

OFFICIAL

COOPERATIVE REGIONAL RESEARCH PROJECT - REVISED PROJECT PROPOSAL

PROJECT NUMBER: NE-176 (Rev.)

TITLE: Characterization and Mechanisms of Plant Responses to Ozone in the Northeastern U.S.

DURATION: October 1, 1995 to September 30, 2000.

STATEMENT OF THE PROBLEM:

Tropospheric ozone (O_3), resulting from man's activities, is the primary air pollutant affecting crops and native vegetation in the eastern U.S. A strong oxidant, O_3 causes foliar injury, accelerates leaf senescence, and reduces growth and yield of many plant species. While the injurious effects of O_3 have been reported for many years and considerable progress has been made toward determining the mechanisms of action, several aspects of its toxicity to plants are not known or are inadequately understood. Foremost among these are 1) environmental and biotic factors favoring or limiting O_3 injury, 2) the specific mode of action of O_3 , and 3) types of plant defense systems (basis for tolerance and sensitivity). This regional project revision addresses each of these areas. A more complete understanding of plant responses to O_3 would allow a more knowledgeable characterization of O_3 effects on vegetation of the Northeast, enable more effective development of resistant varieties, and provide additional information for developing ambient air quality standards.

JUSTIFICATION:

Ozone is the most important and most widespread air pollutant in the northeastern U.S. As the principle component of photochemical smog, it is formed when temperature and incident solar radiation are high. Consequently, ambient O_3 concentrations are greatest during summer months, when plants are actively growing (Krupa & Manning, 1988; Lefohn, 1992). Plant response to O_3 is affected by the concentration and length of exposure, air temperature, soil moisture, solar radiation, plant nutrition, plant growth stage, and pests such as pathogens and insects. All of these factors fluctuate continually under ambient conditions. The growth rate and productivity of crops and forests in the region are determined by interactions between O_3 and these factors during critical stages of plant development (Runeckles & Krupa, 1994).

Despite Clean Air Act legislation, O_3 continues to present a significant threat to agricultural and native vegetation in the Northeast. NESCAUM (1993) reported that the federal O_3 standard (120 nl L^{-1} for 1 hr) was exceeded on 27 days during the summer of 1993, with peak levels at several locations reaching $150\text{-}200 \text{ nl L}^{-1}$. Concentrations of O_3 precursors, such as nitrogen oxides and volatile organic compounds (VOCs, hydrocarbons) are increasing as part of the "greenhouse gas phenomenon" (Enquete Commission, 1992; Krupa & Kickert, 1989). Yield-reducing O_3 episodes, three times their present frequency, have been predicted by the year 2025 (Chameides *et al.*, 1994). The U.S. Congress has expressed high interest in

this situation by declaring that one of its priority research needs is "Improving the understanding of the formation and effects on crops and ecosystems of ground-level O₃ in urban and rural areas" (Air Quality Subcommittee, Committee on Environment and Natural Resources).

The effects of O₃ on some important crops and sensitive tree species have been extensively reviewed (Chappelka and Chevone, 1993; Heck, *et al.*, 1988a, b; Lefohn, 1992). Much of this work has involved empirical analyses of the relationships between O₃ exposures and plant yield losses. Few of the high value crops grown in the Northeast have been investigated in any detail. These include fruits, vegetables and turfgrasses which have significant economic value to the region. In addition, there are many forests and recreational areas in the Northeast which contain sensitive tree species that may be adversely impacted by O₃.

While we have some knowledge about plant physiological and biochemical processes affected by O₃, we know very little about the interactions of O₃ and environmental, edaphic, and biotic factors affecting plant uptake and subsequent response. We are unable, therefore, to relate measured O₃ levels in the ambient air to potential effects due to our incomplete understanding of how these interactions occur. This limited information base has seriously hampered the development of a national secondary air quality standard to protect vegetation from ambient O₃ concentrations.

Researchers participating in NE-176 have begun to utilize current methods of molecular biology and genetic engineering to determine mechanisms that cause cell damage and control O₃ uptake. The use of transgenic plants with enhanced defense systems is a step in this direction (Pitcher *et al.*, 1991; Pitcher *et al.*, 1992; Zilinskas *et al.*, 1993a; Pitcher *et al.*, 1994). The characterization of specific biochemical responses will further our understanding of the regulation of O₃ uptake into plant leaves and the specific sites of cellular injury.

The use of tolerant and sensitive genotypes, in field experiments, will allow project scientists to identify and quantify the interactive effects of environmental factors, O₃ exposure profiles, and developmental stage on plant response to O₃. These types of studies have only recently begun (Krupa *et al.*, 1993, 1994) and are necessary to develop process-oriented models that relate ambient O₃ levels to plant growth and yield losses.

The characterization of O₃ effects on plants requires methods of investigation that range from molecular biology to biochemistry to plant physiology to whole plant growth analysis. No one laboratory has the resources or personnel to adequately address this task. This can be accomplished, however, by using a regional research project approach. This allows for effective use of personnel, facilities and knowledge of all cooperating members. Such an approach has been very successful with NE-176 and it will be equally effective in the new project.

The Technical Committee of NE-176 has a continuing commitment to determine and define the influence of O₃ on plant productivity in the Northeast. The effects of ambient O₃ exposures on plant growth and yield are being investigated in field studies. Plant response to O₃ and defense mechanisms are being examined at the genetic, molecular and biochemical levels. Interactions between plant developmental growth stages, O₃ exposures and environmental factors are being studied. All of these investigations involve collaboration between small groups of scientists within NE-176. Integration of the conclusions resulting from this multidisciplinary research are already being used to provide a more comprehensive understanding of how O₃ affects plant growth, development and yield (Criteria Document,

Photochemical Oxidants, USEPA, in preparation).

RELATED CURRENT AND PREVIOUS WORK

Numerous books, chapters and review articles have been written about the effects of O₃ on plants (Alscher and Wellburn, 1994; Runeckles and Krupa, 1994; USEPA Criteria Document on Oxidants, 1993; Lefohn, 1992; and Manning, 1990). These references should be consulted for detailed information concerning our existing knowledge.

In the following section, research conducted by scientists in NE-176 is summarized and schematically presented in Figure 1. Whole plant experiments have led to the characterization of O₃ tolerant and sensitive cultivars, growth and yield performance of genotypes in field conditions, and interactions of O₃ and CO₂ on plant growth and physiology. Mechanistic studies have focused on biochemical/molecular attributes of genotypes that may be associated with O₃ tolerance. A major accomplishment has been the production of specific genotypes that can serve as model experimental systems. Transgenic tobacco plants that overexpress antioxidant enzymes have been developed by Zilinskas at NJ and O₃ tolerant and sensitive white clover clones have been selected by Heagle and Miller at USDA/ Raleigh, NC. Research proposed by cooperators in this Regional Project revision make extensive use of these model plants.

Whole plant experiments: Characterizing plant responses to ozone.

In MA, Manning continued characterization of plant response to O₃ using a number of species important to the economy of the Northeast, such as strawberry, cranberry, turfgrass, tomato, morning glory, and browailia and native plants, such as white pine, several species of aster, and blackberry. For most species studied, a wide range of O₃ sensitivity among cultivars and within populations was observed.

At USDA/Beltsville, Lee identified several O₃ tolerant and sensitive soybean cvs. and near isolines (Lee *et al.*, 1994; Foy *et al.*, 1995). Some cvs. showing high tolerance to O₃ (Biloxi, Bossier, Lee, and Perry) were also acid soil (AL) tolerant, but two AL tolerant cvs. (Aurora and Brunatna) were O₃ sensitive. Some cvs. (Chief, Salute 216 and Smena) were sensitive to both AL and O₃.

In NY, McGrath examined the effects of natural O₃ exposures on symptom development, yield and powdery mildew infection in cucurbits. Plant developmental stage was the most important determinant of injury occurrence. Both O₃ symptoms and powdery mildew infections were first observed near the start of fruit production in six successive plantings. Younger plants were not sensitive to the high O₃ concentrations that caused extensive injury to plants with fruit.

Open-top chamber studies.

Whole plant, field experiments were conducted at MD, USDA/Beltsville and Raleigh using open-top chambers to examine the interactive effects of O₃ and elevated CO₂ on the growth, yield and physiology of several crop species, including soybean, corn, cotton and wheat. At USDA/ Beltsville (Lee) and MD (Mulchi), high O₃ levels (60 nl L⁻¹) reduced yields up to 20%, however, increased CO₂ alleviated the negative impact of O₃ (Mulchi *et al.*, 1992; Rudorff, 1993; Zakaria *et al.*, 1994a,b). At USDA/Raleigh (Miller), a similar protective effect of elevated CO₂ against O₃ exposure was observed for soybean cv. Essex and white

clover (Heagle *et al.*, 1993). The physiological basis for CO₂-enhanced defense against O₃ does not appear to be limited to stomatal closure alone. Rates of photosynthesis were stimulated when plants were grown in CO₂ enriched atmospheres, even in the presence of moderate levels of O₃ (Slaughter *et al.*, 1993).

Use of antioxidant chemicals.

The application of antioxidant compounds, such as ethylene diurea (EDU), to vegetation is known to prevent leaf damage mediated by O₃. However, the mechanism(s) of protection is poorly understood. Studies with EDU, and other compounds, were continued at several participating stations to further document dose-response relationships, chemical effects on plants and the extent of the protective capacity against O₃ exposures.

At MA (Manning), the urea portion of EDU was compared to the whole molecule for efficacy against O₃ injury using Bel-W3 and Bel-B tobacco seedlings. EDU protected all plants in all experiments. Urea did not provide any protection and urea-treated Bel-B plants, when exposed to O₃, sustained extensive injury. EDU dose-response studies with tobacco cvs. Bel-W3, Bel-B, MD59 and MD872 and clover clones NC-R and NC-S demonstrated that a single foliar spray application at 300 mg L⁻¹ a.i. protected leaves from injury for a 7-day period at peak O₃ concentrations of 50 to 100 nl L⁻¹.

At PA, Pell conducted to assess the role of antisenescent properties of EDU in foliar protection using an O₃ sensitive potato cv., Norland. EDU, at 15 mg L⁻¹ soil treatment, was sufficient to provide O₃ protection (100 nl L⁻¹) with no signs of chemical toxicity. Net photosynthesis, chlorophyll content, Rubisco quantity and relative levels of mRNA for the Rubisco large and small subunits showed O₃-induced decreases (Reddy *et al.*, 1993; Pell *et al.*, 1994). EDU provided complete protection from effects caused by O₃ alone, but did not affect changes resulting from senescence processes.

At TX, Flagler found that both EDU and Ozoban (sodium erythorbate) prevented O₃ injury on field-grown shortleaf pine over experimental periods of two to four years (Flagler and Toups, 1991; Flagler *et al.*, 1994b).

Influence of environmental factors and plant physiological status on the ozone response.

At AL, Chappelka investigated the interaction of O₃ with other environmental stresses (low temperature) on loblolly pine (Chappelka *et al.*, 1990), O₃ effects on assimilate allocation between roots and shoots (Qui *et al.*, 1993), and the occurrence of O₃ injury on native vegetation in wilderness areas (Chappelka *et al.*, 1994). Ozone can enhance the low temperature sensitivity of loblolly pine new needle growth and restrict carbon partitioning to the roots. Visible foliar injury, attributed to ambient O₃, was observed on seedling, sapling and mature trees in the Great Smokey Mountains National Park.

At TX, Flagler investigated the interaction of O₃ and drought stress on growth and physiology of loblolly and shortleaf pine. In loblolly pine, O₃ decreased diameter and biomass (Elsik *et al.*, 1992), and inhibited photosynthesis and needle conductance (Elsik *et al.*, 1993; Flagler *et al.*, 1994). Similar results were observed with shortleaf pine and no interaction between O₃ exposure and drought stress occurred (Flagler *et al.*, 1993).

Characterizing the relationship between natural O₃ exposures and the development of foliar symptoms is an important aspect in assessing the impact of pollutant concentrations on plant growth and yield. Environmental conditions, edaphic factors, plant growth stage, and biotic stresses are all known to influence O₃ effects on plants (Krupa and Kickert, 1990;

Runeckles, 1992). While the uptake of O₃ through open stomata is of primary consideration in correlating exposure concentrations to plant response, the physiological status and developmental stage of the vegetation also influence the degree of injury.

A cooperative study between MA (Manning) and MN (Krupa) has been initiated to assess the relative importance of O₃ and other environmental factors in the development of foliar injury on Bel-W3 tobacco (Krupa *et al.*, 1993). An integrated model is being developed to predict the timing and severity of O₃ injury on this bioindicator species.

Mechanistic studies: Mechanisms of ozone effects on roots.

At USEPA /Corvallis, Andersen has focused on understanding the mechanistic effects of O₃ on the root and rhizosphere systems of plants. Labelling studies were used to construct a carbon budget for a ponderosa pine seedling growing in association with an ectomycorrhizal fungus (Andersen and Rygielwicz, 1991; Rygielwicz and Andersen, 1994) and to follow the effects of O₃ on the carbon budget from all major pools (Andersen and Rygielwicz, 1994, in review). Mycorrhizal and nonmycorrhizal plants generally responded in a similar fashion to O₃; the presence of O₃ made mycorrhizal plants more similar to non-mycorrhizal plants in carbohydrate allocation patterns. The results suggest that O₃ exposure may influence the vigor of the symbiosis and diminish the beneficial effects of mycorrhiza.

Ozone-induced modifications of Rubisco.

At PA, Pell has characterized O₃-induced changes in the Rubisco protein that could influence plant vulnerability to accelerated senescence. The total activity of Rubisco was contrasted in leaves of potato genotypes exposed to O₃ from leaf emergence until senescence. An accelerated loss of enzyme activity was observed in sensitive cvs., Norland and Cherokee, but not in tolerant cvs., Superior and Norgold Russet (Enyedi, *et al.*, 1992). Rubisco protein, in both genotypes, contained a similar number of available and total sulfhydryl groups, chemical moieties that are susceptible to oxidation (Enyedi *et al.*, 1992), and the nucleotide sequence and deduced amino acid sequence for the large subunit of the protein was identical (Enyedi and Pell, 1992). These studies demonstrated that Rubisco structure was probably not responsible for the variation in O₃-induced loss of activity between the genotypes.

Rubisco activity, in purified protein exposed to O₃ *in vitro*, declined in conjunction with a loss in sulfhydryl groups, formation of carbonyl groups and formation of aggregates of the protein (Eckardt, 1995). However, when intact plants were exposed to O₃ and Rubisco was subsequently purified, no structurally modified protein could be detected. Leaf discs of O₃-treated plants incubated at 0°C exhibited aggregates similar to those formed when the protein was treated with oxidant *in vitro*, whereas in discs incubated at 30°C, no aggregates of Rubisco were formed and the protein was present at much lower concentrations than in control tissue (Eckardt, 1993). These results indicate that O₃ induces structural modifications in Rubisco which can lead to accelerated proteolysis. The basis for accelerated loss of the enzyme in sensitive potato cvs. in comparison to resistant cvs. still must be determined.

Ozone exposure of potato resulted in a decrease in the mRNA levels of both the small and large subunit of Rubisco, *rbcS* and *rbcL*, respectively (Reddy *et al.*, 1993). Immature leaves sustained a large loss in *rbcS*, which was not noted in mature leaves. When plants were incubated in the dark, levels of *rbcS* declined regardless of O₃ treatment. Small decreases in Rubisco quantity were detected in both immature and mature foliage incubated in the dark, regardless of treatment. When mature leaves were incubated in the dark following

O₃ treatment, large reductions in Rubisco were detected (Eckardt and Pell, 1994). The latter response is a further indication that O₃ predisposes Rubisco to degradation.

Antioxidant activity and gene expression.

At USDA/ Raleigh, Miller and Heagle selected ten clones of white clover on the basis of visible injury in response to O₃. Stomatal conductance (g_s) of a resistant (NC-R) and a sensitive (NC-S) clone, measured in field and greenhouse low O₃ environments, was not different. The differential sensitivity of the clones, therefore, could not be attributed to variation in O₃ uptake rate.

The basal activities of the antioxidant enzymes SOD, catalase, APX, DHAR, mono-dehydroascorbate reductase (MDHAR), GR and polyphenol oxidase were measured in the resistant and sensitive clones. All enzyme activities were similar between clones. However, further research is required to examine enzyme response under conditions of oxidative stress to assess the role of these antioxidants in O₃ tolerance.

Induction by O₃ of plant defense responses involving phenylpropanoid metabolism was examined in soybean (Booker). In greenhouse studies, the activities of phenylalanine ammonia-lyase and 4-coumarate CoA:ligase were stimulated within 3 h of treatment with 100 nl L⁻¹ O₃ (Booker, 1993). Activities of cinnamyl alcohol dehydrogenase and peroxidase increased after 1.5 d of O₃ treatment, along with the appearance of foliar injury. In field studies, soluble and cell wall-bound hydroxycinnamic acid content rose 14-57% in O₃-treated leaves. The level of polymeric phenolics was increased 2X by O₃, although increased biosynthesis of core lignin was not evident (Booker *et al.*, 1991). In this case, membrane degradation caused by O₃ could allow pre-formed phenolics and polyphenol oxidases to react to initiate formation of phenolic polymers. However, pressure-bomb experiments indicated that cell wall flexibility decreased in leaf tissue from O₃-treated plants (Fiscus *et al.*, 1995). Phenylpropanoid metabolism was apparently stimulated by O₃ and may be related to changes in cell wall structure.

In VA (Chevone), the response of the antioxidant system to O₃ in tolerant and sensitive soybean cvs. was investigated in laboratory studies. In a sensitive cv., 'Dare', stomatal closure induced by O₃ was delayed compared to tolerant cvs. resulting in a 10% greater internal flux of pollutant after a 4 h fumigation (200 nl L⁻¹ O₃). Isozyme activities of SOD were not altered by O₃ treatment in any cv. (Sheng *et al.*, 1993). Endogenous activities of GR, APX and substrate concentrations of ascorbate and glutathione were similar among cvs. and were not affected by O₃ exposure (Sheng, 1992). The general lack of response of the antioxidant system could have resulted from excessive oxidative pressure.

Also at VA, antioxidants in eastern white pine genotypes, susceptible or tolerant to needle tipburn, were characterized over three years in field specimens. Enzyme activity of GR, SOD and APX was lowest during the summer months and highest during the winter (Anderson *et al.*, 1992). Substrate concentrations of ascorbate and glutathione increased 4X to 5X from summer to winter. No differences in antioxidants were observed among the white pine genotypes, precluding endogenous activity as a mechanism of O₃ tolerance. In a preliminary study, moisture stress, high temperatures and O₃ exposure resulted in an increase in the foliar activity of SOD and GR (Anderson, 1991).

At NJ (Zilinskis), molecular tools were developed to elucidate the function of antioxidant enzymes in plant response to O₃. Proteins from pea were purified and characterized, cDNAs encoding these enzymes were isolated and analyzed, antibodies to these enzymes were

produced as molecular probes, and transgenic tobacco and potato plants were constructed that overexpress these enzymes. The enzymes and cDNAs analyzed included the plastidic and cytosolic Cu/Zn-SOD (White and Zilinskas, 1992), mitochondrial Mn-SOD (White *et al.*, 1991; Altomare, 1992), cytosolic APX (Mittler and Zilinskas, 1991 a,b,c; Mittler and Zilinskas, 1992), and cytosolic MDHAR (Murthy and Zilinskas, 1994). Collectively, these enzymes are capable of scavenging superoxide radicals and hydrogen peroxide and regenerating reduced ascorbate. Some of the genes encoding pea antioxidant enzymes were very responsive to O₃, most notably the genes encoding cytosolic proteins (Zilinskas *et al.*, 1993b; Pitcher *et al.*, 1992); however, for most enzymes, increased activity paralleled the development of visible foliar symptoms (Pitcher *et al.*, 1991; Pitcher *et al.*, 1992). Detailed studies of the response of the gene encoding cytosolic APX showed that the regulation of this gene is very complex, involving transcriptional and post-transcriptional control (Mittler and Zilinskas, 1994; Zilinskas *et al.*, 1993a). No EDU-associated increase in any of the SOD isozymes was found in soluble leaf extracts of pea or snapbean, contrary to the prevailing hypothesis for EDU's protective role against O₃ damage (Pitcher *et al.*, 1992).

Transgenic tobacco plants (*Nicotiana tabacum* Bel W3 and W38), which overproduce pea SOD and APX under control of the CaMV35S promoter, were constructed and propagated (Zilinskas *et al.*, 1993; Pitcher *et al.*, 1994). These plants included those that overproduce Cu/Zn-SOD, Mn-SOD, and APX in the cytosol and Mn-SOD and APX in the chloroplast. Transgenic potato (Russet Burbank) were constructed which overproduce pea Cu/Zn-SOD or APX in the cytosol. The tolerance of these transgenic plants to oxidative stress conditions, relative to nontransformed controls, remains to be evaluated.

RELATIONSHIP TO OTHER REGIONAL PROJECTS:

NE-176 is an unique regional research project. No other regional project focuses on characterization and mechanisms of plant responses to O₃ in the Northeast, or elsewhere in the U.S. A search of the CRIS data base did not reveal any projects with similar research objectives. The National Atmospheric Deposition Program (NADP) represent a NATIONAL RESEARCH SUPPORT PROJECT (NRSP-3) with primary emphasis on precipitation chemistry and effects on terrestrial systems. The research support objectives of NRSP-3 and the research objectives of NE-176 complement each other. A number of stations participate in both NE-176 and NRSP-3, enabling effective communication between the two programs.

OBJECTIVES:

The overall objective of this research project is the characterization of plant genotypes that are capable of high growth rates and high productivity under O₃ episodes that occur each summer in the Northeastern U.S. Cooperating regional scientists will study plant species that are important in the Northeast and other selected genotypes that possess attributes applicable to investigating specific hypothesis. These plants will be utilized in a number of studies addressing the following three objectives:

1. Characterize whole plant responses to O₃, including carbon assimilation and

- allocation, growth and productivity;
2. Identify and delineate primary factors, both biotic and environmental, that determine plant response to O₃; and
 3. Determine mechanisms of O₃ action and plant defense systems, using cultivars and genotypes characterized in whole plant experiments.

At the termination of the NE-176 Revised Regional Project, on September 30, 2000, the following principle outcomes should be achieved:

1. Development of a primary scientific data base that relates ambient O₃ concentrations to growth and yield of crop plants and native vegetation. This data base will be used in the writing of the USEPA Oxidant Criteria Assessment Document (year 2000) and in setting a secondary Air Quality Standard to protect vegetation from O₃ damage.
2. Construction of a first-order predictive model for plant response to ambient O₃ concentrations using critical plant developmental, physiological and environmental variables.
3. Characterization of the importance of antioxidant enzymes in plant defense against ambient O₃ levels using transgenic plants that overexpress and underexpress these enzymes.
4. Establishment of the role of antioxidant enzymes, ascorbate and gas exchange processes in plant tolerance to ambient O₃ concentrations using tolerant and sensitive genotypes.
5. Characterization of biochemical and molecular mechanisms involved in O₃-induced Rubisco decline in tolerant and sensitive plant genotypes.

PROCEDURES:

A Regional Research Approach.

The characterization and development of O₃ tolerant cultivars requires an understanding of biological processes that range from whole plant carbon assimilation and allocation patterns, to gas flux from the atmosphere into the leaf, to biochemical and genetic regulation of carbohydrate metabolism and oxidative defense mechanisms. The influence of environmental factors on the O₃ response also must be known to assess the full productive capacity of selected, or genetically enhanced, plant material. The integration of these various components is represented in Figure 1, which also demonstrates interactive aspects of this regional research project. A unifying element of the project is common plant material available to station scientists; O₃ tolerant and sensitive white clover clones from USDA/Raleigh and transgenic tobacco (overexpressors of antioxidant enzymes) from NJ.

Scientists at participating experiment stations and laboratories have developed their own unique expertise and facilities that can be applied to investigate specific hypothesis within the project objectives. The primary cooperative effort is the coordinated research approach to investigate O₃ effects at different levels of plant function, from the leaf canopy to gene expression. No individual station has the personnel or resources available to accomplish all

objectives of this regional project. General research areas among the stations are briefly summarized as follows: Field fumigation/exclusion facilities for growth and yield, canopy gas exchange rates, community structure (USDA/Raleigh, Beltsville/MD, MA, AL, TX, BTI, USEPA/Corvallis); O₃ and CO₂ interactions (USDA/ Raleigh, Beltsville/MD); CSTR/greenhouse systems for laboratory O₃ fumigations (BTI, NJ, MA, PA, VA, USDA/ Raleigh, Beltsville; USEPA/Corvallis); plant disease interactions/mycorrhizal associations (MA, NY, USEPA/Corvallis); leaf gas exchange rates, photosynthesis (VA, MD, BTI, USDA/ Raleigh, TX); antioxidant biochemistry/enzyme activity (NJ, VA, PA, USDA/Beltsville, Raleigh); gene regulation/molecular biology (NJ, PA); plant transformation (NJ, PA); modelling pollutant uptake and plant growth responses (MN, BTI, USDA/Raleigh, USEPA/Corvallis).

A primary consideration to attain the objectives of this revised regional project is to enable the effective flow of information between the different levels of research. This interchange will be accomplished between whole plant studies and mechanistic studies by the exchange of unique plant material. Whole plant experiments will characterize growth and yield responses, examine the influence of environmental conditions on O₃ effects, and further identify genotypes that are sensitive or tolerant to O₃. Plant material identified by such research will be used by other scientists to test one or more of the hypotheses in Figure 1, which are related to the mechanism of O₃ toxicity or defense.

Information derived from mechanistic studies will identify specific proteins and genes that respond to O₃ and enable characterization of enzyme activity and gene expression. Such 'marker' genes can be incorporated into a biochemical screening procedure to assess the relative tolerance of important plant species and genotypes in the northeast. These genes also will be utilized in biotechnology experiments to produce genotypes with enhanced O₃ tolerance. Transgenic plants will be tested for field performance to verify O₃ resistance.

The information obtained from field research and the influence of environmental variables on plant response will increase our understanding of O₃ effects under natural conditions. This knowledge is necessary to develop process-oriented models of O₃ impact on plant productivity which can be utilized to estimate growth and productivity losses under a number of ambient pollutant concentration regimes. Results from all aspects of the project's research will be incorporated into USEPA Criteria Assessment Documents that constitute the scientific bases for establishing Ambient Air Quality Standards.

1. Characterizing Plant Responses to Ozone.

At MD (Mulchi), studies will be conducted on physiological and morphological processes by which CO₂ enrichment alleviates the negative impact of low O₃ concentrations on plant yield. Soybean will be grown to maturity in open-top chambers aspirated with charcoal filtered air (CF) containing two levels of CO₂ (350 or 500 $\mu\text{L L}^{-1}$) and three levels of ozone (25, 40 or 55 nl L^{-1}) under well-watered conditions. The plants will be grown to the third trifoliolate stage of growth before initiating the collection of data.

Specific studies will examine the effects of treatments on the morphology of cells (light microscopy) and membranes (HPLC analysis of lipid fraction) of the vascular system in leaves. Carbohydrate translocation activities and partitioning in plants will be investigated by pulse labeling with highly enriched isotopes of carbon (¹³C or ¹⁴C) as ¹⁴CO₂. These studies will be conducted on leaf tissue of the same age and location on the plants and at different

nodes to span both the vegetative and fruiting phases of growth.

In NY (Long Island), McGrath has reported that sensitivity to high ambient O₃ concentrations and infection by powdery mildew was related to plant developmental stage in cucurbits; fruiting coincided with increased sensitivity to both. Proposed research is to further characterize whole plant responses to O₃ and foliar pathogens, emphasizing alterations in sensitivity to either, in relation to carbon allocation shifts that occur during fruiting. The impact of O₃ on yield will be examined by using foliar applications of EDU to protect plants from natural exposures. Cultivars varying in their sensitivity to O₃ will be compared for foliar injury under natural field conditions and in greenhouse exposure chambers (cooperative study with Zilinskas, NJ). Changes in susceptibility to O₃ injury and to infection by powdery mildew, associated with fruit production in cucurbits, will be investigated by using successive plantings of cultivars that differ in O₃ sensitivity and comparing foliar symptomatology and yield during the growing season. A second approach to be utilized is a comparison among concurrent plantings of cultivars at various sites in the northeast.

At MA, Manning will continue investigations examining the response of cultivated and native plant species to O₃. These studies will be conducted under controlled, greenhouse conditions to identify sensitive and tolerant genotypes. Promising candidates will be further evaluated in the field in open-top chambers. EDU will be used to determine ambient O₃ effects on candidate plants in the field. Potential species include: browallia, turfgrasses, asters, blackberry, viburnum, apple, and white, loblolly, and short-leaf pine. This work will be done cooperatively with researchers from VA, AL, MD and TX where EDU application procedures and data collection will follow protocols developed at MA.

The response of white clover to O₃ will be studied in depth. Little is known about the effects of O₃ on leaf and shoot production. Detailed growth analysis, including RGR, ULR, RSR, LAR, LWR and SLA will be conducted with the NC-R and NC-S clones to determine the morphological and physiological responses to O₃ under greenhouse and ambient conditions. EDU will be used in the field to distinguish O₃ effects on growth parameters.

At TX, Flagler will continue research with antioxidant chemicals in cooperation with Manning (MA). These studies will consist of a multi-field site evaluation of EDU and Ozoban as protectants against O₃ for growth and biomass accumulation of three pine species, white, loblolly and short-leaf. Physiological and biochemical investigations addressing the mode(s) of action of these chemicals will be initiated. Specific measurements for these studies will include foliar injury, height and diameter incremental growth, biomass accumulation, needle gas exchange rates (LiCor Photosynthesis system) and alterations in nitrogen metabolism (total foliar N, nitrate reductase activity) and Rubisco content (PAGE and immuno-blotting). Greenhouse studies, in the absence of O₃, will be conducted to assess the effect of chemical application on growth, physiology and metabolism of the pine species.

At Boyce Thompson Institute, Cornell University, NY, a group of scientists (Laurence, group leader, Weinstein, Topa, MacLean, Jacobson, Comstock) will continue studies of ecosystem and individual plant responses to O₃ and other environmental stresses. At the individual level, researchers will evaluate whether the differences in growth patterns between seedlings and mature hardwood trees (red oak and sugar maple) lead to a difference in sensitivity to O₃. At the community level, studies will examine the effect present and future patterns of O₃ have on the productivity of eight major forest tree species in four regional forest

types of North America. In this project, scientists will be evaluating the degree to which nutrient or soil limitations and inter-tree competition alter the O₃ damage to productivity. At the regional level, researchers will assess, through modelling, the relative significance of O₃ in comparison to other climate change factors in altering the productivity and composition of forests in the southeastern U.S.

Studies with sugar maple are designed to test the central hypothesis that long-term exposure to O₃ reduces allocation of photosynthate to roots of seedlings. In testing this hypothesis, scientists will examine changes in source/sink relationships and a direct inhibitory effect of O₃ on the transport process (inhibition of phloem loading or sieve tube occlusion). Sugar maple seedlings will be grown for three years in open-top chambers at three O₃ levels. Changes in seasonal growth and carbon source/sink relationships will be assessed by examining carbon acquisition, allocation (to various plant organs), partitioning (among various chemical fractions), in conjunction with relative growth measurements. Staggered harvests will be conducted over three growing seasons, and phenological observations, and measurements of net photosynthesis (LiCor photosynthesis system), relative growth rates, and carbon partitioning among starch, sucrose and reducing sugars, will be interfaced with short-term ¹⁴CO₂-labeling experiments. These latter experiments will assess the fate of recently assimilated ¹⁴CO₂ by following partitioning of label into various chemical fractions, including respiratory losses. Ozone inhibition of photosynthate transport will be assessed using both autoradiography on leaf tissue (to determine vein loading) and micro-autoradiography on leaf and stem tissue (to examine the transport pathway). Finally, O₃ effects on N retranslocation in sugar maple saplings will be examined. Foliar samples will be collected bi-weekly from mid-July to the beginning of October and analyzed for N, total protein, and soluble amino acid content. Support for the hypothesis that O₃ interferes with N retranslocation would occur if decreases in foliar N content over time were slowed by high O₃.

At USEPA/Corvallis (Andersen), has reported that O₃ exposure of ponderosa pine seedlings reduces new growth and new root starch concentration the year following exposure (Andersen *et al.*, 1991) and significantly increases the CO₂/O₂ ratio from the root/soil compartment (Scagel and Andersen, 1994). The hypothesis that O₃ accelerates root turnover and stimulates microbial activity in these plant systems is currently being investigated. Efforts are now under way to separate root from microbial respiration. The future direction of the root/rhizosphere research is to further characterize changes in root metabolism as a result of O₃ stress, and determine how metabolic shifts may impact the plants ability to acquire both moisture and nutrient resources, and respond to other co-occurring stresses. In addition, shifts in rhizosphere biology, resulting from O₃ exposure, will be further characterized.

At USDA/Raleigh, Miller and colleagues have selected ten clones of clover based on visible injury response to O₃ exposure (100 nl L⁻¹, 6 h d⁻¹ for 3 days). Field and green-house studies in charcoal-filtered air indicated no differences in stomatal conductance between tolerant and sensitive clones. However, the stomatal response to elevated CO₂ was greater in the a resistant genotype (NC-R) compared to a sensitive genotype (NC-S). Research at Raleigh will continue to investigate stomatal characteristics as a mechanism of O₃ tolerance. Clones will be evaluated under a range of O₃ exposure conditions, in field and laboratory studies, for stomatal response (LiCor diffusion porometer). These studies will include 'real time' measurements of O₃ flux to better quantify physiologically effective O₃ doses.

At VA, Chevone will utilize the NC-R and the NC-S clover clones to continue studies of the mechanisms of O₃ tolerance. Previous experiments, using high O₃ exposures (150 to 200 nl L⁻¹, 4 h) and low light intensities (350 to 600 μE m⁻² s⁻¹), resulted in >50% increases in stomatal resistance within 90 min., limiting internal O₃ flux. In proposed studies, net photosynthesis and stomatal resistance will be measured during O₃ exposures (50 to 100 nl L⁻¹ 6 h d⁻¹ for 4 or 8 days) at low and high light intensities, 500 and 1100 μE m⁻² s⁻¹, respectively. Changes in apparent O₃ flux will be calculated to estimate the internal dose. This information will be utilized to develop fumigation regimes that minimize stomatal closure and provide a more uniform level of oxidative stress to allow for the maximum response of the antioxidant system and other protective, biochemical processes.

2. Factors that Determine Plant Response to Ozone

The importance of the ambient environment in affecting a plant's reaction to O₃ has been long recognized in the air pollution literature, but has been investigated primarily in controlled, experimental situations (Runekels, 1992). Much less is known about plant response in a natural field setting (Grunhage and Jager, 1994; Krupa *et al.*, 1993a). Consequently, a research component that focuses on ambient O₃ exposure dynamics and environmental interactions in relation to whole plant effects has been added to the revised proposal as a third primary objective (Fig. 1).

One of the key issues in ambient O₃ exposure-plant response research is to provide a satisfactory mathematical explanation of stochastic cause-effect relationships. At MN, Krupa (in cooperation with Manning, MA) will continue development of a predictive model of plant response to ambient O₃ that incorporates the influence of other environmental variables. Plant species, such as radish, white clover, snapbean and black cherry (all species with known sensitive cvs. or clones) will be used in modeling the dynamics of ambient O₃ exposures and biomass or yield responses. The comparison of tolerant and sensitive genotypes is an important consideration since the response of sensitive plants often has been found to have little relationship to ambient O₃ concentrations. (Tonneijck and Bugter, 1991; Krupa *et al.*, 1993). The approach used will be an extension of the previous work of Krupa *et al.* (1994a, 1995) where the daily dynamics of ambient photochemical O₃ production, patterns of O₃ flux from the atmosphere into plant canopies and the foliar injury or yield response *per se* were integrated to provide a cohesive view of cause and effect. Such an effort is required in understanding real world exposures and their adverse effects on plants.

At AL, Chappelka has conducted field surveys of foliar O₃ injury in wilderness areas and will continue these surveys in the Great Smokey Mountains National Park. Visible injury response data will be correlated with regional O₃ concentrations and environmental factors such as temperature, soil moisture and vapor pressure deficit to develop exposure-response correlations. Initial studies will be conducted to relate visible foliar injury to growth of forest species, using radial growth of selected species as the primary indicator. Simulated ecosystem studies will be conducted in open-top chambers to investigate O₃ effects on an early successional old-field community. Response measures will include reproductive effort, nutrient cycling, litter decomposition and shifts in species diversity.

3. Mechanisms of O₃ Toxicity and O₃ Tolerance

Several mechanisms of O₃ toxicity or tolerance will be investigated by scientists at cooperating research institutions (Fig. 1). These studies will utilize plants of known O₃ sensitivity, as determined in whole plant studies; or plants that have been genetically altered with respect to their oxidative stress capacity.

At NJ, Zilinskas will continue to examine the role of antioxidant enzymes in providing O₃ tolerance. The necessary molecular probes and transgenic plants have been produced and the proposed studies will provide a better understanding of the genetic and molecular basis of expression and the functional significance of these enzymes in the protection of plants against O₃ and other oxidative stress conditions.

Five specific objectives will be examined during the next five years. These are:

1. to follow the expression in pea of SOD, APX and MDAR in response to O₃;
2. to analyze the effects of O₃ exposure (on the whole plant as well as at the physiological and molecular level) on transgenic plants which overproduce the antioxidant enzymes SOD and APX;
3. to construct transgenic plants which overproduce MDAR and to examine the tolerance of these plants to O₃;
4. to construct (using antisense technology) and analyze the phenotype of transgenic plants which underexpress SOD, APX and MDAR; and
5. to construct plants (or obtain by sexual crossing) which overproduce all three enzymes (SOD, APX, MDAR) and examine the tolerance of these plants to O₃.

With regard to objective 1, data obtained thus far has shown that there is clearly a response of these enzyme systems to O₃, and that the overall regulation of their expression appears to be complex. Previous experiments were conducted at O₃ doses (300 nl L⁻¹ for 1.5 to 6 h), which differentially affected the expression of SOD and APX genes. Proposed research will utilize these high concentrations and lower ones that are comparable to high ambient concentrations (100 to 150 nl L⁻¹ for 4 to 6 h). Other laboratories have shown that total APX activity increased considerably under low to moderate O₃ exposures; the situation with SOD activity is not as clear, likely being influenced by the inadequacy of the indirect solution assays that have routinely been applied. With the availability of specific cDNA and antibody probes, the problem can be addressed more systematically and quantitatively, in addition to examining the more fundamental question of the level of regulation (*i.e.* transcription or post-translational).

The overall working hypothesis for objectives 2 through 5 is that a balanced set of enzymes is necessary to minimize oxidative damage from O₃. However, since it is not known which enzymes might be rate-limiting, the approach proposed is to overexpress each enzyme individually, as well as in concert. Likewise, it is known that an excessively high level of overproduction of SOD in the chloroplast provides no protection against O₃, whereas a relatively low level of overexpression provides protection against oxidative stress. Thus it is necessary to analyze a number of independent plants where transgene expression varies from low to very high levels of overproduction of these antioxidative enzymes. As antisense technology allows one to address the same question in a converse manner, these experiments will provide an independent means to examine the role of these enzymes in O₃ tolerance.

Construction of transgenic plants will follow protocols and design previously used in

Zilinskas' laboratory. Analysis for the expression and stable integration of the transgenes will be performed as in the past. Once plants have been identified that have single gene insertions and express known (low, medium and high) levels of the transgenes, their physiological response to O₃ and other oxidative stress conditions will be assessed. Collaboration with other cooperating scientists (Miller, USDA/Raleigh; Lee, USDA/Beltsville and Mulchi, MD) will enable measurement of physiological responses of the genetically altered plants under field conditions and in precisely controlled fumigation chambers.

At PA, Pell will continue to explore the nature of protein oxidation by O₃. Of major interest is the capacity of O₃ to induce carbonyl formation on proteins and which proteins may be most susceptible. Antibodies are available for carbonyl groups to allow detection of such modifications. Another interest concerns the rapid loss of the Rubisco small subunit mRNA induced by O₃ exposure. Studies will examine whether this loss in message can be detected for other important proteins, and the mechanism by which such losses occur. One critical area of this research is to determine whether O₃ influences the stability of key mRNA transcripts. Also at PA, work is progressing on transforming potato plants to prevent production of O₃-induced ACC synthase. Once this transformation is completed, ethylene production in response to O₃ exposure will be examined to determine whether O₃-induced emission of ethylene and the hypothesized acceleration of foliar senescence is a completely negative response, or perhaps partially compensatory.

A key question arising from research at PA is whether O₃-induced nuclear encoded events, e.g. decline in *rbc*s and induction of ACC synthase leading to ethylene emission, regulate chloroplastic events. Alternatively, is the reduction in Rubisco a direct result of oxidation phenomena occurring within the chloroplast? Transformed lines of tobacco (developed by Zilinskas, NJ) which overexpress antioxidants at specific locations within the cell, viz. cytoplasm, chloroplast or both, will be used by researchers at PA to determine:

1. Whether O₃-induced nuclear events, e.g. drop in *rbc*s or induction of ACC synthase, will occur in tobacco plants with elevated protection from oxidation *vis a vis* overexpression of plastid AP; and whether these plants will exhibit any chloroplastic effect on concentration of Rubisco; and
2. If similar O₃-induced nuclear events occur in plants exhibiting increased cytoplasmic antioxidizing potential.

At USDA/, Beltsville, Robinson will examine the role of ascorbate (AA) and natural sulfhydryl reagents in ameliorating O₃ damage in crop plants. In regard to the ability of AA to serve as an antioxidant, many questions remain to be answered. Among these are 1) the magnitude of carbohydrate status in relation to AA synthesis, transport and redox status, 2) the magnitude of detoxification of pollutant products in relation to AA content, 3) the mechanisms by which oxidized and reduced AA are retained within the chloroplast or transported to sites of oxidative damage, and 4) the dependence of AA synthesis, transport and redox status upon leaf starch, sucrose and hexose sugar levels, and the impact this may have on crop productivity in ambient air environments. The proposed research should provide information to answer these questions and define the role of AA in plant tolerance to O₃.

The research approach will be to utilize genetic lines of soybean, snapbean and spinach which are sensitive or tolerant to O₃ and to induce increased foliar carbohydrate production via elevated CO₂ and nitrogen limitation. Control plants will be maintained with

limited or normal hexose sugars. Photosynthetically competent chloroplasts will be isolated from test and control plants, and synthesis of AA from ^{14}C -labeled precursors, as well as the transport of oxidized and reduced AA across the chloroplast envelope will be examined. Intact leaves will be selected from O_3 -treated and control plants, lyophilized, subjected to the nonaqueous technique for leaf organelle separation and the fractions will be analyzed for photosynthates and 'real-time' AA synthesis, redox status, and intracellular transport patterns.

At USDA/Beltsville, Lee will continue studies on mechanisms of O_3 tolerance using resistant and susceptible genotypes of soybean and snap bean to investigate the protective mechanism(s) of ethylene diurea (EDU). This research will involve the development of methods and techniques (e.g. high-performance liquid chromatography-electrochemical detection [HPLC-EC], electron paramagnetic resonance [EPR], or spectrophotometry) to detect oxyradicals in plant tissues. Studies will include physiological and biochemical responses of antioxidant protective mechanisms and assessment of identified resistance components for improved crop performance.

At USDA/, Raleigh, Miller and Booker will examine mechanisms of plant tolerance to O_3 using genotypes of white clover and snap bean that have a wide range of sensitivity to O_3 . Preliminary studies with clover clones have shown that basal levels of several antioxidant enzymes (GR, APX, DHAR, MDHAR, and SOD) are not different in sensitive and tolerant genotypes. Enzyme activities will be characterized before and after O_3 exposure. Changes in lignin derivatives and phenolic polymers in response to O_3 treatment will be examined in other genotypes of clover and snap beans..

At VA, Chevone will continue studies of plant antioxidant systems as a mechanism of O_3 tolerance. Clover clones NC-S and NC-R will be used in CSTR fumigations in a step-function concentration profile that simulates ambient conditions (O_3 range of 40 - 120 nl L^{-1} , 8 h d^{-1} , 4 or 10 d) to allow full response of antioxidant enzymes. The activity of SOD, APX and catalase (CAT) will be characterized in the clover clones (solution assays), prior to and after O_3 exposure. Isozyme activity of SOD will be analyzed using native PAGE and the NBT activity stain and mRNA levels of plastid SOD will be examined using probes from pea (B. Zilinskas, NJ). Protein concentration of enzymes and isozymes will be assayed using specific antibodies as these are available. A second area of research is the role of stress-response proteins that include the heat-shock and chilling proteins, dehydrins and LEAS. Using specific antibody probes, the endogenous concentrations and induction of these proteins will be examined in the NCR and NCS clover clones before and during O_3 exposure.

Table 1. Summary of specific cooperative research projects among participating stations.

<u>Station/Cooperators</u>	<u>Research Responsibilities</u>
1. NY-McGrath = NJ-Zilinskas	Field testing pumpkin varieties for developmental response to O ₃ and powdery mildew. Characterize O ₃ sensitivity of pumpkin varieties in controlled, CSTR fumigation studies.
2. NJ-Zilinskas USDA/Raleigh-Miller, Heagle PA-Pell VA-Chevone	Development of antioxidant molecular probes (pea), production of transgenic tobacco overexpressing antioxidant enzymes. Open-top chamber, field testing of transgenic tobacco. Rubisco sensitivity to O ₃ of transgenic tobacco. Application of pea antioxidant molecular probes to white clover gene transcription analysis.
3. USDA/Raleigh-Miller,Booker VA-Chevone	Comparative response to O ₃ of gas exchange rates and antioxidant enzyme activity of white clover clones in field (USDA/Raleigh) and laboratory (USDA/Raleigh,VA) using different varying O ₃ profiles.
4. MA-Manning MN-Krupa	Field O ₃ exposures of selected crop species, environmental monitoring. Environmental data analysis, model development of plant response to O ₃ exposure profiles.
5. MA-Manning TX-Flager	Comparative effects of EDU on growth and foliar O ₃ symptoms of three pine species at two locations.

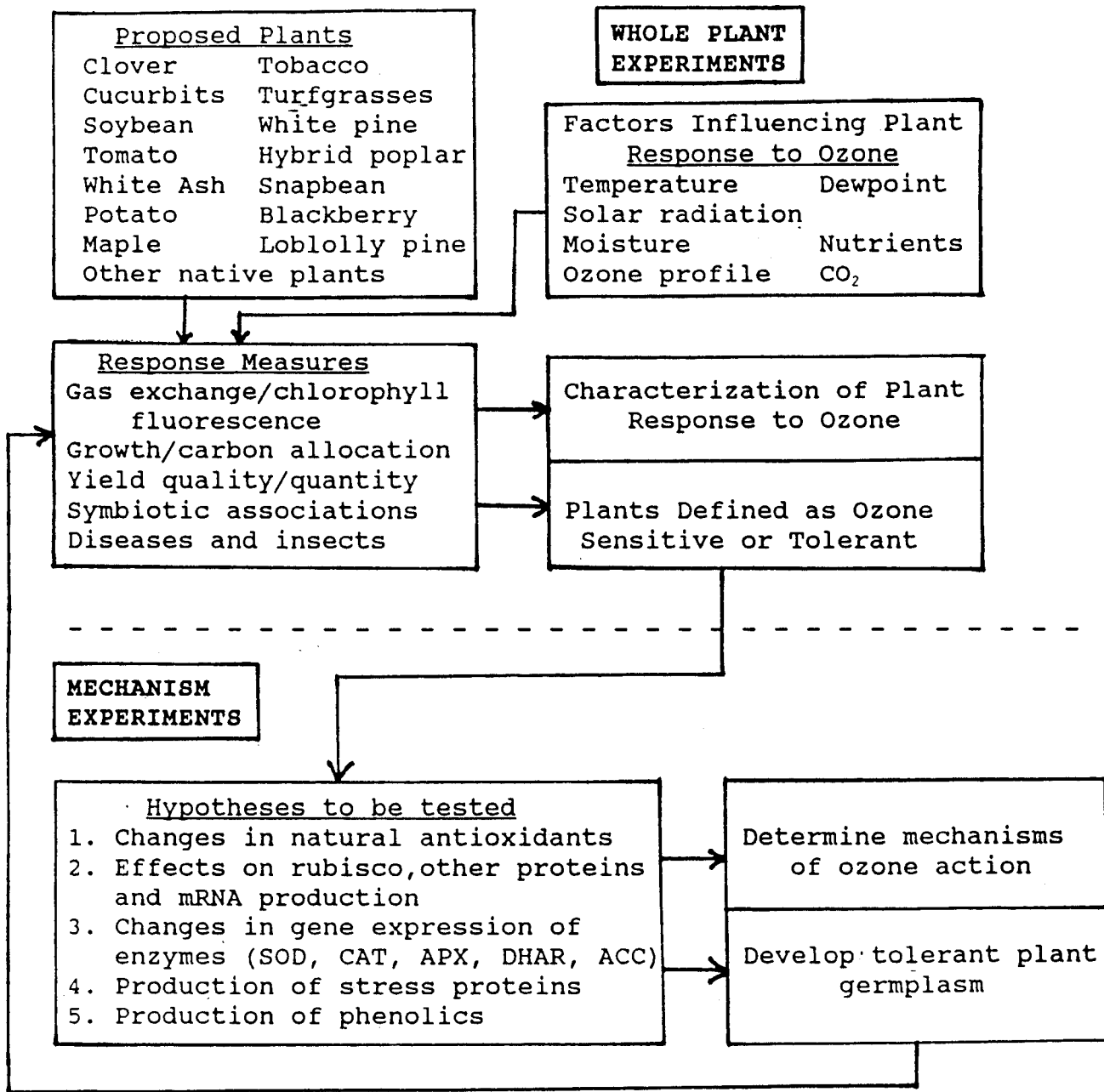


Figure 1. Flow diagram of interrelationships in the Regional Research Project 'Characterization and Mechanisms of Plant Responses to Ozone in the Northeastern U. S.'.

ORGANIZATION:

The NE-176 Technical Committee is organized according to the revised 1986 Manual for Cooperative Regional Research (USDA/CSRS)^W. Membership is in accordance with pages 18-21 of the Manual with each participating station and agency limited to one vote. All voting members are eligible for office. A Chairperson and a secretary are elected annually. Their duties are outlined on page 20 of the Manual. Meetings of the Technical Committee are held in the winter of each year in accordance with pages 21-21 of the Manual.

SIGNATURES:

Regional Project Title: Characterization and Mechanisms of Plant Responses to Ozone in the Northeastern U.S.

Robert Miller
Administrative Advisor

6-26-95
Date

Janet Rossi
Chairman, Regional Association of
Directors

8/9/95
Date

Dee F. Vonderheid
Chairman, Committee of Nine

9/28/95
Date

George E. Cook
Administrator,
Cooperative Research, Education and Extension Service

9/28/95
Date

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