

Temporal processes that contribute to nonlinearity in vegetation responses to ozone exposure and dose

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ABSTRACT

Ozone interacts with plant tissue through distinct temporal processes. Sequentially, plants are exposed to ambient O₃ that (1) moves through the leaf boundary layer, (2) is taken up into plant tissue primarily through stomata, and (3) undergoes chemical interaction within plant tissue, first by initiating alterations and then as part of plant detoxification and repair. In this paper, we discuss the linkage of the temporal variability of apoplastic ascorbate with the diurnal variability of defense mechanisms in plants and compare this variability with daily maximum O₃ concentration and diurnal uptake and entry of O₃ into the plant through stomata. We describe the quantitative evidence on temporal variability in concentration and uptake and find that the time incidence for maximum defense does not necessarily match diurnal patterns for maximum O₃ concentration or maximum uptake. We suggest that the observed out-of-phase association of the diurnal patterns for the above three processes produces a nonlinear relationship that results in a greater response for the higher hourly average O₃ concentrations than from the lower or mid-level values. The fact that these out-of-phase processes affect the relationship between O₃ exposure/dose and vegetation effects ultimately impact the ability of flux-based indices to predict vegetation effects accurately for purposes of standard setting and critical levels. Based on the quantitative aspect of temporal variability identified in this paper, we suggest that the inclusion of a diurnal pattern for detoxification in effective flux-based models would improve the predictive characteristics of the models. While much of the current information has been obtained using high O₃ exposures, future research results derived from laboratory biochemical experiments that use short but elevated O₃ exposures should be combined with experimental results that use ambient-type exposures over longer periods of time. It is anticipated that improved understanding will come from future research focused on diurnal variability in plant defense mechanisms and their relationship to the diurnal variability in ambient O₃ concentration and stomatal conductance. This should result in more reliable O₃ exposure standards and critical levels.

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1. Introduction

Ozone interacts with plant tissue through distinct processes: (1) ambient O₃ moves through the leaf boundary layer, (2) O₃ is taken up into plant tissue primarily through stomata, and (3) O₃ undergoes chemical interaction within plant tissue, first as unregulated attack and injury to the tissue components, where O₃ is not immediately detoxified by antioxidants already present, and then as activation of further plant detoxification processes of repair. These processes temporally and often independently change throughout each day and generally occur out of phase from one

another. For example, maximum O₃ concentration often occurs in the late afternoon, while maximum stomatal aperture and uptake occurs during the late morning to early afternoon. Biochemical processes, which occur within plant tissue, produce antioxidants to detoxify O₃. These biochemical processes change throughout the day and may be most active during the time when maximum photosynthesis is occurring and antioxidants are produced; these processes are often less active during the evening and early morning hours. Fig. 1 illustrates the conceptual phasing of the temporal relationship among the three processes of external O₃ concentration, uptake, and detoxification. The interrelationships have not been the focus of most investigations and therefore little work has been done on the sequential timing of their occurrence.

The differences in temporal changes and the out-of-phase relationship among these processes may be responsible for the

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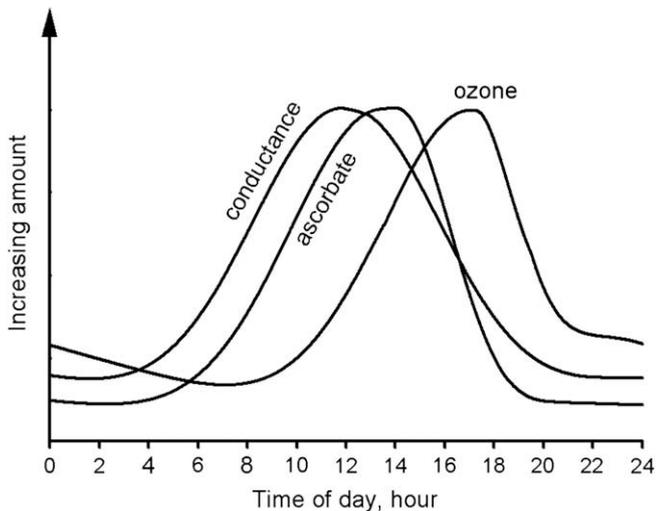


Fig. 1. Conceptual temporal relationship among ozone stomatal conductance, ozone concentration, and apoplastic ascorbate (i.e., surrogate for defense). The curves illustrate the phasing relationship among the three processes (see text).

nonlinearity of the response observed in research experiments which examined plant response to O_3 (Amiro et al., 1984; Amiro and Gillespie, 1985; Lefohn and Tingey, 1985; Fredericksen et al., 1995; Massman, 2004; US EPA, 2006; Musselman et al., 2006). The nonlinear response was observed when the higher hourly average concentrations elicited a greater effect than that elicited by the mid- and lower-levels. Initially, evidence for nonlinearity was observed in laboratory research (Musselman et al., 1983, 1986, 2006; Hogsett et al., 1985; US EPA, 2006) performed using controlled fumigation experiments. Exposures with the same O_3 concentration multiplied by time product (i.e., concentration times period of experiment), using differing numbers of peak O_3 concentrations, resulted in a difference in growth losses to vegetation. Additional evidence for the nonlinearity of exposure and vegetation response was observed from measurements of biological responses to uncontrolled ambient O_3 concentrations in a conifer forest ecosystem of the San Bernardino National Forest in California (Lee et al., 2003; Tingey et al., 2004; Musselman et al., 2006).

Because plant response is thought to be closely related to the amount of O_3 absorbed into leaf tissue, recent research has been focused on flux-based O_3 parameters. Although flux-based indices appear to have advantages over the exposure metrics due to their linkage to uptake into vegetation, experimental results are needed to identify and quantify possible defense mechanisms and their relationship to O_3 uptake in order to make flux-based indices a reliable predictor of plant response (Musselman and Massman, 1999; Massman et al., 2000, 2006; Tausz et al., 2007; Wieser and Matyssek, 2007; Matyssek et al., 2008).

Plants do not respond to O_3 in a simple linear relationship of either dose (moles area⁻¹) or entry rate (moles area⁻¹ sec⁻¹) with any multiple pathway system such as early senescence, growth, stomatal closure, wound-response, or tissue injury. At higher O_3 concentrations, one might expect a multiplicity of processes, which may initiate a trigger or a decline in protection. Biochemical processes vary temporally and thus, may be dependent upon the temporal changes associated with O_3 concentration, uptake, and defense. Environmental conditions, such as temperature, radiation, and moisture stress, also influence the biochemical processes.

Nonlinearity of biochemical reactions exists in O_3 response. In this paper we concentrate on possible reasons for the observed nonlinearity in exposure- and dose-response of vegetation to O_3 .

We expand upon the important contributing factors to the nonlinearity that have previously been discussed in the literature and focus on the temporal phasing of the ambient O_3 concentration, entry of O_3 into leaf tissues and cells of the plant, and especially the status of apoplastic antioxidant detoxicants within plant tissue with respect to time of day of exposure. This paper discusses additional research efforts that will contribute to the understanding of the processes and will lead to the development of more reliable exposure and dose metrics for the protection of vegetation in the standard-setting and critical levels process.

2. The identification and description of important temporally related processes

2.1. Introduction

In exploring nonlinearity between O_3 exposure/dose and vegetation injury and growth, the following processes and their temporal properties are important: (1) O_3 exposure, which includes the concentration of O_3 in the atmosphere with magnitudes of duration and frequency of exposure and persistence of the leaf boundary layer thickness; (2) the entrance of the O_3 into the tissues and cells of the plant, which includes stomatal and cuticular behavior, water potential of the leaf and its affect upon the stomatal behavior, and (3) the aspect of apoplastic antioxidants, especially the highly reactive compound of ascorbate, within the leaf and their possible regeneration and the further cellular events and changes to total metabolism. The final end point is the observable response of the plant, such as the loss of productivity, generation of chlorosis or necrosis, temporal shifts in development, and the plant's ability to tolerate other stresses.

Ozone can affect plants in different ways. Plants can experience a decline in assimilation and hence a slowing of growth. Plants can also experience less ability to reproduce, leading to less production of viable seeds or fruits. Ozone exposures can result in plants having less ability to mount a defense against pathogens or insects, leading to weaker plants more liable to be overcome by other stresses. Ozone exposures can result in the induction of early senescence, leading to lowered total productivity. While all these O_3 effects on plants are detrimental, they are not at all similar, neither in their expressed physiology nor in their triggering mechanisms. Each effect has a different but distinct end-point, depending upon the amount of "effective" O_3 dose (Musselman et al., 2006). Therefore, the specific exposure or dose that elicits an effect is dependent upon the end product (e.g., visible injury or damage) being described. Visible injury is the production of an event which can be visibly observed such as water-logging (loss of internal water from cells surrounding an air space within the tissue), chlorosis (loss of the chlorophyll of a region by either a decline in chlorophyll within a living cell or the death of a cell leading to bleaching of the chlorophyll), necrosis the death of a cell or a small region of cells, or bronzing (the formation of anthocyanine and polymerized quinones and protein within a living cell). Damage is a reduction in the intended value or use of the plant, including reductions in economic, ecologic, or aesthetic value (Musselman et al., 2006). The identification and quantification of the important mechanisms responsible for driving the biological processes of detoxification and repair are necessary because these mechanisms impact the ability of air quality standards to adequately protect vegetation.

Plants can alleviate stress from toxins such as O_3 either by avoidance through exclusion or restriction of that stress (e.g., O_3 uptake can be limited by stomatal closure) and/or by direct confrontation using metabolic detoxification of the species within the leaf (e.g., ascorbate or other antioxidants within the leaf cell's

wall). Confrontation, which is referred to by others as defense or repair (see Musselman et al., 2006), suggests the production of an antiozonide compound, such as ascorbate or a coherent sequence of reactions, to reestablish the status of the cells prior to detrimental O₃ impact.

Repair is the use of excess energy or carbon species to replace an altered component of the cell. While repair/replacement is an on-going process of metabolism, there are rogue processes, which yield altered compounds that cannot be changed by any form of metabolism of the cell. Radiation-induced cross-linkage of proteins is one such process, while some events can be reversed, such as radiation-induced T–T dimers of nucleotides (Vaisman et al., 2003). Repair suggests the presence of mechanisms to “undo” the high reactivity of O₃ and eliminate the altered biochemicals. The biochemical ability to carry out each separate step is distinct and has a very different cost to the cell. The defense mechanism is likely more rapid than the repair mechanism because some biochemicals of constitutive detoxification (see Musselman et al., 2006) are pre-existing (Mahalingam et al., 2006). Development or initiation of non-constitutive repair mechanisms suggests a time delay for these more complex processes to be activated. In addition to confrontation, work has been published describing how O₃ affects stomata responses, which can limit further uptake of O₃ (see summary by Heath, 1996; Heath, 2008).

Effective O₃ dose (Musselman et al., 2006) takes into consideration the temporal nature of conductance and concentration and involves the integration of O₃ uptake with internal physiological responses in order to properly evaluate plant performance under O₃ stress. If the biological mechanisms responsible for confrontation are not taken into consideration, predicted vegetation effects will be overestimated (Massman et al., 2000; Massman, 2004; Musselman et al., 2006). Confrontation mechanisms, which tend to be temporally dependent, serve to neutralize the initial dosage received, and as such, are dependent on the O₃ dosing rate or cumulative loading (Musselman and Massman, 1999). During late morning hours, plant photosynthesis rates are high and detoxification chemicals are produced (Musselman et al., 2006). Thus, morning O₃ fluxes may contribute relatively less to effective flux due to enhanced detoxification. However, in the late afternoon and evening hours, when photosynthesis is reduced, defenses may become overwhelmed, resulting in additional accumulation of injury or damage (Musselman et al., 2006). The diurnal patterns of stomatal uptake and apoplastic antioxidants are both critical in determining plant response for the understanding of nonlinearity in concentration- and dose-response for O₃ effects in vegetation. Reaction of O₃ or its product with ascorbate and possibly other antioxidants present in the apoplastic space of the mesophyll cells serves to decrease the amount of O₃ or product available to alter the plasma membrane of the cells. Not all the initial reactions of O₃ within the leaf are known but the involvement of hydrogen peroxide is apparent (Heath, 2008). In this section, we investigate the diurnal patterns for three processes of O₃: concentration, conductance, and defense mechanisms.

2.2. Diurnal variability of O₃ concentrations

At most low-elevation locations in the United States, the ambient O₃ concentration diurnal pattern varies during the day and location (Lefohn and Foley, 1993; Rombout et al., 1986; Plopper et al., 2000; US EPA, 2006), depending on the balance among the many factors affecting O₃ formation, transport, and destruction. At lower elevation monitoring sites, diurnal patterns for O₃ typically show a rise in concentration with an afternoon peak. As can be seen from Fig. 2, daily 1-h maxima in rural areas tend to occur in mid to late afternoon and daily 1-h minima tend to occur during the early

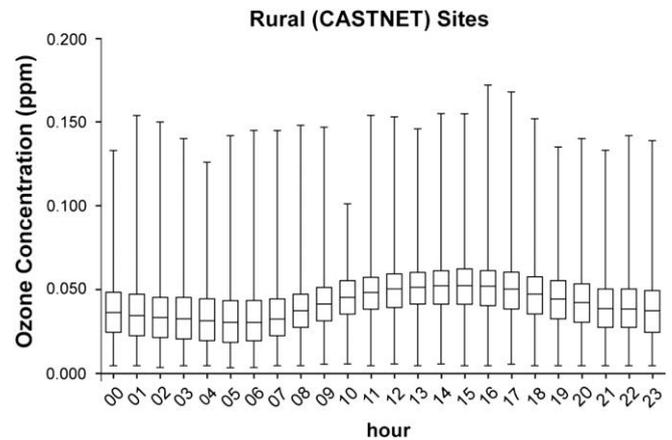


Fig. 2. Composite diurnal variability in hourly O₃ concentrations observed at CASTNET sites. Values shown are averages from April to October 2000 to 2004. Boxes define the interquartile range and the whiskers, the minima and maxima. Source: US EPA (2006).

morning, but exceptions do occur. Typically, high values (i.e., ≥ 0.10 ppm) occur during the daylight hours in the mid to late afternoon. However, at high-elevation sites or sites removed from the areas of O₃ production, high values can occur later in the evening (US EPA, 2006).

2.3. Temporal variability of the movement of ozone into the internal leaf space

Gas exchange through the stomata into the leaf is by passive diffusion, in which the flux into or out of the leaf is proportional to the gradient of the gas from outside to inside or vice versa with a proportionality constant of conductance. Ozone, which is reactive, moves into the leaf and through the mesophyll and enters on the abaxial surface. Chameides (1989), Moldau (1998), and Kollist et al. (2000) have attempted to calculate how much O₃ may be in the cell's water space and how reactive that space is towards the O₃. Their data provide an estimate of the concentration and replenishment rate of O₃ that might be observed within the cell. Even if the external concentration is constant, the flux of the varied passive diffusion pathways and solubility of O₃ may not yield an adequate replacement rate internally. In other words, rapid reactions within the cell's wall with O₃ may deplete the actual amount that can be present at the cell membrane, thereby changing the speed and types of reactions possible. This would then make a higher external concentration seemingly more reactive because it could continuously supply the internal amount of O₃ to the cell's membrane and thus keep the reactions going. A more detailed discussion of O₃ uptake and its concentration within the leaf is included in Musselman et al. (2006) and Matyssek et al. (2008).

Stomata conductance varies with time of day (Fig. 1). Light increases stomata conductance in the morning, but its high midday value decreases with higher afternoon temperature and soil moisture stress. The stomatal conductance drops still further as darkness falls, and may be near zero during the night. Evidence exists that for many species, stomata remain partially open at night (Musselman and Minnick, 2000; Grulke and Retzlaff, 2001). However, the uptake is less during the nighttime than during the daytime. Stomatal conductance is governed by the operation of the guard cells and is affected by a great number of variables, including light, water potential, temperature, accumulation of assimilates, and hormonal concentrations (Lu et al., 1997; Assmann, 2003; Klüsener et al., 2003; Christmann et al., 2006; Kang et al., 2007). Thus, it is difficult *a priori* to determine actual O₃ uptake.

2.4. Temporally related important defense processes: antioxidants within the apoplastic space

2.4.1. Introduction

Defense is believed to be associated with a range of antioxidants, which are highly reactive to O₃. If present in sufficient concentrations, antioxidant metabolites and enzymes, such as ascorbate, glutathione, and superoxide dismutase (Matters and Scandalios, 1987; Sasaki-Sekimoto et al., 2005; Pignocchi et al., 2006; Yoshida et al., 2006) trigger effective detoxification reactions. After O₃ enters the leaf tissue, it first reacts with extracellular antioxidants and that interaction appears to stimulate ascorbate production and/or its movement from one compartment to another (Heath, 2008). The production of H₂O₂ next to the membrane appears to both trigger a pathogen-like response and the generation of more H₂O₂. For example, several antioxidant proteins are stimulated by O₃ in *Nicotiana plumbaginifolia*, such as glutathione peroxidase, superoxide dismutase, and catalase (Willekens et al., 1994). The time scales for their changes are somewhat variable (Heath, 2007). Although the linkage of these chemical reactions is poorly understood, there are some basic observations regarding some of the significant compounds (Heath, 1984, 1987; Wellburn, 1990).

2.4.2. Ascorbate

Most of the recent results indicate that ascorbate within the cell wall is the primary line of cellular defense (Lyons et al., 1999a,b; Zheng et al., 2000; DeTemmerman et al., 2002; Heath, 2008; Matyssek et al., 2008), but other antioxidants contribute to detoxification of O₃ (van Hove et al., 2001; Booker and Fiscus, 2005; Cheng et al., 2007; Matyssek et al., 2008). Ascorbate has been used as a surrogate to model and quantify O₃ detoxification (Plöchl et al., 2000; US EPA, 2006; Heath, 2008). Ozone can induce the production of ascorbate (Kubo et al., 1995; Hofer et al., 2008). Apoplastic ascorbate has been shown to increase with an increase of stomata conductance and increasing flux of O₃ (Eller and Sparks, 2006), although that variation is quite variable (linear rate of flux with ascorbate has an r^2 of 0.252 for $N = 18$). Haberer et al. (2006) also report that apoplastic ascorbate is related to O₃ concentration and to air temperature and solar radiation. They suggest that the O₃ and ascorbate correlation may be related to the independent effects of radiation on O₃ and on ascorbate.

Elevated extracellular ascorbic acid is likewise related to O₃ tolerance (Lee et al., 1984; Luwe et al., 1993; Burkey and Eason, 2002). Burkey and Eason (2002) and Burkey et al. (2003) reported that elevated extracellular ascorbic acid was associated with O₃ tolerance in snap bean genotypes that exhibited a wide range of O₃ sensitivity and concluded that ascorbic acid in the leaf apoplast plays a role in plant response to O₃ stress. Hofer et al. (2008) reported that higher concentrations of ascorbate in spruce needles occurred in a year that experienced high temperature and high ambient O₃ concentrations compared to a second year with lower temperature and lower ambient O₃ exposure. Even though ambient O₃ was lower in the second year, stomatal conductance and O₃ uptake were higher, resulting in greater potential for O₃ depletion of the apoplastic ascorbate pool. However, it is unclear whether the lower ascorbate observed during the second year resulted from O₃ depletion of ascorbate or lower ascorbate production.

Ascorbate within the wall declines when the tissue is exposed to O₃ (Luwe et al., 1993; Moldau, 1998; Turcsányi et al., 2000; Zheng et al., 2000) and that decline appears to be somewhat related to the amount of O₃ penetrating the leaf tissue. Using diffusion theory, Chameides (1989) calculated that almost all of the O₃ entering plant leaves through stomata would react with ascorbic acid in the walls of plant cells without reaching the cell membranes. In this sense, ascorbic acid functions as a sacrificial antioxidant. Chameides (1989)

hypothesized that ascorbate in the apoplasm of the mesophyll cells would react with O₃. He based his calculations upon an internal concentration of O₃ of about 20 ppb and found that the length of the pathway from the surface of the apoplast to the plasmalemma would influence to what extent O₃ would react with ascorbate. Because ascorbic acid reacts more rapidly with singlet oxygen than with O₃ (Giamalva et al., 1986; Rougée and Bensasson, 1986) almost all of the singlet oxygen generated by a decomposition of O₃ would be expected to react with ascorbic acid in the cell wall or be quenched by water before reaching the cell membrane (Kanofsky and Sima, 1995). The increased tolerance of O₃ for some plant leaves having high ascorbic acid contents (Lee et al., 1984; Luwe et al., 1993) is consistent with this model of O₃ inactivation by ascorbate.

Ascorbate is water-soluble and is highly reactive to oxidative agents. Pools of ascorbate exist throughout most cells and so measurements within the total leaf tissue can provide only an average, which can lead to inaccurate conclusions. For example, ascorbate is present within the wall, within the cytoplasm (Moldau, 1998; Burkey, 1999), and within the chloroplasts (Law et al., 1983). Turcsányi et al. (2000) measured about 38 times higher concentration of ascorbate in the apoplast than that in the remainder of the cell, yet the volume of the wall is a small percentage of the total volume of the cell. Thus, a 50% loss of apoplastic ascorbate can be converted into only few percent loss of total ascorbate, due to the volume ratios. Lyons et al. (1999b) indicate that apoplastic ascorbate accounts for only about 1% of total ascorbate in leaf tissue, but the turnover rate of apoplastic ascorbate still provides significant protection from O₃. Total ascorbate may not be as important as apoplastic ascorbate because total ascorbate represents an average of all the pools in varied organelles of the cell (Burkey, 1999; Kollist et al., 2000). The rates of replenishment of apoplastic ascorbate are crucial; cytoplasmic ascorbate can be rapidly transported into the apoplasm (Bichele et al., 2000) and there is a mechanism to reduce dehydroascorbate back to ascorbate in the apoplastic space (Kollist et al., 2001). The speed of these reactions is very dependent upon species and the leaf's metabolic state.

The family of ascorbate peroxidase (which uses ascorbate to detoxify peroxides by forming dehydroascorbate) consists of at least five different isoforms, with isozymes in the apoplastic and cytosolic space (Yoshimura et al., 2000). Since ascorbate (in most reduction/oxidation forms) can move through the plasma membrane (Bichele et al., 2000), the levels of all forms of ascorbate are interdependent and moderately influenced by each other. Dehydroascorbate (DHA) can also be broken down, representing a continuous loss of ascorbate from varied parts of the cell. The turnover rate in leaves has been estimated to be from 2 to 13% per hour, depending upon species and developmental age (Smirnoff et al., 2001).

In addition, the level of total ascorbate is influenced by illumination of the leaves. It has been found that plants grown in high light conditions have more ascorbate present than those grown in low light (Tausz et al., 2004). This is more than likely due to a stimulation of the pathway caused by light; the last enzyme step in the synthesis of ascorbate is controlled by a light-activated gene (Tamaoki et al., 2003). The level of all of the ascorbate species is highest during midday; these concentrations are decreased by about 50% at night (Peltzer and Polle, 2001). Also, the redox potential of ascorbate declines in midday; the percent of dehydroascorbate then rises. Ascorbate declines in the evening and the decline is probably linked to oxidative and replacement processes. Any changes in apoplastic ascorbate and reductive power for maintenance of reduced ascorbate levels must come from the cytoplasm processes (Horemans et al., 2000). Ascorbate has a baseline level during all portions of the day, but increases during the daylight hours; its level appears to be dependent upon assimilation. Although this might suggest the use of photosynthesis as an indication of defense capability (Musselman

and Massman, 1999; Massman, 2004), we caution that photosynthesis is not equivalent to apoplastic ascorbate as a surrogate for defense. In the absence of diurnal information about apoplastic ascorbate, the substitution of photosynthesis data as a surrogate for apoplastic ascorbate (and perhaps other antioxidants) may introduce greater uncertainty for estimating detoxification.

Apoplastic ascorbate concentrations of barley and wheat were considerably higher at 0900 and 1300 h than at 1700 h (Kollist et al., 2000), indicating a late afternoon depletion. Burkey et al. (2003) reported apoplastic ascorbate of beans to be higher in early afternoon (1300–1500 h) compared to early morning (0600–0800 h). Fig. 1 shows an example of a conceptual diurnal pattern of apoplastic ascorbate. The apoplastic ascorbate curve shown in the figure is a composite derived from data from Kollist et al. (2000), Peltzer and Polle (2001), and Burkey et al. (2003). While there are other data sets (e.g., Verdaguer et al., 2003) that show no diurnal variation during the day, these studies describe total activity rather than diurnal variation of each individual pool of antioxidants.

2.4.3. Other reactions

Ascorbate has the potential to react with much of the entering O_3 until the ascorbate concentration becomes very low; O_3 then begins reacting with components of the membrane. We do not know exactly what compounds are formed once O_3 enters the tissue. However, hydrogen peroxide is certainly one of the major species. Other possible species can affect the rates and possible reaction pathways (Chelkowska et al., 1992; von Gunten, 2003).

In the past, hydrogen peroxide was thought to be a purely toxic compound for cells. However, plant cells generate hydrogen peroxide (called Reactive Oxygen Species—ROS) for specific purposes (see Neill et al., 2002), especially to attack other invading organisms in plant defense during most wounding events (Mehdy, 1994; Simon-Plas et al., 1997). The presence of higher than normal levels of H_2O_2 within the apoplastic space could be a signal that the well-studied pathogen defense pathway has been triggered. Fig. 3 illustrates this

pathway with some of its varied processes; these processes are suggestive that many, if not all the events which activate pathways or genes, should be observed upon O_3 fumigation of plants. The pathway begins with O_3 generating H_2O_2 in the apoplastic space, followed by these oxidative compounds interacting with the plasma membrane, presumably by changing its permeability to Ca^{2+} (Heath, 2008; Matyssek et al., 2008). That “step” then triggers a wounding-like response by generating Reactive Oxidative Species (ROS), which leads to a build up of H_2O_2 within the tissues and in sequence a spread of messages increasing the activity of MAP (Mitogenetic Activating Protein) kinases and other protein kinases (protein cascades). These diverging agents change metabolism and lead to a rise in the production of ascorbate and other wound responsive elements, such as ethylene and other phytohormones (Matyssek et al., 2008). These elements lead to more general responses, such as resistance to virulent pathogens, programmed cell death, and increased senescence and all the varied cellular responses linked to these larger effects. Many of the events illustrated in Fig. 3 have been shown to be events that O_3 exposure triggers, leading some to suggest a common inducer event (Sandermann et al., 1998; Heath, 2007). Additional information is available in Matyssek et al. (2008).

3. Discussion

3.1. Integrating the temporal association of ozone concentration, uptake, and defense to improve vegetation response relationships in standard-setting and critical levels analyses

The observed nonlinearity in exposure- and dose-response of vegetation to O_3 appears to be related to the temporal out-of-phase occurrence of the (1) the highest hourly average O_3 concentrations, (2) the maximum flux of O_3 into the tissues and cells of the plant, and (3) the maximum detoxification of O_3 , as indicated by the highest level of apoplastic ascorbate. Ascorbate within the cell wall appears to be the primary line of cellular defense. Fig. 4 presents

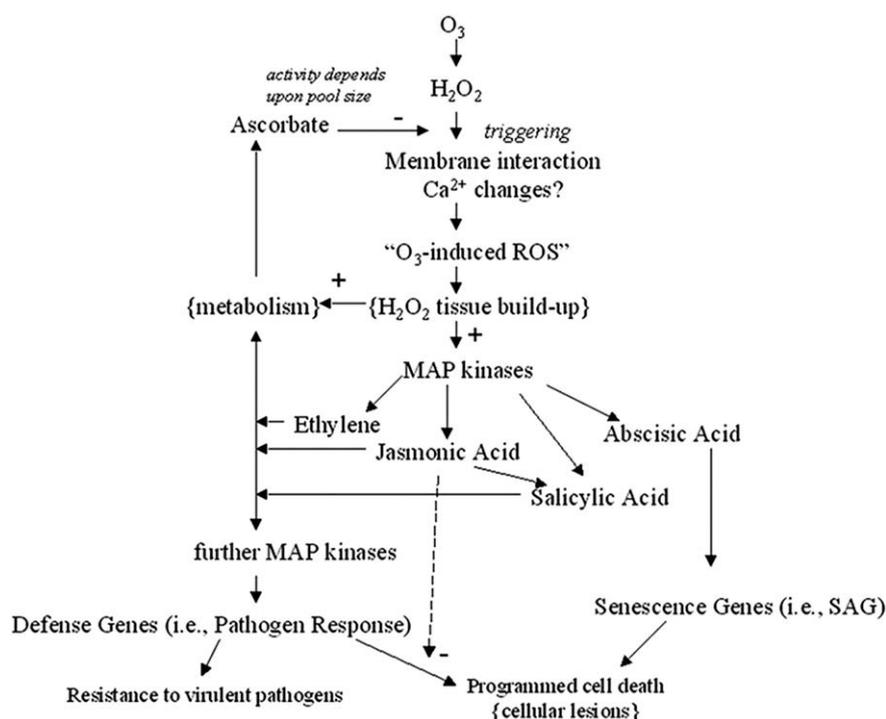


Fig. 3. A schematic representation of the interactions of the first step of O_3 -triggered reactions within the cell with other diverse secondary reactions. This figure is adapted from Conklin and Barth (2004).

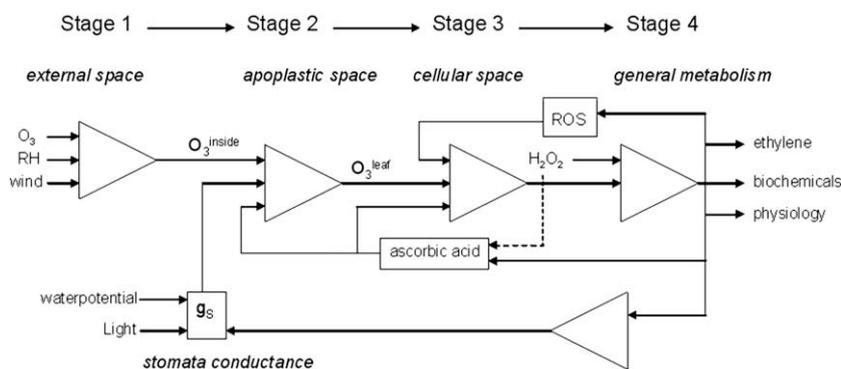


Fig. 4. The interaction of the processes of O₃ entry into the leaf, reactions within the apoplasts and initial reactions within the cell. The triangles represent conversion and reactions transforming O₃ in the area. The lines represent discussed interactions. The dotted line is used for clarity and does not represent other processes. For more details, see text.

a conceptual summary of the possible stages that occur when O₃ is exposed to the plant. Stage 1 involves the plant being exposed to O₃ with the sensitivity of the plant to the exposure being affected by edaphic conditions, such as temperature, relative humidity (RH) and wind, and possible reactions on the leaf's surface. Stage 2 involves the conductivity of the plant's stomata being affected by light and the water potential of the leaf. We have not discussed these stages in depth. Stage 3 involves additional processes that provide additional defense capabilities, such as the generation of H₂O₂ from O₃ and its interaction with Reactive Oxygen Species (ROS). Stage 4 involves the "wounding" process that results in changes in general metabolism, which includes both photosynthesis to provide the needed carbon skeletons and energy for the other processes and the emissions of ethylene. In their review of O₃ entry and biochemical changes, Matyssek et al. (2008) have pointed out that extensive shifts occur in biochemistry and carbon flow through varied pathways. These shifts likely force the end products of Stage 4 reactions, as described in Fig. 4, to affect homeostasis and to be dependent upon time of day as well as the season.

Although ethylene is not strictly considered to be part of the defense system, the temporal relationship of increased ethylene production can be an important indicator of the efficiency of the defense/repair processes. If the defense process is overcome, then "wounding" results. Ethylene release is consistent with any sort of wounding response or damage to leaf tissue. Ethylene production was identified early on as a plant response to O₃ exposure (Tingey et al., 1976; Wellburn, 1990). Tingey et al. (1976) showed that for a variety of plants, the observed foliar injury (induced by up to 750 ppb O₃ for 4 h) correlated with the production of stress ethylene and the amount of ethylene released was exponentially related to the external concentration of O₃ to which the plants were exposed. In addition, authors reported that the amount of O₃-induced ethylene release was reduced by repeated exposure, indicating a type of "acclimatization" or increased defensive reactions to O₃. It has been suggested that the isoprene emission by some plants would tend to react with or "soak up" O₃ within the leaf, similar to ethylene release (Loreto and Velikova, 2001). A reaction with ethylene would be expected to dominate the reaction with O₃, when extracellular ascorbate concentration was low.

The observed temporal out-of-phase occurrence of the (1) maximum O₃ concentrations, (2) the maximum flux of O₃ into the tissues and cells, and (3) the maximum detoxification of O₃, affects the application of flux-based indices in predicting vegetation effects. Flux-based indices have an advantage over exposure indices because they are linked to uptake. However, Yun and Laurence (1999) showed that conductance alone was not adequate for predicting plant response. Defense and repair components are very important in protecting the vegetation from O₃ exposures. Pleijel

et al. (2002) have applied an instantaneous flux threshold that attempts to take into consideration defense mechanisms. However, a problem in using a statistically derived threshold as a surrogate for defense processes is evident in this work. Most of the flux calculated by the authors was associated with concentrations below 0.06 ppm because the conductance was highest when the concentrations were below 0.06 ppm (Musselman et al., 2006). Pleijel et al. (2002) associated most of the measured effects with concentration at the lower end of the distribution. These results do not agree with the observations from controlled and uncontrolled experiments that show the importance of the higher O₃ concentrations in plant response.

Depending upon the sensitivity of the plant, it is likely that low concentrations of O₃ result in little internal effect beyond depletion of apoplastic ascorbate. Above a certain level of O₃ replenishment or loading, the cell will shift into a new state of response that is different than it was under a low O₃ concentration. Given the inherent complexity of the reactions, the continuous changing of O₃ loading, the age-related changes in plant sensitivity, and the continually changing environmental conditions, there can be no temporally related constant threshold that is relevant to the changing states of plant responses. Similar conclusions about the use of thresholds have been discussed for human response to O₃ (Hazucha and Lefohn, 2007). For vegetation exposed to O₃, the trigger for response varies with species, plant stage of development, and physiological and biochemical condition, as influenced by environmental stresses which include O₃ loading.

Vegetation models, which relate to the standard-setting and critical level process, do not currently take into consideration the combination of uptake and detoxification processes. These models do not provide sufficient predictive power when applied under ambient ecosystem conditions. Detoxification processes are dynamic and cannot be represented in response modeling by a constant threshold value. Flux-based models that use a fixed threshold do not allow for the temporal (i.e., daily and seasonal) variability of defense mechanisms and the predicted results associated with these models may not provide consistent results. Musselman et al. (2006) show in an example that employing a flux threshold preferentially weights the daylight hours between 1000 h and 1500 h. The use of a flux threshold reduces the cumulative dose during the morning hours, during which plant photosynthesis is high and detoxification chemicals are being produced. This is in agreement with the expectation that the morning O₃ fluxes should contribute relatively less to the cumulative total. However, in the afternoon hours, when photosynthesis is reduced (from stress or carbohydrate loading) and defenses may become overwhelmed, additional accumulation of dose is occurring. The application of a constant flux threshold does not take into consideration the additional accumulation occurring

during the late afternoon, nighttime, and early morning hours. The application of a flux threshold *underemphasizes* or eliminates the fluxes occurring at these biologically important times. As a result of reviewing the evidence for the temporal variability associated with the apoplastic ascorbate, we suggest that the temporal variability of ascorbate can be used as a surrogate to estimate the diurnal patterns for O₃ detoxification potential. We recognize other antioxidants contribute to detoxification of O₃ (van Hove et al., 2001; Booker and Fiscus, 2005; Cheng et al., 2007; Matyssek et al., 2008). Because the production of antioxidants is related to the photosynthetic process, the diurnal pattern of the other antioxidants that may be associated with the primary line of cellular defense may be similar to the apoplastic ascorbate pattern. We suggest that by linking the diurnal information associated with apoplastic ascorbate with the diurnal patterns of uptake and hourly O₃ concentrations, a better quantification of effective flux (Musselman et al., 2006) may be achievable and thus, a better estimate of plant response to O₃ attainable. In the absence of relevant antioxidant data, one might consider using the diurnal pattern of ambient light as a surrogate for changes in antioxidants. However, using light is indirect, resulting in increased uncertainty in quantifying the temporal antioxidant level.

Effective flux is the balance between stomatal flux and intra-leaf detoxification (Musselman et al., 2006). With the inclusion of the diurnal pattern for detoxification in effective flux-based models, it would be anticipated that the observed nonlinearity between O₃ exposure and vegetation effects would be exhibited within the predictive characteristics of the model. This improvement of the modeling predictions has the potential for providing better input for establishing relevant O₃ exposure–response and dose–response standards and critical levels.

Nonlinearity is not only observed for O₃ exposure and vegetation effects. Results from human experiments, using controlled laboratory exposures of volunteers, indicate a difference in hour-by-hour human physiologic response to variable (i.e., triangular) exposures and constant concentration O₃ exposures. Results by Hazucha et al. (1992) and Adams (2003, 2006a,b) suggest that the higher hourly average concentrations elicit a greater effect than the lower hourly average values in a nonlinear manner (Hazucha and Lefohn, 2007). Similar to the vegetation experiments, the results from human experiments, using controlled laboratory exposures, showed that the absolute value of the high (i.e., O₃ ≥ 100 ppb) hourly average concentrations (US EPA, 2006) affect the dynamic FEV₁ (forced expiratory volume at 1 s) responses more than the mid- and lower-level concentrations, resulting in a nonlinear relationship between dose and FEV₁ response. FEV₁ is a routine spirometric measure of lung function impairment which is employed systematically in human studies to assess the effects of exposure. Hazucha and Lefohn (2007) concluded that the nonlinear dose–response relationship for human health would result in the current 8-h average O₃ standard not adequately describing the hour-by-hour pattern of response of FEV₁.

3.2. Future research direction

Future research is needed to focus on the plant defense mechanisms and diurnal variability associated with the important mechanisms discussed in this paper. Specifically, more research is needed on quantifying the diurnal pattern of apoplastic ascorbate and other important antioxidants associated with O₃ detoxification. There are two general methodologies of testing plant responses to O₃ exposure. One method focuses on understanding the biochemical mechanisms and involves applying high levels of O₃ for a short period of time (i.e., 3–8 h). The processes disrupted after applying these exposures are deemed to be part of the total mechanisms, which result in “injury” to the plant and its systems.

Much of the current information described in this paper on defense mechanisms has been obtained using high O₃ exposures. A second exposure method, with the goal of understanding the long-term physiological response to ambient O₃, involves levels and timing of O₃, which closely match those observed under ambient conditions. More research is needed that focuses on the responses of vegetation to long-term O₃ exposures at ambient concentrations. The long-term ambient exposures are more closely linked to the developmental processes of the plant and are often ultimately responsible for changes to the net production of the plant.

Which exposure method is best depends upon what questions are being addressed. For regulatory purposes, the ambient type of exposure is required because it matches those exposures that occur under normal conditions. However, besides observing declines in total productivity using long-term exposures, additional exposure protocols are often required in order to better understand the processes affecting biochemical mechanisms. Although we cannot necessarily assume that the same biochemical reactions and physiological processes will proceed under both the high concentration laboratory exposures and the ambient-level exposures, the higher laboratory exposures provide us insight into the physiological and biochemical processes that may occur, particularly when vegetation is exposed to peak O₃ concentrations under ambient conditions.

Few long-term biochemical studies have been undertaken because of the additional complications identifying responses specific only to O₃. Because the whole plant's physiology is continually shifting during these long-term experiments, both control and enhanced O₃ exposed plants should be tested and compared throughout the entire time of the experiment. Normal changes in the baseline physiological condition of the control plants during these long-term experiments should be quantified when comparing response to experimental O₃ treatments.

The underlying short-term biochemical studies provide insights on what reactions might be expected from longer-term O₃ exposures. Biochemical data currently available suggest that coherent research on long-term exposure should be designed using those data, if we can sort out which reactions are most likely to echo or predict production alterations. For example, if high O₃ exposure for a short-time period results in an increase in a specific m-RNA, one could identify whether a similar response is observed under ambient exposure. Thus, a good understanding of the results from a laboratory biochemical exposure is critical to begin the formulation and understanding of physiological responses to O₃ under ambient exposure.

Many of the changes observed under ambient but short-term exposure, such as those for total ascorbate and superoxide dismutase, have been classified as “non-significant”. However, these compounds may be associated with long-term changes; the total pools may change over the long term and result in developmental alterations over weeks of exposure. Thus, a balance of both short-term, high concentration and longer-term ambient-type exposure experiments are required to adequately identify and quantify the changes that occur to those important biochemical mechanisms that are responsible for O₃ detoxification.

4. Summary and conclusions

The concept of the out-of-phase relationships among the three important processes: conductance, O₃ concentration, and detoxification (i.e., apoplastic ascorbate), is important in flux modeling. The out-of-phase relationship among the three processes appears to explain the observed nonlinearity associated with the greater weighting of the higher hourly average O₃ concentrations versus the mid- and lower-level values in affecting vegetation injury and

damage. The focus on detoxification variation should not be separated from conductance and O₃ temporal variation; rather all three processes should be evaluated together. While conductance and O₃ variation are included in flux-based models, the current threshold-based flux models do not predict the greater weighting of the higher hourly average O₃ concentrations that has been observed in the laboratory and in the field under ambient conditions. While research has quantified information on the temporal variability of conductance and O₃ concentrations, a paucity of information exists on the temporal variability of apoplastic ascorbate or other important antioxidants. Because of the importance of the temporal variability of detoxification processes, both long-term and short-term research is needed to better quantify the temporal variability of the important detoxification processes that affect vegetation response.

The dramatic strides in understanding the genetic make-up of plants, gene control, and signal transduction/control over the last few years will continue to accelerate in the future. That knowledge will translate into better models and more detailed schemes of how O₃ alters the basic metabolism of plants. While additional work is required on a larger variety of species under varied exposure conditions, the following conclusions can be drawn:

1. The penetration of O₃ into the leaf through the stomata remains the critical step of O₃ sensitivity. Not only does O₃ appear to modify the opening of the stomata, partially closing it, but O₃ also appears to alter the response of stomata to other stressful situations, including a lowering of water potential within the leaf and ABA responses. The level of O₃ within the leaf is considerably lower than that of the external concentration due to varied reactions within leaf tissue. However, its value is above “zero” if O₃ uptake is continuing;
2. The initial reactions of O₃ within the leaf are still unclear. However, reaction of O₃ or its product with ascorbate and possibly other antioxidants present in the apoplastic space of the mesophyll cells is clear and serves to lower the amount of O₃ or product available to alter the plasma membrane of the cells. The detoxification of O₃ taken up by the leaf is clearly complex and temporal. In the absence of relevant antioxidant data, one might consider using the diurnal pattern of ambient light or rates of photosynthesis as surrogates for changes in antioxidants. However, using light or photosynthesis is indirect, resulting in increased uncertainty in quantifying the temporal antioxidant level;
3. There is not a simple threshold level of O₃ for plant response because of the diurnal and seasonal variability of detoxification processes;
4. For relevance of standard setting, laboratory biochemical experiments should utilize environmental, developmental, and temporal conditions that mimic ambient-type exposures over longer periods of time versus the high concentrations applied over short-time periods;
5. The short-term, high O₃ concentration experiments provide insight into the physiological and biochemical processes that may occur, particularly when vegetation is exposed to peak O₃ concentrations under ambient conditions;
6. The nonlinear behavior between O₃ exposure and vegetation response appears to be associated with the out-of-phase differences of the temporal relationship among elevated hourly average O₃ concentrations, uptake, and detoxification mechanisms;
7. Additional research is needed to quantify the temporal variability of apoplastic ascorbate and other antioxidants; and
8. By linking the diurnal information associated with apoplastic ascorbate with the diurnal patterns of uptake and hourly O₃

concentrations, a better quantification of *effective flux* may be achievable and thus, a better estimate of plant response to O₃ attainable, resulting in more reliable inputs in the standard-setting and critical levels processes.

Over the last few decades, understanding of how O₃ interacts with the plant at a cellular level has been dramatically improved. However, the translation of defense mechanisms into better understanding how O₃ is involved with the altered cell metabolism, whole plant productivity, and other physiological facts has not yet been fully solved and will require additional and better-coordinated work. Combining the results derived from laboratory biochemical experiments using short but elevated O₃ exposures alongside results from experiments performed under ambient-type exposures over longer periods of time will provide new and potentially useful insights. The two exposure protocols may be different but with proper scrutiny, their results should provide us with a better understanding of how best to apply exposure and dose indices to protect vegetation.

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