

# Biochemical Reactions of Ozone in Plants<sup>1</sup>

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## Abstract

*Plants react biochemically to ozone in three phases: with constitutive chemicals in the apoplastic fluid and cell membranes; by forming messenger molecules by the affected constitutive materials (ethylene); and by responding to the messenger molecules with pathogenic RNAs and proteins. For instance, plant reactions with ozone result in constitutive molecules such as the ozonolysis of ethylene in the gas spaces of the leaf, and the reaction with ascorbic acid in the apoplastic fluid. Formation of messenger molecules include the stimulation of ethylene production. Responses to the messenger molecules include the formation of the pathogen related proteins and their mRNAs. Reactions of ozone with biological molecules also frequently result in classical ozonolysis of double bonds, with the production of various aldehydes and peroxides, such as ethylene, isoprene, fatty acids, tryptophan, and some phenylpropenoic acids. Some reactions of ozone with biological molecules do not fit the classical ozonolysis mechanism, such as the oxidation of methionine and some phenylpropenoic acids.*

## Introduction

Although ozone has toxic effects on both plants and animals, the mechanisms by which the toxic effects are elicited are inadequately understood. However, plants react biochemically to ozone in three phases: with constitutive cells and materials; by generating messenger molecules; and by responding to the messenger molecules.

Reactions with constitutive cells and materials result in substances in the gas phase, (e.g., ethylene) that are dissolved in the apoplastic fluid, (e.g., ascorbic acid) and that react with the lipids and proteins of the plasma membrane of cells surrounding the sub-stomatal cavity. The concentration of ozone in the leaf spaces inside the leaf is zero (Laisk and others 1969). This indicates that ozone passing through the stomata is immediately consumed by oxidizable substances lining the leaf spaces. Because lesions characteristic of ozone damage occur in the palisade parenchyma, palisade cells are part of the sink for ozone.

Reactions of messenger molecules cause stimulation of the synthesis of molecules such as ethylene, while the response of messenger molecules results in the formation of messenger RNA and the proteins for which they provide the genetic code. However, because these three stages of response to ozone are simply an analysis of the problem, a practical method of protecting plants needs to be developed.

This paper discusses the biochemical reactions of plants to ozone, focusing on the reactions of constitutive cell materials, the synthesis of messenger molecules, the responses of the messenger molecules, and various methods to protect plants from ozone damage.

## Discussion

### *Ozone Reaction with Constitutive Materials*

#### **Ethylene**

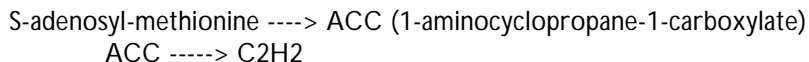
Ethylene has been known as a "ripening hormone." The arrival of the ripening is coincident with a "climacteric" in ethylene production. In addition to this

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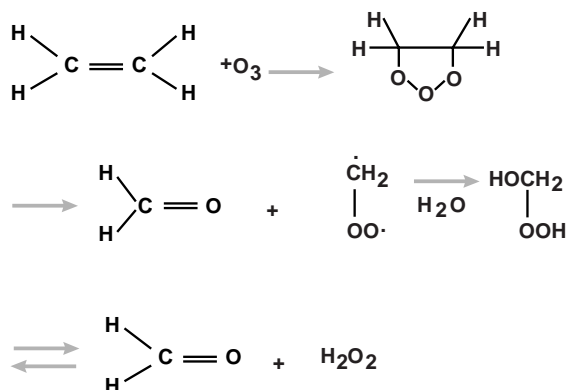
function in normal development, ethylene is known to be produced under various forms of stress. The biosynthesis of ethylene has posed a problem that has consumed many years of research, but it is now clearly understood as the following function:



The two critical enzymes are ACC synthase (which is the rate-limiting enzyme), and ethylene synthetase.

The reaction of ozone with ethylene in the gas phase involves a classical ozonolysis (*fig. 1*). The degradation products include formaldehyde, hydroxymethylhydroperoxide (HMHP), and hydrogen peroxide. Mehlhorn and Wellburn (1990) and Hewitt and Kok (1991) have suggested that ozone toxicity is a consequence of the reaction of ozone with ethylene. Mehlhorn and others (1990) attribute radicals detected after exposure of plants to ozone to the reaction of ozone with ethylene. Are these reactions taking place in the atmosphere? Hellpointer and Gab (1989) measured the peroxide content in rainwater collected on different days (*table 1*). The major peroxide usually found was hydrogen peroxide. Small amounts of HMHP were also detected. These measurements show that the reaction of ozone with ethylene in the atmosphere is a clear possibility. In the gas phase reaction it was found that hydroxyl radicals could be detected by the conversion of cyclohexane to cyclohexanol and cyclohexanone (Atkinson and others 1992). The yield of radicals was 0.12 per mole of ethylene reacted.

**Figure 1** — Reaction of ozone with ethylene.



**Table 1** — Peroxides in rainwater (Hellpointer and Gab 1989).<sup>1</sup>

| H   | H <sub>2</sub> O <sub>2</sub> <sup>2</sup> | HMHP <sup>3</sup> | HEHP <sup>4</sup> | CH <sub>3</sub> OOH <sup>5</sup> |
|-----|--|-------------------|-------------------|----------------------------------|
| 5.9 | 7  | 0.2               | 0.1               | 0.2                              |
| 6.7 | 63.1                                       | nd                | nd                | 0.3                              |
| 6.4 | 110.6                                      | nd                | nd                | 0.4                              |

<sup>1</sup> Data are expressed as μmoles/liter.

<sup>2</sup> H<sub>2</sub>O<sub>2</sub>: hydrogen peroxide.

<sup>3</sup> HMHP: hydroxymethyl hydroperoxide.

<sup>4</sup> HEHP: hydroxyethyl hydroperoxide.

<sup>5</sup> CH<sub>3</sub>OOH: methyl hydroperoxide.

### Isoprene

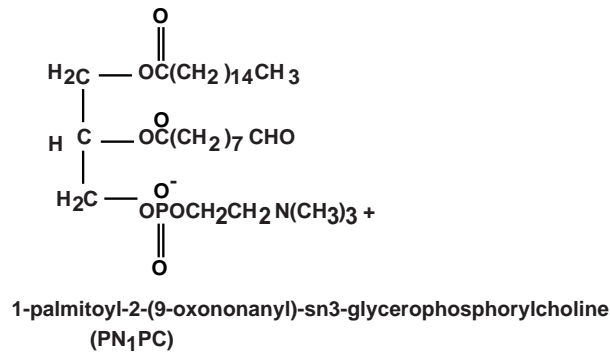
Isoprene is the basic member of the group of plant natural products known as isoprenoids. Important classes of these compounds are formed from multiples of the basic five carbon unit: monoterpenes (C<sub>10</sub>), sesquiterpenes (C<sub>15</sub>), and diterpenes (C<sub>20</sub>). These compounds are important articles of commerce because of



fatty acids is that they are most often esterified to form phospholipids, which are a universal feature of biological membranes, forming a lipid bilayer. After ozonolysis the product of phospholipid oxidation has only a nine-carbon chain (1-acyl-2-(9-oxononyl) sn-glycerophosphocholine (Santrock and others 1992) (*fig. 4*). This compound has the capability to lyse biological membranes.

Even though these reactions can be measured in solutions of phospholipid, it is doubtful that ambient ozone has access to the double bonds in the phospholipid bilayer of the biological membrane.

**Figure 4** — 1-palmitoyl-2-(9-oxononyl)-sn3-glycerophosphorylcholine.



### Amino Acids and Proteins

All parts of plant cells contain proteins. They may be structural in nature, or they may be catalytic. They may be soluble or membrane-bound. The plasma membrane is close to 50 percent lipid and 50 percent protein. The proteins of the membrane are responsible for various catalytic activities, including pumps for sodium, potassium, and calcium. The proteins of the plasma membrane are probably also responsible for the reception of chemical stimuli (natural and unnatural). The reaction of ozone with membrane proteins can be visualized as having a devastating effect.

Reaction of ozone with a few amino acids and proteins has been well documented (Mudd and others 1969, Previero and others 1963). The reactive amino acids include tryptophan, histidine, and methionine (*fig. 5*). The product of tryptophan ozonolysis is N-formylkynurenine (Kuroda and others 1975, Previero and others 1963), which is consistent with a classical ozonolysis breaking the double bond. The product of ozonolysis of histidine, when it is in peptide linkage, is aspartic acid which also implies a classical ozonolysis of the double bond of the imidazole ring (Berlett and others 1996). The product of oxidation of methionine is methionine sulfoxide (Johnson and Travis 1979, Mudd and others 1969) showing a pathway of ozone oxidation that is clearly not a classical ozonolysis. Some other amino acids that are oxidized by ozone include cysteine, tyrosine, and phenylalanine. Proteins that are inactivated by ozone include lysozyme (Kuroda and others 1975), glyceraldehyde-3-phosphate dehydrogenase (Knight and Mudd 1984), and glutamine synthase (Berlett and others 1996).

### Ascorbic Acid

Ascorbic acid (vitamin c) is a widely distributed constituent of plant tissue. It has long been known that fruits can prevent and correct the results of vitamin c deficiency in humans. Ascorbic acid is an outstanding antioxidant. It is fundamental important in the detoxification of superoxide produced during the reactions of photosynthesis. Ascorbic acid is also an important cofactor in hydroxylation reactions such as the formation of hydroxyproline. The presence of ascorbic acid in apoplastic fluid can be viewed as a protection against ozone, but it is incomplete since the effects of ozone on plant cells is obvious.

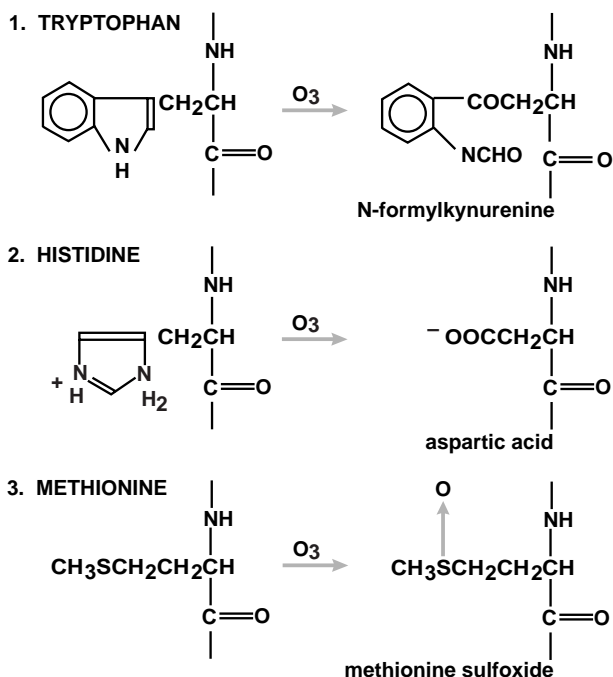


Figure 5 — Reaction of ozone with amino acids.

Foliar spray of ascorbic acid can prevent ozone damage to plants (Freebairn 1957). Ascorbic acid in the apoplastic fluid of plant leaves has also been shown to be oxidized by ozone (Luwe and others 1993). The reaction of ozone with ascorbic acid is stoichiometric, and the reaction is more rapid at pHs above the first pK (pH 4.7) (*fig. 6*) (Giamalva and others 1985). The reactivity of ascorbic acid may also be caused by the cleavage of the double bond in the ring (Chameides 1989), and the oxidation product may be an epoxide formed at the double bond (Kanofsky and Sima 1991). There are precedents for both reactions. An examination of the product of oxidation of ascorbic acid by ozone using nuclear magnetic resonance, mass spectrometry, and enzymic re-reduction using DHA reductase (*fig. 7*) has shown beyond doubt that the product is dehydroascorbic acid. Since dehydroascorbate reductase is common in plants, the oxidation of ascorbic acid by ozone in the apoplastic fluid is not a suicide-protection mechanism but rather a renewable form of defense. This defense does depend on the ability of the plant to take up DHA, re-reduce it in the cytoplasm and then export AA to the apoplastic fluid.

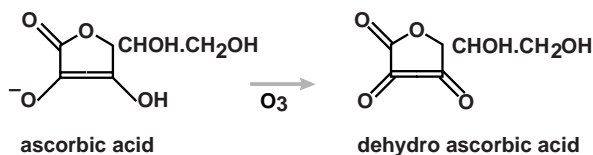


Figure 6 — Reaction of ozone with ascorbic acid.

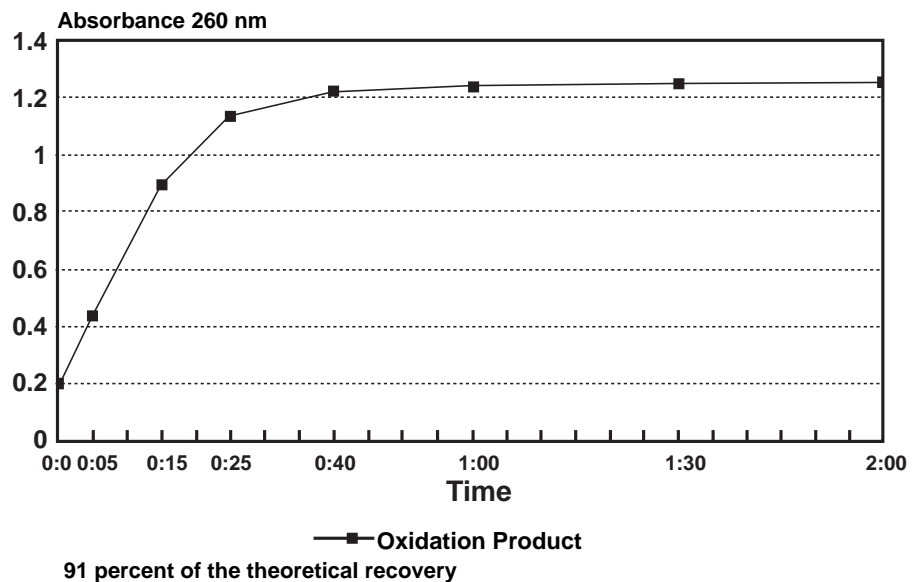
### Phenylpropenoids

The phenylpropenoids are unique plant natural products. They have been recognized and studied for many years because of their abundance and their characteristic reactions. The phenylpropenoids are synthesized by way of shikimic acid and phenylalanine. Phenylalanine is the direct precursor of the simplest phenylpropenoic acid: cinnamic acid, catalyzed by phenylalanine-ammonia lyase (PAL). PAL has received a great deal of attention because it responds to many stimuli, including irradiation, pathogen infection, and ozone. Phenylpropenoic acids are common in plants. They comprise cinnamic acid coumaric acid, caffeic

acid, ferulic acid and sinapic acid. These compounds are important because they are precursors of lignin and flavanoids. They may also have a role in protection of plants from the damaging effects of ultraviolet-B radiation.

The phenylpropanoic acids are susceptible to oxidation by ozone.<sup>3</sup> In some cases the major pathway of oxidation is ozonolytic cleavage of the double bond in the propenoic acid side chain. In other cases the oxidation seems to be directed to the phenolic ring. Pryor (1974) has suggested a mechanism for the attack of ozone on the phenolic compounds that involves radical attack on the phenolate ion. However, the reaction of ozone with the propenoic acid moiety is clearly important.

**Figure 7** — Re-reduction of ozone-oxidized ascorbic acid by DHA reductase.



## Messenger Molecule Synthesis

### Ethylene

The production of ethylene after the exposure to ozone is one of the fastest responses known. The speed of the response is consistent with the role of ethylene as a messenger molecule in the succession of events after ozone exposure (Langbartels and others 1991). The messenger RNA for aminocyclopropane carboxylic acid synthase has also been shown to rapidly increase after exposure to ozone (Schlagnhauer and others 1995). Gunderson and Taylor (1988) have shown that ethylene causes stomatal responses similar to those when plants are exposed to ethylene. Other messenger molecules could be examined such as jasmonic acid and salicylic acid. In the case of salicylic acid the response is too slow to account for responses to ozone (Yalpani and others 1994).

### Responses to the Messenger Molecules

Clear responses to messenger molecules are the formation of chitinase and b-glycosidase (Schraudner and others 1992). The formation of these proteins may be a beneficial response to the challenge of ozone, or merely a response to the stress by production of proteins analogous to "pathogen related proteins." It is probable that the late production of these proteins are an indication that damage has been done.

### Protection from Ozone

In the early 1950's the protection of plants from ozone was accomplished by application of ascorbic acid (Freebairn 1957). But the protection was short-lived because of the ready oxidation of ascorbic acid in the atmosphere. The protection afforded by ascorbic acid points to a critical distinction among protectant chemicals:

<sup>3</sup> Unpublished data on file at the Department of Botany, University of California, Riverside.

those that are simply sacrificed and lower the effective dose of ozone to the plant, such as foliar applications of ascorbic acid, and those that alter the plant physiologically and biochemically in such a way as to protect the plant from ozone. There are no convincing examples of the second class of protectants at present. Many compounds have been tested for protection, and several have been found effective. The most interesting and most effective of these compounds is N-[2 2-oxo-1-imidazolidinyl)ethyl]-N'-phenylurea (EDU). EDU was first synthesized and tested for protection of plants against ozone by chemists at DuPont Chemical Company (Carnahan and others 1978). EDU has been found to be effective both as a foliar spray and as a soil drench (Kostka-Rick and Manning 1993). It has been stated that EDU does not react with ozone (Pitcher and others 1993), and it is therefore a candidate for effectiveness by changing the physiology and biochemistry of the plant. Lee and Chen (1982) have reported that EDU has cytokinin-like activity, which could be related to its activity as an antiozonant. Lee and Bennett (1982) found that the application of EDU increased the activities of superoxide dismutase, catalase, and peroxidase, but these findings have been contradicted by Pitcher and others (1993). The mode of action of EDU is therefore still controversial and worthy of much greater research effort.

Another method for plants to resist the effects of ozone is to develop resistant varieties. Resistant varieties of several agricultural crops include tobacco (Heggestad 1993), alfalfa (Graumann 1972), and maize (Cameron and others 1970). These plants can be studied to understand the reasons for resistance, and breeding programs can be initiated to introduce the desirable trait into all commercially available lines. One method to detect differences in resistant and susceptible plants is to analyze the DNA by restriction fragment length polymorphism (RFLP). Neale and Harry (1995) have discussed the uses of these technologies with particular attention to coniferous trees. The identification of DNA fragments associated with resistance could be useful to breeders, especially since the DNA testing for resistant crosses can be made on seedlings rather than mature plants.

## Conclusion

The reaction of ozone with many types of compounds found in plants have been studied *in vitro*. It is difficult to connect these known chemical reactions to reactions which are responsible for the succession of events in the plant which eventually result in characteristic lesions of ozone damage. How are we going to make the connection between chemical reactions and visual symptoms? One approach is to compare the DNA of genetically related plants that are either ozone-resistant or ozone susceptible. Methods of analysis include the use of DNA probes for known enzymes that may be involved in ozone resistance. Another method is the comparison of DNA fragments derived by action of restriction endonucleases. Techniques of molecular biology offer the most promise for understanding the differences between ozone-resistant and ozone susceptible plants.

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