# THE PHYTO3 TECH CROP PROTECTION TECHNOLOGY FOR MICROORGANISM AND INSECT CONTROL USING OZONE, UV AND DIPOLE-ELECTRICAL AIR JET SPRAY TECHNOLOGIES - TECHNICAL BASIS AND CHEMISTRIES INVOLVED

**Hanspeter Steffen**
*Dorfstrasse 84, CH-3473 Alchenstorf, Switerland*

**Rip G. Rice**
*RICE International Consulting Enterprises, Sandy Spring, MD 20860, USA*

**Keywords:** Ozone, UV-Radiation, Agricultural Crops, Systemic Acquired Resistance, Negatively Charged Water, Electrolyzed Water.

## Contents

**Summary**

Plants can't walk away when they are attacked by pathogenic microorganisms and insects, or if they are exposed to any form of stress. They do not have a central nervous system that allows them to defend themselves or to ask for help! In the early part of the $20^{th}$ century, it was discovered that growing plants can be stimulated to respond to stresses by developing a Systemic Acquired Resistance (SAR) to microorganisms and insects. Since the 1960s, and especially during the 1990s and early 2000s, significant advances in this agronomical technology have been made. SARs have been proven to result from the application of many types of chemical formulations. Plants respond by generating their own chemicals internally that subsequently prevent attacks by microorganisms and insects.

Early in the 21st century, it was shown that sequential treatment of growing plants with

(1) an aqueous spray of high voltage, pulsed negatively charged water, followed immediately with
(2) a spray of ozone-containing water containing 8 mg $L^{-1}$ of ozone generated from oxygen, and that followed immediately by
(3) high energy UV-C radiation, also causes plants to develop SARs to microorganisms and insects, but without the use of chemicals.

The primary advantages of this new ozone-UV-based technology are
a) there are no harmful effects on the plants,
b) no toxic chemical residues remain on the plants,
c) the technology can be used in rainy weather, when crop protection is most necessary,
d) the technology is environmentally friendly (no chemical residues), and
e) the technology is cheaper for crop growers compared to current chemical approaches.

In this chapter will be summarized the technical background of Systemic Acquired Resistance, how it is believed the three-step PhytO3 Tech approach performs to stimulate SARs in crops, a description of field-operating equipment in use today in several European countries, and a cost analysis in comparison to current chemical treatments.



## 1. Introduction

Just as the human body can be immunized to certain microorganism attacks and can recover from physical wounds, so growing agricultural plants have evolved a number of inducible defense mechanisms against attacks by pathogens (disease-causing agents) and physical wounding (by sucking and chewing insects) by producing and distributing resistance-inducing chemicals within the plants themselves. Ross (1961) showed that tobacco plants challenged with tobacco mosaic virus (TMV) subsequently developed increased resistance to secondary infection in localized and distal tissues (tissues

farthest from the point of origin). This spread of resistance throughout the plant's tissues was termed "Systemic Acquired Resistance" (SAR).

Since the discovery by Ross (1961) of the SAR-triggering effect ofTMV in tobacco plants, many other materials have been shown to produce similar SAR triggers. These agents include microorganisms, pathogens, some chemicals, some reactive oxygen species (ROS) such as hydrogen peroxide (Alvarez *et al.*, 1998) and recently ozone (Mahalingam *et al.*, 2003) and UV-C radiation (Nawrath *et al.*, 2002). The resistance conferred is long-lasting, sometimes for the life of the plant, and is effective against a broad band of pathogens, including viruses, bacteria, fungi, and oomycetes (oospore-forming, non-photosynthetic fungi) (Ryals *et al.*, 1996; Sticher *et al.*, 1997).

Kachroo *et al.* (2003) introduced the subject of SAR with the statement that plants resist pathogen infection by inducing a defense response that is targeted specifically to combat invasion by the pathogen (Keen, 1990; Van der Hoorn *et al.*, 2002). In many cases, the induction of these responses is accompanied by localized cell death at the site of pathogen entry, which often is able to restrict the spread of pathogens to cells within and immediately surrounding the lesions. This phenomenon, known as the Hypersensitive Response (HR), is one of the earliest visible manifestations of induced defense response, and resembles programmed cell death in animals.

Concurrent with hypersensitive response development, defense mechanisms are triggered locally and in parts distant from the site of primary infection. This phenomenon, known as Systemic Acquired Resistance (SAR), is one of the most studied induced defense responses and is accompanied by a local and systemic increase in endogenous salicylic acid and a concomitant up-regulation of a large set of defense genes, including genes that encode pathogenesis-related (PR) proteins (Ward *et al.*, 1991; Gaffney *et al.*, 1993; Uknes *et al.*, 1993; Dong, 2001).

Although the stimulation of SAR in growing agricultural plants has been known to occur for many years, a complete understanding of the “whys” and “hows” of SAR has not yet been revealed. Research efforts have increased, and during the 1990s significant strides have been made. The most recent review article on the subject was published by Durrant and Dong (2004).

## 2. Nature of Systemic Acquired Resistance

Early experiments indicated that an infected plant leaf produces a systemic signal for SAR, and that this signal is not species-specific (Dean and Kuc, 1986; Jenns and Kuc, 1979). The nature of the systemic signal has been a subject of controversy for years, with evidence to support many theories. As of this writing, the most simplistic explanation for the observed and effective observation of systemic acquired resistance in plants involves the affected plant responding to attack by generating certain chemicals internally. These chemicals then are distributed rapidly throughout the plant, where they provide a defense against further attack by outside forces.

### 2.1. Plant-Secreted SAR-Causing Chemicals

Chemicals that are secreted internally in major amounts by plants and that appear to have an effect in developing and procreating SAR include salicylic acid (o-hydroxybenzoic acid), ethylene ($H_2C=CH_2$), and jasmonic acid (3-oxo-2-penteny1cyclopentylacetic acid), but additional chemicals are found to be produced in later studies. Research studies on these three major chemicals are numerous. A study by Maldonado *et al.* (2002) suggests that a lipid-based molecule might be the mobile signal for SAR. Kumar and Lessig (2003) found that SAR activity of lipid molecules is increased several times by the presence of salicylic acid.

Two additional chemicals are known to be secreted in response to attacks on growing plants by various agents, including ozone, hydrogen peroxide, and UV-C radiation - these are ethylene, $H_2C=CH_2$, a gas that is quite reactive with ozone, and jasmonic acid. These two pathways, plus those involving salicylic acid, may occur individually, or concurrently.

Steffen (2005a,b) points out that as of this date, more than 41 genes are known in plants responding to the elicitor ozone via the ethylene pathway. Ethylene as well as salicylic acid is used by the exposed plant as a messenger and elicitor of pathogenic defense gene expression, activating the same defense systems as salicylic acid.

The jasmonic acid generated in a plant in response to ozone and other elicitors, triggers the secretion of defense genes that are especially important in root zones of plants (Steffen, 2005a,b). Elicitors can be generated by physical attack (chewing or sucking insects), microbiological (pathogenic) microorganisms, or chemicals (pesticides, herbicides, Reactive Oxygen Species).

### 2.2. Transport of the Systemic Signal

How does the SAR signal travel throughout the plant? Again, the complete answers are not known, and the topic is the subject of much research. Girdling experiments have suggested that the SAR signal produced in inoculated leaves travels through the phloem (plant sieve tubes that conduct synthesized food substances from leaves to plant parts where they are needed) to upper leaves (Guedes *et al.*, 1980; Ross, 1966). Later research has indicated that the phloem is the major conduit for SAR signals, but some fraction of the signal also may be able to move by a different route (Durrant and Dong, 2004).

### 2.3. Roles of Ozone, Hydrogen Peroxide, and Other Active Oxygen Species

Numerous studies indicate the importance of "Active" or "Reactive" Oxygen Species in causing SAR as well. Alvarez *et al.* (1998) found that $H_2O_2$ accumulates in small groups of cells in uninoculated leaves of *Arabidopsis* after infection with a virulent strain of *P. syringae.* These "microbursts" of active oxygen species are produced within two hours after an initial oxidative burst in the inoculated tissue and are followed by the formation of microscopic hypersensitive response lesions. Using catalase to scavenge the peroxide, it was demonstrated that both the primary and secondary oxidative bursts are required for the onset of SAR. Alvarez et al. (1998) proposed that microbursts of reactive oxidative species may activate defense responses at a low level throughout the plant, and that this contributes to the SAR-induced state.

A study by Paolacci *et al.*, (2001) showed that exposure to gaseous ozone could elicit plant defense responses abiotically in leaves of bean plants. These authors suggested that these effects might rest on a sequence of molecular events leading to the hypersensitive response during plant pathogen-incompatible interactions. In that study, Paolacci *et al.*, (2001) also refer to several earlier studies demonstrating the ozone-induced synthesis of phenylpropanoid molecules, of related isoflavanoid and stilbene phytoalexins (of which salicylic acid is a member), as well as of catechin (a flavin allelochemical and antioxidant that is synthesized downstream of other reactions) in several plant species.

The transcription of certain of the corresponding genes (following exposure to ozone) also has been studied [reviewed by Kangasjärvi *et al.*, 1994; Langebartels *et al.*, 1997; Schraudner *et al.*, 1997; Sandermann *et al.*, 1998)]. On ozone exposure, the activity of chalcone synthase (CHS) was found to increase in soybeans (Keen and Taylor, 1975) and in pine (Rosemann *et al.*, 1991). The expression of phenylalanine lyase and CHS, together with those of other defense genes, enzymes and metabolites (among these flavone glycosides), was found to be enhanced in parsley after three to eight hours from the beginning of ozone exposure (Eckey-Kaltenbach *et al.*, 1994a,b). Such defense responses were found to overlap at least in part with those elicited by UV-irradiation and by pathogens (Sandermann, 1996).

A review by Fermin-Muñoz (2000) found that the production of active oxygen species, such as superoxide anions, hydroxyl free radicals, and hydrogen peroxide (all formed when ozone is dissolved in water and exposed to UV-C light) have been observed in many plant-pathogen interactions, and are known to play an important role in plant defense (Wu *et al.*, 1997). Plants have been engineered to continuously produce active oxygen species, for example, expression of a defective calmodulin gene (Oh *et al.*, 1999) or a less active catalase (Chamnongpol *et al.*, 1998) in transgenic tobacco led to increased accumulation of $H_2O_2$ and to an activated expression of pathogenesis-related (PR) proteins.

Levine *et al.* (1994) showed that hydrogen peroxide plays an important role in establishment of both localized and systemic defense responses in plants. It has been recognized for some time that when plant leaves are contacted by hydrogen peroxide, their stomata react by closing. More recently, however, Desikan *et al.* (2005) have discovered that a previously uncharacterized function for the *Arabidopsis (Arabidopsis thaliana)* ethylene receptor (ETR1) is that of mediating $H_2O_2$ signaling in stomatal guard cells, allowing the stomata to remain open, at least for a longer period than had been observed.

It should be borne in mind, however, that over-exposure of growing plants to ozone can cause cell death to occur (Rao and Davis, 1999; Rao *et al.*, 2000a,b). Rao *et al.* (2002) discuss this eventuality, but proposed a schematic model to illustrate that ozone-induced, Hypersensitive Response cell death in *Arabidopsis* is the net result of extensive cross-talk between multiple acting signaling pathways that converge to modulate the type and magnitude of ozone-induced defense responses. Mechanisms in this model involve salicylic and jasmonic acids, as well as ethylene.

According to the Rao *et al.* (2002) model, upon entering leaf tissue through stomata, ozone generates excess active oxygen species (including $H_2O_2$) resulting in increased biosynthesis of signaling molecules (such as salicylic acid), which, in turn, potentiates the feedback amplification loop of runaway cell death cycle that induces the biosynthesis of signaling molecules such as ethylene. This chemical (ethylene) has been shown to induce lipases known to promote senescence, a slow form of cell death (Hong *et al.*, 2000). As yet, it is unclear whether salicylic acid alone is sufficient to induce the production of ethylene.

However, ozone, either by reacting directly with membrane lipids (Mudd, 1997) and/or by generating excess active oxygen species, induces the biosynthesis of jasmonic acid or methyl jasmonate, which has been shown to reduce ozone-induced lesions both by attenuating salicylic acid-dependent lesion initiation (Rao *et al.*, 2000b) and ethylene-dependent lesion propagation processes (Overmyer *et al.*, 2000).

## 2.4 The Role of Salicylic Acid in SAR

Salicylic acid (o-hydroxybenzoic acid) and its possible roles in developing and transporting SAR throughout plants has been the subject of a number of review articles (Dempsey *et al.*, 1999; Dong, 2001; Ryals *et al.*, 1996; Shah and Klessig, 1999). In many plants, but not all, SAR is preceded by an increase in salicylic acid within the plant. It is generally conceded today that salicylic acid is an essential signal for SAR across a range of plants, although the mechanism by which this acid induces SAR might differ with the plants. Salicylic acid is believed to be synthesized internally by the plant in response to attacks or wounds. Bacterial enzymes and proteins in the plants are believed to be responsible for generating salicylic acid, while many other biochemicals within the plant take part in controlling its synthesis (Durrant and Dong, 2004).

Enhancement of the salicylic acid signal also occurs through a signal amplification loop involving reactive oxygen species (Shirasu *et al.*, 1997). Salicylic acid has been observed to bind the $H_2O_2$-scavenging enzymes catalase and ascorbate peroxidase, and inhibits their activities. This finding has led to the proposal that increases in $H_2O_2$ were responsible for signal transduction leading to pathogenesis-related gene induction and resistance (Chen *et al.*, 1993; Dumer and Lessig, 1995). Later studies have suggested that $H_2O_2$ functions upstream of salicylic acid (León *et al.*, 1995; Neuenschwander *et al.*, 1995). Low concentrations of salicylic acid also have been shown to potentiate the production of reactive oxygen species and cell death. In soy beans inoculated with *P. syringae*, the addition of salicylic acid dramatically enhanced the oxidative burst and cell death (Shirasu *et al.*, 1997; Tenhaken and Rubel, 1997). It is therefore hypothesized that in systemic tissues, the accumulation of low levels of salicylic acid, together with the development of microbursts of reactive oxygen species, could amplify responses to secondary infections (in plants) and contribute to SAR (Draper, 1997; Shirasu *et al.*, 1997).

There is ample evidence to indicate that SAR is conferred by expression of a collection of genes (Durrant and Dong, 2004). The sequencing of the *Arabidopsis* genome has allowed global analysis of gene expression changes during SAR to be conducted using DNA microarray technology. A promoter analysis study conducted on 1058 genes

induced by pathogen infection, salicylic acid, methyl jasmonate, or ozone, suggested a role for all of these materials in stress responses, but did not identify which were important during SAR (Mahalingam *et al.*, 2003).

### 2.5 Interaction between SAR and Other Plant Defense Pathways

Durrant and Dong (2004) point out emphatically that it is impossible to understand SAR fully without studying its interaction with other biological processes that occur within growing plants. It has been hypothesized that plant defense pathways interact synergistically or antagonistically to fine-tune responses according to challenging organism(s). Different responses may confer resistance to the same pathogen. On the other hand, activation of one pathway may lead to cross-talk: inhibition of another that is less effective against the challenging pathogen. Cross-talk between different defense pathways is reviewed by Bostock (1991).

Examples of cross-resistance have been found wherein insect feeding can induce aspects of SAR (Heil and Bostock, 2002). This has been observed in response to aphids and whiteflies (sucking insects, which therefore do minimal damage to plant tissues). Plants perceive some insects as pathogens rather than herbivores, and this concept is supported by the identification of a gene that confers resistance to aphids and nematodes (Milligan *et al.*, 1998; Rossi *et al.*, 1998). Evidence for co-regulation by salicylic and jasmonic acids comes from a gene expression profiling study in which 55 genes were induced by treatment by either salicylic or jasmonic acid (Schenk *et al.*, 2000).

Besides synergism between the three chemical pathways to SAR (salicylic acid, jasmonic acid and ethylene), there is also evidence for antagonism between the three. The induction of SAR has a negative effect on the jasmonic acid and ethylene pathways, normally induced by chewing insects and wounding of the plants (Felton *et al.*, 1999). A challenge for the future will be to understand how the three responses are coordinated and to unravel the downstream signaling network in each case (Durrant and Dong, 2004).

Figure 1 shows the sequence of events that take place in a plant from the time of recognition of the pathogen to defense gene induction, based on current knowledge (Durrant and Dong, 2004). Note the prominent role of salicylic acid, and the alternate role of reactive oxygen species (including $H_2O_2$**).**

Our understanding of SAR has increased considerably since the late 1990s and early 2000s as we have begun to elucidate the molecular mechanisms underlying this response. Many of the processes contributing to SAR are clearly required in both local and systemic tissues and contribute to basal disease resistance. These include the synthesis of salicylic acid, changes in redox status, and the induction of defense gene expression. …. there is evidence for negative and positive feedback of salicylic acid signaling and cross-talk between different signaling pathways, adding to the complexity of the defense response ..... Induction of SAR to control infection of crop plants is already being used in the field by application of BTH. Better understanding of the SAR signaling pathway will certainly lead to new environmentally friendly methods of crop protection (Durrant and Dong, 2004).

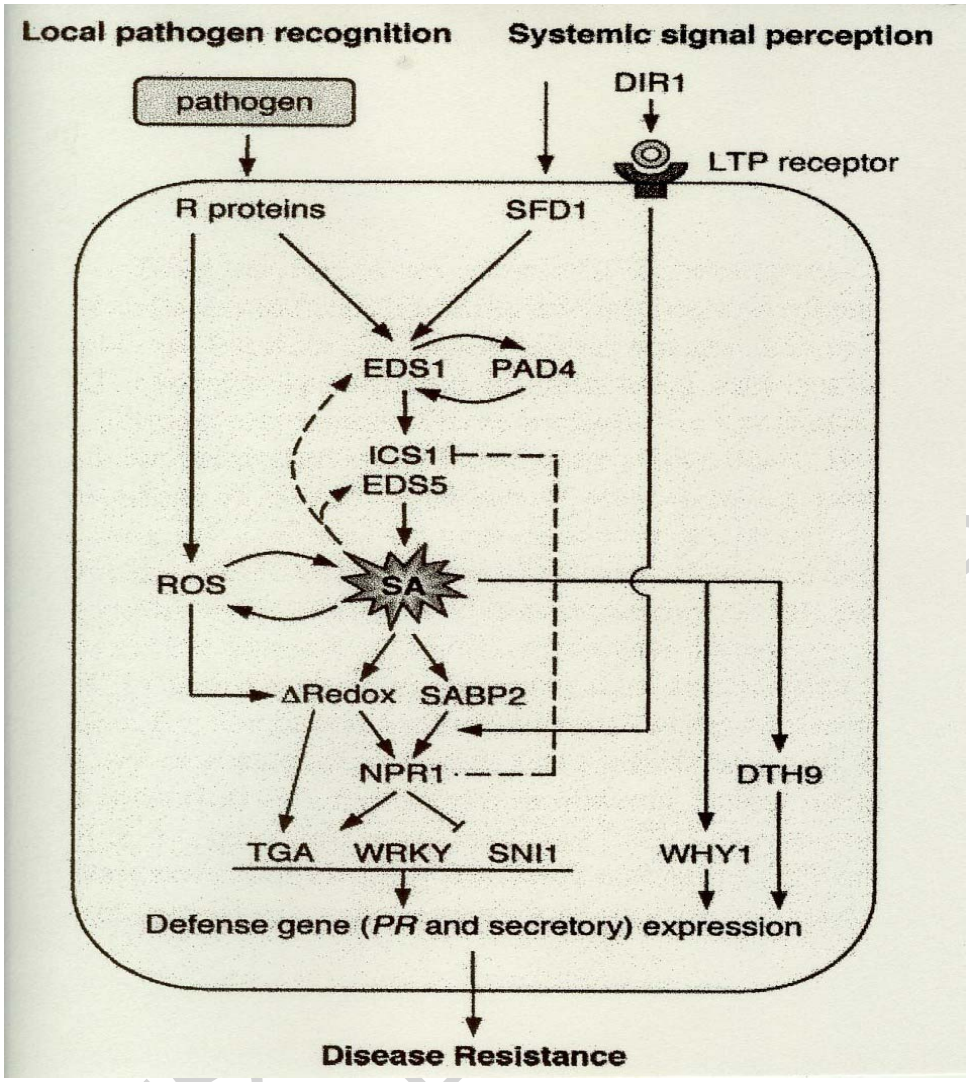


Figure 1. Sequence of plant events from pathogen recognition to defense gene induction (Durrant and Dong, 2004).

Durrant and Dong (2004) concluded their excellent review of SAR with the following comments:

"The current authors conclude that, given the known SAR-stimulating effect of hydrogen peroxide, $H_2O_2$, and of ozone (Mahalingam *et al.*, 2003), there is potential for a procedure that can provide low levels of $H_2O_2$ and/or ozone, to agricultural crops.

If such a system can be developed, even though the chemistries and biochemistries that must occurring within plants are still many years away from being understood, the most significant advantages of a successful SAR-triggering system would be increased crop yields without resorting to chemicals that leave residues on the crops or in the soil."

Such a system is the PhytO3 Tech technology, developed during the 1990s, described in the next section, and field-tested in several European countries and Brazil.

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**Biographical Sketches**

**Hanspeter Steffen** is a Swiss citizen who graduated from the Swiss Federal Institute of Technology, Zürich (1978), with a Dipl-Ing degree in Agronomy, then earned a diploma of the Swiss Federation as a Fruit and Vegetable Specialist over the following two years. He has worked as an Agro-Consultant for a number of businesses, including Steffen-Ris AG, Utzenstorf, Switzerland (fruit and vegetable trading), Gamma Consult, Winterthur, Switzerland, Blendfine Farm Pvt. Ldt., Sharajah, U.A.E. (Farm Manager), Green Star Farms, Nairobi, Kenya, Geistlich AG, Wolhausen and Peter Ritzmann, Hong Kong and Saigon, Vietnam. Mr. Steffen also established the companies Fruttadoro and Pescadero, Cagayan de Oro City, the Philippines, for the export of fresh fruits, vegetables and seafood to Europe, Hong Kong and Japan. In the early 2000s, Mr. Steffen began applying Advanced Oxidation Technologies to the development of the packaging technologies Ventpack I and Ventpack II, Ventafresh, and the new Crop Protection Technology (PhytO3 Tech) and Redox Technology.

His achievements and recognitions are numerous: Agricultural Prize of the Arab Agricultural Magazine, Bahrain and FAO, Rome Blendfine Farm, "Best Horticultural Operation in the Middle East", 1986; International Invention Exhibition Geneva, Switzerland, three major prizes for the invention VENTPACK in the field of packaging of perishables (Gold Medal, with special recognition of the jury; 1st Prize of the International Inventors Association IFIA; State Research Prize of Rumania – 2001); International Invention Exhibition Geneva, Switzerland, two major Prizes for the new Crop Protection Technology PhytO3 (Gold Medal with special recognition of the jury; State Research Prize of Rumania – 2004); and International Invention Exhibition Geneva, Switzerland, Gold Medal for the Project "The Chemical-Free House". Washing and cleaning processes in a house with Electrolyzed Rainwater produced with Solid-Diamond Electrode Technology – 2008).

Mr. Steffen is a member of the Agro-Technical Society of Switzerland, the International Ozone Association, and the International Ultraviolet Association.

**Dr. Rip G. Rice** is President/CEO of Rice International Consulting Enterprises, located in Sandy Spring, Maryland, USA, and specializing in ozone technologies, particularly with respect to Agri-Foods as well as water and wastewater treatment. He served as Ozone Resource to the Electric Power Research Institute Expert Panel which declared ozone to be Generally Recognized As Safe for food applications in 1997. He was the senior author of the Food Additive Petition submitted by the EPRI to the FDA to request approval of Ozone as an Antimicrobial Agent for direct contact with and treatment of all types of foods. This petition was approved June 26, 2001. Dr. Rice advises food processors and other interested parties about how to evaluate ozone for various purposes in the Agri-Food industries, including the many combinations of ozone with other technologies (UV radiation, ultrasound, electrolyzed waters, modified air packaging, etc.), in both gas and aqueous phases, in food processing and handling plants. He is a frequent lecturer on these subjects at meetings of the International Ozone Association (IOA) and other trade associations.

Dr. Rice co-founded the International Ozone Institute (now the IOA) in 1973, was its President during 1982-1983, Editor-in-Chief of *Ozone: Science & Technology*, the Journal of the IOA and Editor-in-Chief of *Ozone News*, the newsletter of the IOA. He has authored more than 120 papers on various aspects of ozone technology, and has edited or coedited 21 books, proceedings or monographs in ozone technology. In 1995, Dr. Rice received the Morton J. Klein Memorial Award for outstanding service to the IOA. He has chaired the IOA-Pan American Group Agri-Food Task Force since its formation in 2003.

In 1999, Dr. Rice co-founded the International Ultraviolet Association, and served as Editor-in-Chief of *IUVA News*, IUVA's newsletter, for its first several years.