 1988. Leakage of electrolytes and phenols and altered activity profile of peroxidase and its isoenzymes from wheat leaves inoculated with *Puccinia recondita*. Biologia Plantarum 30:387-392.

Salamini, F., H. Özkan, A. Brandolini, R. Schäfer-Pregl, W. Martin, H. Özkan, A. Brandolini, R. Schäfer-Pregl, and W. Martin. 2002. Genetics and geography of wild cereal domestication in the near east. Nat Rev Genet 3:429-441.

Sandermann, H., D. Ernst, W. Heller, and C. Langebartels. 1998. Ozone: An abiotic elicitor of plant defence reactions. Trends Plant Science. 3:47-50.

Santino, A., M. Taurino, S. De Domenico, S. Bonsegna, P. Poltronieri, V. Pastor, and V. Flors. 2013. Jasmonate signaling in plant development and defense response to multiple (a)biotic stresses. Plant Cell Reports 32:1085-1098.

Sasaki, M., R. Morris, J.W. Schmidt, and B.S. Gill. 1963. Metaphase I studies on F1 monosomics from crosses between the Chinese Spring and Cheyenne common wheat varieties. Canadian Journal of Genetics and Cytology 5:318-319.

Schenk, P.M., K. Kazan, I. Wilson, J.P. Anderson, T. Richmond, S.C. Somerville, and J.M. Manners. 2000. Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proceedings of the National Academy of Sciences of the United States of America 97:11655-11660.

Scherm, H., and S.M. Coakley. 2003. Plant pathogens in a changing world. Plant Pathology 32:157.

Scholthof, K.G. 2007. The disease triangle: Pathogens, the environment and society. Nature Reviews Microbiology 5:152 156.

Schwartz, J. 2016. The year of ozone. American Journal of Respiratory and Critical Care Medicine 193:1077.

Selldén, G., and H. Pleijel. 1995. Photochemical oxidant effects on vegetation response in relation to plant strategy. Water, Air, & Soil Pollution 85:111-122.

Sethi, S. 2015. Bread, wine, chocolate: The slow loss of foods we love. HarperOne, San Francisco.

Shiferaw, B., M. Smale, H. Braun, G. Muricho, M. Smale, B. Shiferaw, E. Duveiller, M. Reynolds, E. Duveiller, M. Reynolds, and G. Muricho. 2013. Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security. Food Sec. 5:291-317.

Short, E.F., K.A. North, M.R. Roberts, A.M. Hetherington, A.D. Shirras, and M.R. McAinsh. 2012. A stress-specific calcium signature regulating an ozone-responsive gene expression network in arabidopsis. The Plant Journal 71:948-961.

Sicard, P., R. Serra, and P. Rossello. 2016. Spatiotemporal trends in ground-level ozone concentrations and metrics in France over the time period 1999–2012. Environ. Research 149:122-144.

Singh, R.P., and J. Huerta-Espino. 2003. Effect of leaf rust resistance gene *Lr34* on components of slow rusting at seven growth stages in wheat. Euphytica 129:371-376.

Singh, R., and N. Jwa. 2013. Understanding the responses of rice to environmental stress using proteomics. Journal of Proteome Research 12:4652.

Singh, R.P., D.P. Hodson, J. Huerta-Espino, Y. Jin, S. Bhavani, P. Njau, S. Herrera-Foessel, P.K. Singh, S. Singh, and V. Govindan. 2011. The emergence of Ug99 races of the stem rust fungus is a threat to world wheat production. Annual Review of Phytopathology 49:465-481.

Singh, S., and S.B. Agrawal. 2010. Impact of tropospheric ozone on wheat (*Triticum aestivum* L.) in the eastern Gangetic plains of India as assessed by ethylenediurea (EDU) application during different developmental stages. Agriculture, Ecosystems and Environment 138:214-221.

Steuernagel, B., S.K. Periyannan, I. Hernández-Pinzón, K. Witek, M.N. Rouse, G. Yu, A. Hatta, M. Ayliffe, H. Bariana, J.D.G. Jones, E.S. Lagudah, and B.B.H. Wulff. 2016. Rapid cloning of disease-resistance genes in plants using mutagenesis and sequence capture. Nature Biotechnology 34:652-655.

Stevens, R.B. 1960. Cultural practices in disease control. p. 357–429. *In* D.A. Horsfall JG (ed.) Plant pathology: An advanced treatise. New York, NY: Academic Press.

Stevenson, D.S., F.J. Dentener, M.G. Schultz, K. Ellingsen, v. Noije T.P.C, O. Wild, G. Zeng, M. Amann, C.S. Atherton, N. Bell, D.J. Bergmann, I. Bey, T. Butler, J. Cofala, W.J.

Collins, R.G. Derwent, R.M. Doherty, J. vet, H.J. Eskes, A.M. Fiore, M. Gauss, D.A. Hauglustaine, L.W. Horowitz, I.S.A. Isaksen, M.C. Krol, J.F. Lamarque, M.G. Lawrence, V. Montanaro, J.F. Muller, G. Pitari, M.J. Prather, J.A. Pyle, S. Rast, J.M. Roiguez, M.G. Sanderson, N.H. Savage, D.T. Shindell, S.E. Strahan, K. Sudo, and S. Szopa. 2006. Multimodel ensemble simulations of present-day and near-future tropospheric ozone. Journal of Geophysical Research: Atmospheres 111:D08301.

Stubbs, R.W., J.M. Prescott, E.E. Saari, and H.J. Dubin. 1986. Cereal disease methodology manual. CIMMYT., Mexico, DF.

Tester, M., and P. Langridge. 2010. Breeding technologies to increase crop production in a changing world. Science 327:818-822.

Thatcher, F.S. 1943. Cellular changes in relation to rust resistance. Canadian Journal of Research 21c:151-172.

Thomason, W.E., C.A. Griffey, M.M. Alley 1947, E.L. Stromberg, Herbert, D. Ames (David Ames), 1949, and Hagood, E. S. (Edward Scott), 1952. 2009. Growing bread wheat in the mid-atlantic region.

Tiedemann, A. 1992a. Ozone effects on fungal leaf diseases of wheat in relation to epidemiology: II. biotrophic pathogens. Journal of Phytopathology 134:187-197.

Tiedemann, A.v. 1992b. Ozone effects on fungal leaf diseases of wheat in relation to epidemiology: I. Necrotrophic pathogens Journal of Phytopathology 134:177-186.

Tiedemann, A.v., and K.H. Firsching. 2000. Interactive effects of elevated ozone and carbon dioxide on growth and yield of leaf rust-infected versus non-infected wheat. Environmental Pollution 108:357-363.

Tiedemann, A.v., and K.H. Firsching. 1998. Combined whole-season effects of elevated ozone and carbon dioxide concentrations on a simulated wheat leaf rust (puccinia recondita f. sp. tritici) epidemic / kombinierte saisonale wirkung erhöhter ozon- und kohlendioxidkonzentrationen auf eine simulierte weizenbraunrostepidemie (*Puccinia recondita* f. sp. *tritici*). Zeitschrift Für Pflanzenkrankheiten Und Pflanzenschutz / Journal of Plant Diseases and Protection 105:555-566.

Tiedemann, A.v., and B. Pfähler. 1994. Growth stage-dependent effects of ozone on the permeability for ions and non-electrolytes of wheat leaves in relation to the susceptibility to *Septoria nodorum* berk. Physiol. Mol. Plant Pathology 45:153-167.

Tiedemann, A.v., H.-. Weigel, and H.-. Jäger. 1991. Effects of open-top chamber fumigations with ozone on three fungal leaf diseases of wheat and the mycoflora of the phyllosphere. Environmental Pollution 72:205-224.

Tottman, D.R. 1987. The decimal code for the growth stages of cereals, with illustrations. Annals of Applied Biology 110:441-454.

Tuominen, H., K. Overmyer, M. Keinänen, H. Kollist, and J. Kangasjärvi. 2004. Mutual antagonism of ethylene and jasmonic acid regulates ozone-induced spreading cell death in arabidopsis. The Plant Journal 39:59-69.

US EPA, O. 2016. Table of historical ozone national ambient air quality standards (NAAQS).

Vahisalu, T., I. Puzõrjova, M. Brosché, E. Valk, M. Lepiku, H. Moldau, P. Pechter, Y. Wang, O. Lindgren, J. Salojärvi, M. Loog, J. Kangasjärvi, and H. Kollist. 2010. Ozone-triggered rapid stomatal response involves the production of reactive oxygen species, and is controlled by SLAC1 and OST1. The Plant Journal 62:442-453.

Van Breusegem, F., E. Vranová, J.F. Dat, and D. Inzé. 2001. The role of active oxygen species in plant signal transduction. Plant Science 161:405-414.

Van Dingenen, R., F.J. Dentener, F. Raes, M.C. Krol, L. Emberson, and J. Cofala. 2009. The global impact of ozone on agricultural crop yields under current and future air quality legislation. Atmospheric Environment 43:604-618.

Vaultier, M., and Y. Jolivet. 2015. Ozone sensing and early signaling in plants: An outline from the cloud. Environmental & Experimental Botany 114:144-152.

Velissariou, D., J.D. Barnes, and A.W. Davison. 1992. Has inadvertent selection by plant breeders affected the O_3 sensitivity of modern Greek cultivars of spring wheat? Agriculture, Ecosystems and Environment 38:79-89.

Volz, A., and D. Kley. 1988. Evaluation of the Montsouris series of ozone measurements made in the nineteenth century. Nature 332:240-242.

Wang, K.L.C., H. Li, and J.R. Ecker. 2002. Ethylene biosynthesis and signaling networks. Plant Cell 14:S151.

Wang, X., Q. Zheng, Z. Feng, Z. Feng, J. Xie, Z. Ouyang, and W.J. Manning. 2008. Comparison of a diurnal vs steady-state ozone exposure profile on growth and yield of oilseed rape (*Brassica napus* L.) in open-top chambers in the Yangtze delta, China. Environmental Pollution 156:449-453.

Wattal, R.K., and Z.H. Siddiqui. 2015. Effect of elevated levels of carbon dioxide on the activity of RuBisCO and crop productivity. p. 241-256. *In* K.R. Hakeem (ed.) Crop production and global environmental issues. Springer International Publishing.

West, J.S., B.D.L. Fitt, J.A. Townsend, M. Stevens, J.A. Townsend, M. Stevens, and B.D.L. Fitt. 2012. Comparative biology of different plant pathogens to estimate effects of climate change on crop diseases in Europe. European Journal of Plant Patholology 133:315-331.

Wilkinson, S., and W.J. Davies. 2010. Drought, ozone, ABA and ethylene: New insights from cell to plant to community. Plant, Cell & Environment 33:510-525.

Wilkinson, S., and W.J. Davies. 2009. Ozone suppresses soil drying- and abscisic acid (ABA)-induced stomatal closure via an ethylene-dependent mechanism. Plant, Cell & Environment 32:949-959.

Wilkinson, S., G. Mills, R. Illidge, and W.J. Davies. 2011. How is ozone pollution reducing our food supply? Journal of Experimental Botany 63:527-536.

Wrzaczek, M., M. Brosché, J. Salojärvi, S. Kangasjärvi, N. Idänheimo, S. Mersmann, S. Robatzek, S. Karpiński, B. Karpińska, and J. Kangasjärvi. 2010. Transcriptional regulation of the CRK/DUF26 group of receptor-like protein kinases by ozone and plant hormones in Arabidopsis. BMC Plant Biology 10:1-19.

Xu, H., D.K. Biswas, W.D. Li, S.B. Chen, L. Zhang, G.M. Jiang, W.G. Li, H. Xu, D.K. Biswas, Y.-. Li, S.-. Chen, L. Zhang, and Y.-. Li. 2007. Photosynthesis and yield responses of ozone-polluted winter wheat to drought. Photosynthetica 45:582-588.

Yanez-Lopez, R., I. Torres-Pacheco, R.G. Guevara-González, M.I. Hernández-Zul, J.A. Quijano-Carranza, and E. Rico-García. 2012. The effect of climate change on plant diseases. African Journal of Biotechnology 11:2417-2428.

Yu, L.X., H. Barbier, M.N. Rouse, S. Singh, R.P. Singh, S. Bhavani, J. Huerta-Espino, and M.E. Sorrells. 2014. A consensus map for Ug99 stem rust resistance loci in wheat. Theoretical and Applied Genetics 127:1561-1581.

Zadoks, J.C., T.T. Chang, and C.F. Konzak. 1974. A decimal code for the growth stages of cereals. Weed Research 14:415-421.

Zeven, A.C. 1971. Fifth supplementary list of wheat varieties classified according to their genotype for hybrid necrosis and geographical distribution of ne-genes. Euphytica 20:239-254.

Zhang, H., B. Zhu, B. Qi, X. Gou, Y. Dong, C. Xu, B. Zhang, W. Huang, C. Liu, X. Wang, C. Yang, H. Zhou, K. Kashkush, M. Feldman, J.F. Wendel, and B. Liu. 2014. Evolution of the BBAA component of bread wheat during its history at the allohexaploid level. Plant Cell 26:2761-2776.

Zhang, W., C. Wang, C. Qin, T. Wood, G. Olafsdottir, R. Welti, and X. Wang. 2003. The oleate-stimulated phospholipase D, PLDδ, and phosphatidic acid decrease H_2O_2-induced cell death in Arabidopsis. Plant Cell 15:2285-2295.

Zheng, Y.H., Y.G. Li, W.R. Xia, H. Xu, B.Y. Su, G.M. Jiang, T.Y. Ning, Y.H. Zheng, G.M. Jiang, Y.G. Li, W.R. Xia, H. Xu, and B.Y. Su. 2011. Responses of gas exchange, cellular membrane integrity, and antioxidant enzymes activities of salinity-stressed winter wheat to ozone pollution. Photosynthetica 49:389.

Zuccarini, P. 2009. Tropospheric ozone as a fungal elicitor. Journal of Biosciences 34:125 138.