To the Graduate Council:

I am submitting herewith a thesis written by Robert Michael Barni entitled "Effects of the Herbicide Paraquat on Growth and Superoxide Dismutase Levels in Four Species of Southern Pine Callus." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

Karen W. Hughes, Major Professor

We have read this thesis
and recommend its acceptance:

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Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

EFFECTS OF THE HERBICIDE PARAQUAT ON GROWTH AND SUPEROXIDE DISMUTASE LEVELS IN FOUR SPECIES OF SOUTHERN PINE CALLUS

A Thesis

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ABSTRACT

The effect of the herbicide paraquat on growth, SOD levels and SOD banding patterns was observed in four species of southern pine callus. Short leaf pine callus cultures exhibited a mean increase in growth over that of the controls when grown on medium supplemented with 10^{-8}M and 10^{-6}M paraquat. Average growth of loblolly, Virginia and white pine tissue was inhibited when grown on paraquat supplemented media.

Crude extracts of the pine callus were prepared and subjected to electrophoresis and enzyme assays. Multiple bands of Cu-Zn SOD were found in all the lines tested and 1 band of Mn SOD activity was also present in all four species. Banding patterns varied from species to species, but band number was not affected by paraquat treatment.

Quantitation of SOD levels through densitometer scan analysis of polyacrylamide gels as well as enzyme assays indicated a significant increase in short leaf SOD content over that of the controls when grown on 10^{-8}M and 10^{-6}M paraquat. SOD levels in loblolly, Virginia and white pine callus neither increased nor decreased significantly in response to paraquat treatment.

These results may indicate that the increased levels of SOD in short leaf calli may be providing protection against the paraquat toxicity and thus allowing growth to continue as normal.

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Bis	N,N'Methylenebisacrylamide
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium Bromide
NBT	3-3'-(3,3'-Dimethoxy-4,4'-biphenylene)bis [2(p-nitrophenyl)-5-phenyltetrazolium Chloride]
PMS	Phenazine Methosulfate
SOD	Superoxide dismutase
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	Hydroxymethyl nitromethane

CHAPTER I

INTRODUCTION

I. PURPOSE

The aims of this study were threefold: 1) To determine the effects of the herbicide paraquat on callus cultures of four species of pine, Pinus taeda L. (loblolly pine), Pinus strobus L. (Eastern white pine), Pinus virginiana Mill. (Virginia pine), and Pinus echinata Mill. (short leaf pine), 2) To examine electrophoretic banding patterns and levels of the enzyme superoxide dismutase (SOD) in cell cultures exposed to paraquat. 3) To correlate, if possible, changes in superoxide dismutase levels or banding patterns with growth response of the cell cultures to paraquat.

II. BACKGROUND

Paraquat

Uses. The herbicide paraquat (1,1-dimethyl-4-4'-bipyridylium salt) or methyl viologen has been shown to be a rapid, non-specific and non-residual contact herbicide and has broad horticultural and agricultural usage. Practically all species of broad leaf weeds and grasses have been shown to be susceptible to the herbicidal action. As a result of this universal activity, paraquat is commonly used as a pre-planting herbicidal defoliant. In addition to its non-specificity, paraquat has the advantage of being inactivated upon contact with soil particles.

This inactivation, resulting from the tenacious binding of paraquat to soil organics and clays, enables the planting of crops immediately following field treatment, without the interference of deleterious herbicidal residues. Other uses of paraquat have included the pre-wilting of animal fodder and the desiccation of foliage to aid in the harvesting of crops such as cotton and potatoes (Calderbank and Slade, 1976).

Recently nonherbicidal uses of paraquat have received much attention. Research by Roberts (1973) and subsequent workers (Brown and Nix, 1975; Falco and Finnerty, 1976; Kiatgrajai, Rowe, Conner, Peters and Roberts, 1976; Miniutt, 1977) have shown that the application of paraquat to a number of southern pine species causes the induction of resin soaking or lightwood formation. This increase in production of oleoresins and associated metabolic products may in the future provide a renewable source of varied and useful hydrocarbon compounds.

Chemical characteristics. Paraquat is a highly polar molecule which is rapidly broken down by exposure to light into a number of non-herbicidal compounds; however, in acid and neutral solutions of pH 10 or lower, it exhibits unusual stability (Slade, 1966). Boiling in sulfuric acid or autoclaving results in no appreciable degradation of paraquat's integrity. Destruction begins to occur in basic solutions between pH 10 and pH 12 (Calderbank and Slade, 1976). Degradation of paraquat has been shown to occur in the soil as a result of microbial metabolic activity but metabolism and breakdown of paraquat by higher plants has been shown to be virtually nonexistent (Calderbank and Slade, 1976; Calderbank, Morgan, and Yuen, 1961; Funderburk, 1969).

Mechanism of action. The application of paraquat to the aerial portions of a plant usually results in rapid membrane damage, desiccation of the leaf and death. Approximately six hours after paraquat treatment, the double membrane surrounding the chloroplast becomes leaky and eventually ruptures. During this time the tonoplast bursts and mixing of the vacuolar contents and the cytoplasm occurs. Finally, the plasmalemma ruptures releasing the cell's contents and resulting in rapid leaf desiccation and death (Farrington, Ebert, Land, and Fletcher, 1973).

This high herbicidal activity of paraquat lies in the fact that it is a flat, coplanar molecule consisting of two pyridine rings. Upon reduction by a single electron, the molecular configuration permits a stable free radical to be formed. The odd electron is delocalized over the entire molecule, occupying any one of the 18 possible resonance forms. The stability of the free radical depends upon this delocalization and upon the position of the two nitrogen atoms in the pyridine rings (Homer, et al., 1960).

Early investigations have indicated that paraquat's reduction to the free radical form and subsequent herbicidal activity was primarily associated with the light reaction of photosynthesis. Mees (1960) observed that paraquat treated plants kept in the light were killed much more rapidly than similarly treated plants which remained in darkness. Black and Myers (1966) have also shown that paraquat is reduced to the free radical form when illuminated in a solution of isolated chloroplasts. Continued work has shown that the oxidized herbicide is capable of scavanging an electron from photosystem I in

the region of the primary electron acceptor; but it has also been shown that paraquat can cause an increase in the oxidation of NADPH as well as inhibit reduction of NADP (Bus, Aust, and Gibson, 1974). Therefore, the exact source of electrons for the reduction of paraquat is not known.

Paraquat's toxicity has been shown to require oxygen (Bus, Aust, and Gibson, 1974; Calderbank, 1972; Farrington, Ebert, Land, and Fletcher, 1973; Mees, 1960). The paraquat radical is believed to react with oxygen, resulting in the formation of superoxides ($O_2^{\cdot -}$) and other highly reactive free radicals. It is these free radicals which are thought to be the actual toxic agents responsible for paraquat's activity (Bus, Cagen, Olgaard, and Gibson, 1976; Giannopolitis and Ries, 1977a). Hassan and Fridovich (1978) observed that paraquat was much more toxic to E. coli in the presence of oxygen than in its absence. Mees (1960) demonstrated that a decrease in the speed of the herbicidal activity occurred under low partial pressures of oxygen. The actual toxic agents responsible for paraquat toxicity are formed upon the rapid reoxidation of the herbicide in the presence of oxygen. This immediate reoxidation of the paraquat radical was initially believed to reduce oxygen to water via the intermediate production of hydrogen peroxide (Farrington, et al., 1973; Calderbank, 1972). It was formerly believed that this hydrogen peroxide was the sole agent of paraquat's toxicity; however, more recent studies indicate that H_2O_2 toxicity in cells is accompanied by additional destructive intermediates which can account for the rapid destruction observed upon paraquat treatment. It is fairly well established that electron transfer and subsequent proton uptake

between reduced paraquat and oxygen results in the formation of the superoxide radical (Dodge, Harns, and Baldwin, 1970; Farrington, et al., 1973; Bus, et al., 1974, 1976; Fridovich, 1974b, 1976; Giannopolitis and Ries, 1977a). Farrington, et al. (1973) have shown that the $O_2^{-}\cdot$ and associated transient intermediates are capable of surviving long enough to reach the various cell membranes where damage is first observed in paraquat treated plants. This membrane damage, resulting from lipid peroxidation induced by highly reactive free radicals, is thought to be paraquat's primary mode of action (Dodge, et al., 1970).

In addition to its direct action upon cellular membrane components, the $O_2^{-}\cdot$ can undergo a reaction with H_2O_2 producing a much more reactive free radical; .OH, the hydroxyl radical. In the Haber-Weiss reaction:

$$O_2^{-}\cdot + H_2O_2 \longrightarrow \ .OH + OH^- + O_2$$

the resulting hydroxyl radical produced is the most powerful oxidant known. It is capable of attacking any cellular organic molecule and is responsible for a high degree of biological damage (Farrington, et al., 1973; Fridovich, 1976).

Oxygen Toxicity in Cells

Source of free radicals. The presence of oxygen offers respiring organisms a distinct advantage over the less efficient anaerobic mode of metabolism; however, the presence of molecular oxygen and its constant reduction to water has been shown to produce extremely toxic intermediates (Halliwell, 1974; Fridovich, 1972, 1974b, 1975a, 1975b, 1976, 1978; Gregory and Fridovich, 1973b; Gregory, Goscin and Fridovich, 1974; Hassan and Fridovich, 1978; McCord and Fridovich, 1978; Rolfe, Hentges,

Cambell, and Barrett, 1978; Yost and Fridovich, 1976). These intermediates, if left unchecked, are capable of rapid cell destruction.

In all aerobic organisms, molecular oxygen is constantly being reduced to water through the acceptance of four electrons. This reduction, occurring either a) univalently, b) divalently, c) trivalently, or d) tetravalently, results in the formation of $O_2^{-}\cdot$, H_2O_2, .OH and H_2O, respectively.

a) $O_2 + e^- \longrightarrow O_2^{-}\cdot$

b) $O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$

c) $O_2 + 3e^- + 3H^+ \longrightarrow H_2O + .OH$

d) $O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$

Due to its electron configuration involving spin restrictions molecular oxygen in the ground state prefers the univalent pathway of reduction (Taube, 1965). Univalent reduction of oxygen is known to yield a toxic, highly reactive intermediate the superoxide radical ($O_2^{-}\cdot$). Enzymes such as cytochrome oxidases have evolved which are capable of compensating for the electron spin restrictions presented by molecular oxygen. These enzymes accomplish the divalent and tetravalent reduction of O_2 without the evolution of harmful intermediates; however, despite these enzyme systems, the univalent reduction of O_2 and subsequent production of superoxide radicals does occur.

Various biochemical reactions as well as specific biological activities have been shown to generate $O_2^{-}\cdot$. Autoxidation of reduced flavins, hemoproteins and ferredoxins as well as the aerobic action of various oxidative enzymes are known to yield $O_2^{-}\cdot$ (Massey, et al., 1969;

McCord and Fridovich, 1969; Fridovich, 1976; Halliwell and Ahluwalia, 1976). Mitochondrial fragments, chromatophores, illuminated chloroplasts and phagocytic activity of leucocytes also produce substantial amounts of the free radical.

It has also been shown that superoxide radicals can be induced through the action of outside agents. Increases in the partial pressure of oxygen result in damage to bacteria and higher organisms due to the accumulation of high levels of $O_2^{-\cdot}$ (Gregory and Fridovich, 1973). Streptonigrin is known to exert its effect on E. coli through the production of the superoxide radical (Gregory and Fridovich, 1973a and 1973b; Hassan and Fridovich, 1977). As discussed in the previous section, the herbicide paraquat has also been shown to exert its lethal effect through the enhancement of intercellular $O_2^{-\cdot}$ concentrations (Farrington, et al., 1973).

Obviously the presence of $O_2^{-\cdot}$ and H_2O_2 can be extremely detrimental to cellular components. It would seem to be quite advantageous for an aerobic organism to possess a mechanism of preventing or limiting the availability of these two substances. Such enzymatic mechanisms do exist. Catalases and peroxidases are both capable of reducing H_2O_2 to H_2O. The scavenging of $O_2^{-\cdot}$ is accomplished through the activity of a fairly prevalent enzyme, superoxide dismutase.

Superoxide Dismutase

Protection against free radicals. Superoxide dismutases are enzymes which catalyze the removal of $O_2^{-\cdot}$ from respiring cells. The dismutation of the free radicals is accomplished by the following reaction:

$$O_2^{-}\cdot + O_2^{-}\cdot + 2H \longrightarrow O_2 + H_2O_2$$

This dismutation will occur in the absence of superoxide dismutase but the resulting oxygen molecule formed is in the excited singlet state which is also a reactive species. The enzymatic dismutation results in the production of the far less reactive ground state of molecular oxygen (Halliwell, 1974).

Forms of superoxide dismutase. Superoxide dismutases (SOD) are metalloproteins whose enzymatic activity was first discovered by McCord and Fridovich (1969). Since its discovery, three distinct forms of SOD have been described and studied from a number of various organisms. These different forms of SOD contain either iron, manganese, or copper and zinc as their prosthetic group; however, each type of SOD catalyzes the same dismutation reaction with comparable efficiencies (Fridovich, 1978).

Most aerobic eukaryotic cells have been found to contain a Cu-Zn form of SOD in their cytoplasm (Fridovich, 1974a). This SOD has an average molecular weight of 32,000 daltons and is composed of two identical, noncovalently bound subunits. Each subunit contains one Cu^{+2} atom and one Zn^{+2} atom (Fridovich, 1975b). It has been shown to be an unusually stable enzyme capable of withstanding drastic purification procedures such as acetone and lead citrate precipitation as well as heating to temperatures in excess of 70°C (Fridovich, 1976). In the presence of H_2O_2 and low concentrations of cyanide, the Cu-Zn SOD is inactivated. The susceptibility of this form of the enzyme toward cyanide and H_2O_2 provides an efficient means of distinguishing between the Cu-Zn and other forms of SOD (Beauchamp and Fridovich, 1973;

Fielden, Roberts, Bray, and Rotilio, 1973). The Cu-Zn SOD has been isolated and characterized from a wide variety of eukaryotic cells including higher plants (Table I), algae (Table II), fungi and yeast (Table III), bovine erythrocytes (McCord and Fridovich, 1969), chick liver and other higher animals (Bus, et al., 1976; Autor, 1976).

The iron containing and manganese containing SODs have been shown to exist in prokaryotic cells (Table IV). Generally the Fe SOD is located in the periplasmic space whereas the Mn SOD is found primarily in the cell matrix (Gregory, Yost, and Fridovich, 1973; Yost and Fridovich, 1973). In addition to the Mn SOD being contained within a prokaryotic cell matrix, it is also prevalent within the mitochondrial matrix and chloroplasts of eukaryotic cells (Fridovich, 1974; Asada, Kanematsu, and Uchida, 1977; Bridgen, Harris, and Northrop, 1975).

Typically the Fe SOD is composed of two identical subunits each of which contain one Fe^{+3} atom. The molecular weight of both non-covalently bound subunits is approximately 39,000 daltons. The amino acid sequence homology between E. coli Fe SOD, Mn SOD and mitochondrial Mn SOD is 80% indicating the close relationship between these forms (Steinman, 1978).

The Mn SOD from prokaryotic cells is also composed of two similar subunits, each containing one atom of manganese; Mn^{+3}. The molecular weight of this enzyme is about 40,000 daltons; however, the mitochondrial Mn SOD differs from the others in that it contains four identical subunits per molecule instead of the usual two. It also differs in having a molecular weight of 80,000. Both Fe SOD and Mn SOD are resistant to cyanide and hydrogen peroxide; however, treatment with chloroform-ethanol

TABLE I

A PARTIAL SUMMARY OF PERTINENT LITERATURE ON SUPEROXIDE DISMUTASE FROM HIGHER PLANTS

Date	Author	Organism	Contribution
1972	Sawada, Ohyama, and Yamazaki	Pisum sativum	Purification and properties of Cu-Zn SOD
1973	Asada, Urano, and Takahashi	Spinacia oleracea	Reported preparation of crystalline Cu-Zn SOD from chloroplasts and cytosol.
1973	Beauchamp and Fridovich	Wheat germ	Characterized two Cu-Zn SODs and one Mn SOD
1975	Hai, Kovacs, Matkovics, and Matkovics	29 various seeds	Observed levels of SOD in seeds from various taxonomic units.
1975	Lumsden and Hall	Spinacia oleracea	Reported Cu-Zn SOD from stroma and Mn SOD from lammella of chloroplasts.
1976	Arron, Henry, Palmer, and Hall	Helianthus tuberosus	Reported Mn SOD from mitochondrial matrix, Cu-Zn SOD from intermembrane space and cytosol.
1976	Baker	Musa sapientum Persea americana Malus sylvestris Lycopersicon esculentum	Observed soluble SOD levels in pre- and post-climacteric fruits.
1977	Asada, Kanematsu, and Uchida	27 various photosynthetic organisms	Extensive survey of SOD from numerous taxonomic units.
1977b	Giannopolitis and Ries	Pisum sativum Zea mays Avena sativa	Observed changes in SOD levels of roots, shoots, and leaves during maturation.

TABLE I (Continued)

Date	Author	Organism	Contribution
1977c	Giannopolitis and Ries	Pisum sativum Zea mays	Purification of SOD showing increase in activity during shoot greening.
1978	Del Rio, Sevilla, Gomez, Yanez, and Lopez	Pisum sativum	Reported induction of Cu-Zn SOD activity at low Mn concentration in nutrients.
1978	Harper and Harvey	Lolium perenne	Reported increased levels of soluble and chloroplastic SOD in paraquat resistant cultivars.
1978	Jackson, Dench, Moore, Halliwell, Foyer, and Hall	Spinacia Medicago sativa	Observed high specific activity of Mn SOD in mitochondria and chloroplasts of leaves.
1978	Matkovics, Novac, Szabo, Vargas, and Farkas	Lycopersicon esculentum	Reported reduction in SOD levels after infection of plant with tobacco mosaic virus.
1979	Baum and Scandalios	Zea mays	Reported 5 isozymes of SOD in corn tissues

TABLE II

A PARTIAL SUMMARY OF PERTINENT LITERATURE ON SUPEROXIDE DISMUTASE FROM ALGAE

Date	Author	Organism	Contribution
1974	Abliovich, Kellenberg, and Shilo	*Anacystis nidulans*	Demonstrated the inducability of Mn SOD by high O_2 concentrations.
1975	Asada, Yoshileawa, Takahashi, Maeda, and Enmanji	*Plectonema boryanum*	Reported the purification of both Mn SOD and Fe SOD from blue-green algae.
1975	Lumsden and Hall	4 species of blue-green algae, 1 species of red algae and numerous green algae species	Performed an electrophoretic survey of both prokaryotic and eukaryotic algal SODs
1976	Henry, Halliwell, and Hall	*Chara* *Nitella* *Spirulina* *Scenedesmus*	Partial purification of Mn SOD from various eukaryotic algae.
1978	Henry, Gogotou, and Hall	*Anabaena cylindrica*	Reported partial purification of Mn SOD from heterocysts and vegetative cells.
1978	Kanematsu and Asada	*Spirogyra* *Chara* *Nitella*	Described Cu-Zn SOD found only in phragmoplast algae.

TABLE III

A PARTIAL SUMMARY OF PERTINENT LITERATURE ON SUPEROXIDE DISMUTASE FROM FUNGI AND YEAST

Date	Author	Organism	Contribution
1972	Goscin and Fridovich	*Saccharomyces cerevisiae*	Isolated and purified Cu-Zn SOD from yeast.
1972	Misra and Fridovich	*Neurospora crassa*	Described the purification of 1 isozyme of Cu-Zn SOD.
1973	Rapp, Adams, and Miller	*Fusarium oxysoprum* *Neurospora crassa*	Reported extensive characterization of Cu-Zn SOD.
1973a	Weisiger and Fridovich	*Saccharomyces cerevisiae*	Reported electrophoretic variants of purified Cu-Zn and Mn SOD.
1974	Lavelle, Durosay, and Michelson	*Pleurotus olearin*	Reported the absence of a Cu-Zn SOD from fungi. Contains only a Mn SOD.
1974	Gregory, Goscin, and Fridovich	*Saccharomyces cerevisiae*	Observed induction of both Cu-Zn SOD in cytosol and Mn SOD in mitochondria through increased O_2 tension.
1975	Ravindranth and Fridovich	*Saccharomyces cerevisiae*	Reported the crystallization of a tetrameric Mn SOD from mitochondria.
1976	Arron, Henry, Palmer, and Hall	*Neurospora crassa*	Reported isolation of Mn SOD from mitochondrial matrix and Cu-Zn SOD from intermembrane space.
1978	Shatzman and Kosman	*Dactylium dendroides*	Observed a coordination of Cu-Zn SOD and Mn SOD synthesis in response to altered Cu levels in media

TABLE IV

A PARTIAL SUMMARY OF PERTINENT LITERATURE ON BACTERIAL SUPEROXIDE DISMUTASE

Date	Author	Organism	Contribution
1970	Keele, McCord, and Fridovich	*E. coli*	Reported the first discovery of Mn SOD from a prokaryote.
1972	Vance, Keele, and Rajagopalan	*Streptococcus mutans*	Reported first discovery of 2 different forms of SOD from the same organism.
1973	Gregory and Fridovich	*E. coli* *Streptococcus faecalis*	Reported induction of Mn SOD in response to increase O_2 pressures.
1973	Yost and Fridovich	*E. coli*	Reported first purification of Fe SOD.
1973	Gregory, Yost, and Fridovich	*E. coli*	Observed localization of Fe SOD in periplasmic space and Mn SOD in cell matrix.
1974	Puget and Michelson	*Photobacterium leiognath*	Observed and purified the first Cu-Zn SOD from a marine bacterium.
1974	Hewitt and Morris	*Chlorobium* *Closteridium*	Reported high concentration of Mn SOD in obligate anaerobes.
1975	Bridgen, Harris, and Northrop	*Bacillus*	Reported partial sequencing of Mn SOD.
1976	Beem, Richardson, and Richardson	*E. coli*	Performed crystallization of Mn SOD
1976	Kusunose, Ichihara, Noda, and Kusunose	*Mycobacterium tuberculosis*	Reported first purification and isolation of a tetrameric Fe SOD.
1976	Lumsden, Cammack, and Hall	*Rhodopseudomonas spheroides*	Isolation and purification of Mn SOD.

TABLE IV (Continued)

Date	Author	Organism	Contribution
1976	Yamakura	*Pseudomonas ovalis*	Observed presence of Fe SOD
1977b	Hassan and Fridovich	*E. coli*	Observed induction of Mn SOD by herbicide paraquat application.
1977	Hatchikian and Henery	*Desulfovibrio desulfuricans*	Purified a Fe SOD from a strict anaerobic bacteria.
1978	Niekus, Wouters, DeVries, and Stouthamer	*Campylobacter sputorum*	Reported increase in levels of cytoplasmic Mn SOD in response to raised O_2 concentration.
1978	Baldensperger	*Thiobacillus dentrificans*	Performed purification of Fe SOD dimeric form from a chemotrophic bacteria.
1978	Steinman	*E. coli*	Reported the first complete amino acid sequencing of a Mn SOD.
1978	Kanematsu and Asada	*Chromatium vinosum*	Characterized an Fe SOD from an obligate anaerobic purple sulfur bacteria.
1979	Hofman, Krieg, and Smiberb	*Campylobacter fetus*	Reported inducability of Mn SOD with increased O_2 concentration.
1979	Welch, Sword, Brehm, and Dusanic	*Listeria monocytogenes*	Reported the partial purification of Fe SOD.

during extraction, the Tsuchihashi precipitation, results in their rapid denaturation. This susceptibility allows the distinction of the Mn SOD and Fe SOD from the Cu-Zn form (Beauchamp and Fridovich, 1973).

SOD induction. Increases in the levels of SOD have been accomplished through various means. Since enzymes are often induced or derepressed by their substrates, increases in $O_2^{\cdot -}$ concentrations could result in greater accumulation of cellular SOD. Intercellular increases in $O_2^{\cdot -}$ have been attempted through increases in external O_2 concentrations, exposure to streptonigrin or by the application of the herbicide paraquat (Fridovich, 1976). Experiments with E. coli, Streptococcus faecalis, and Saccharomyces cerevisiae have shown that growth under high concentrations of oxygen does result in elevated SOD levels. S. faecalis exhibited a 16-fold increase in Mn SOD and E. coli showed a 25-fold increase in Mn SOD in response to hyperbaric O_2; however, the Fe SOD present in both prokaryotes showed no alteration in synthesis due to the presence or absence of hyperbaric O_2 (Gregory and Fridovich, 1973a,b). Gregory, et al. (1974) reported the increase in the concentration of both Cu-Zn SOD and Mn SOD in Saccharomyces cerevisiae grown in high O_2 tension.

The herbicide paraquat has also been shown to augment the production of $O_2^{\cdot -}$ in cells (Farrington, et al., 1973). Hassan and Fridovich (1977b and 1978) observed a dramatic increase in the synthesis of Mn SOD in E. coli after paraquat application. The Fe SOD was not affected. Other studies with paraquat tolerance and increased SOD levels were carried out by Harper and Harvey (1978). In these experiments paraquat tolerance

in cultivars of perennial ryegrass was found to be the result of increased concentrations of SOD over that of the susceptible varieties.

CHAPTER II

MATERIALS AND METHODS

I. CULTURE METHODS

All tissue culture procedures were carried out in a sterilized transfer room or contamination control hood using sterile technique. Dissections were performed on paper towels saturated with 70% ethanol and instruments were autoclaved and flame sterilized before using. Cultures were grown in either 25 X 150 mm test tubes plugged with cotton and capped with Bellco KAPUTS or 125 ml Erlenmeyer flasks plugged with cotton and capped with aluminum foil. All media and media supplements were autoclaved for 20 minutes at 30 psi. All cultures were incubated at 25°C and exposed to 16 hours of daily illumination. Light intensity was maintained at 2.2×10^4 ergs-cm^{-2}-sec^{-1}.

Establishment of Pine Callus

Fresh seeds of loblolly (*Pinus taeda* L.), white (*Pinus strobus* L.), Virginia (*Pinus virginiana* Mill.) and short leaf (*Pinus echinata* Mill.) pines were supplied by the West Tennessee Regional Tree Nursery, a division of the Tennessee Department of Conservation.

Excision and culture of embryos. Seed coats of Virginia, white and short leaf pine were eroded by emersion in concentrated sulfuric acid for 30-45 minutes. Due to their exceedingly hard seed coats, loblolly seeds required an extensive acid treatment of 3-4 hours. After the initial acid bath the seeds were placed in full strength Clorox under a

fume hood for 5 minutes in order to remove the remaining seed coat and to further sterilize the seed surface. The seeds were then removed from the Clorox and washed in sterile double distilled water. Any remaining seed coat was dissected away from the female gametophyte using a dissecting microscope and sterile technique. The gametophytic tissue surrounding the embryo was carefully removed and the excised embryo was planted upright in a 25 X 150 mm test tube containing 25 ml of a modified Knop's medium (Appendix A-1).

Establishment of callus. After the embryos had been incubated for 30 days, the seedlings were removed from the Knop's medium and dissected into radical, hypocotyl, and cotyledonary segments. These three segments were separated and placed in test tubes containing 25 ml of Brown's gymnosperm medium (Appendix A-2) for callus induction. Explants were incubated for 6 weeks and callus was allowed to proliferate without transfer. This callus was subsequently divided and subcultured to produce clonal lines. Each clonal line was the product of a single embryo explant. The resulting subcultures were used for testing of paraquat toxicity and subsequent determination of superoxide dismutase levels.

Response to Paraquat Supplemented Media

Media. Paraquat dichloride (29.1%) was supplied by the Ortho division of the Chevron Chemical Company. Due to its extremely high heat stability, paraquat was added directly to media and autoclaved (Slade, 1966). Clonal explants of previously established pine callus tissue were transferred to test tubes containing 25 ml of Brown's

gymnosperm media supplemented with 0, 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M, 10^{-4}M, and 10^{-3}M paraquat. In a separate series of experiments for determination of SOD induction on paraquat supplemented media, callus was subcultured on media containing 0, 10^{-8}M and 10^{-6}M paraquat only.

Growth response of pine callus. All initial explant sizes were kept as uniform as possible throughout the duration of the experiments. Maximum uniformity was obtained by dissecting callus into small cubes and adjusting all of their sizes prior to placing them onto media. Average dry weight of initial callus explants was 5.0 mg. Cultures were incubated for 42 days after which the cells were dried at 60°C for 24 hours and dry weights recorded. Mean dry weights of initial explants were also determined and used for determining the proportional increase in growth.

Growth response of pine callus on medium containing various levels of paraquat was analyzed in two phases: 1) The growth response of replicates of clonal lines was analyzed by a modified randomized block design analysis of variance (ANOVA). 2) Data from each clonal line were averaged and included in analysis of growth response of different clonal lines. Differences between these lines were also analyzed by a randomized block design ANOVA (Li, 1959).

II. SUPEROXIDE DISMUTASE ANALYSIS

Effect of Paraquat on Pine SOD Levels

An experiment utilizing paraquat supplemented Brown's medium was performed to determine the effect of the herbicide on pine callus SOD levels. The size of explants obtained from a single callus culture per

species was standardized as described previously. Each explant was then transferred to two replicates of 25 ml Brown's medium containing 0, 10^{-8}M and 10^{-6}M paraquat. After a 14 to 21 day period of growth, cultures grown on each of the three media were harvested and crude extracts prepared as described below. Electrophoresis and spectrophotometric assays were carried out to determine any changes in SOD banding patterns or quantitative changes in SOD in response to paraquat treatments.

SOD Extraction

Crude extracts of SOD were obtained by grinding weighed samples of callus with a pestle in a chilled mortar containing a .05M potassium phosphate buffer at pH 7.8 (1 ml/1 gm tissue) (Giannopolitis and Ries, 1977b). Insoluble PVPP (0.1 gm/gm tissue) was added to remove excessive phenolic substances and quartz sand was included to aid in cell disruption. The homogenate was centrifuged at 14,000 Xg for 10 minutes in a Sorval RC2-B centrifuge at 0-5°C. The resulting supernatant was recentrifuged at 14,000 X g for 10 minutes to remove any remaining cell debris. It was then utilized for electrophoresis and further diluted for spectrophotometric assay.

Protein Determination

The amount of total protein in the crude extracts was measured by a modified method of Lowery, Rosebrough, Farr, and Randall (1951) (Appendix B-1). Bovine serum albumin was used as a standard at 50, 100, 200, 300, 400, and 500 µg concentrations.

Electrophoresis

Electrophoresis with 7-1/2% polyacrylamide gels was performed according to Davis (1964) (Appendix C-1 and C-2) and utilized to survey the SOD banding patterns in various cell lines of stock pine callus. Electrophoresis was performed using extracts from duplicate cultures in order to confirm banding patterns obtained.

SOD Localization

SOD was localized on the gels by a modification of the method of Edwards, Hopkinson, and Harris (1978) (Appeneix C-3). Gels were first placed in a solution containing 0.1 mg/ml MTT, 0.05 mg/ml PMS in a 0.1M Tris-HCl buffer at pH 8.0. The gels were kept cold and dark and allowed to remain in the stain for approximately 30 minutes. They were then illuminated with two fluorescent lights for 45 minutes. When desired, Cu-Zn SOD bands were eliminated from the gels by including 1 mM KCN in the staining solution. Similarly Mn SOD was precipitated from the crude extracts by the addition of cold chloroform-ethanol mixture before loading onto gels (Beachamp and Fridovich, 1973). Sketches were made of the gels, and they were then scanned with a Gilford densitometer. These scan peaks were traced, cut out and weighed to determine relative areas under each curve. Adjustments were made to standardize the peak area to 100 µg of total protein per gel.

SOD Assay

SOD from crude extracts was assayed by a modification of the method described by Giannopolitis and Ries (1977b). The assay was based on the ability of SOD to inhibit the rate of reduction of NBT to blue

formazan by photochemically produced superoxide radicals. Assays were performed at 30°C using a Beckman-Gilford spectrophotometer. One unit of SOD activity is defined as the amount of enzyme needed to reduce the rate of the given reaction by 50%. Reagents were added to a cuvette in the following concentrations: 1.3 µM riboflavin, 13 mM methionine, 63 µM NBT, 0.05 M sodium carbonate pH 10.2 and either SOD, crude extract or double distilled water in proportions yielding a final volume of 3 ml. The reaction was initiated by placing the cuvette beside a G.E. Cool White fluorescent tube and illuminated for 1, 5, 7, 10, 12, and 15 minutes.

Bovine SOD was used as a standard to determine the maximum inhibition possible under the given conditions and to determine the areas of greatest linearity.

Other assay systems were attempted in the course of this research but were not found to be useful for SOD quantitation in crude pine callus extracts. The assay of McCord and Fridovich (1969), utilizing the action of xanthine oxidase upon xanthine as the $O_2^{\cdot -}$ generator and cytochrome C as the detector of the radical, proved to be highly affected by impurities in the crude extracts. Neither dialysis nor filtration eliminated those interferring components. Similarly, the assay of Marklund and Marklund (1974) was not useful with the crude extracts. The autoxidation and subsequent free radical chain oxidation on pyrogallol was accelerated instead of inhibited by the addition of the crude extracts. Simple dialysis or filtration also failed to remove the interfering substances.

CHAPTER III

RESULTS

Tissue Morphology

The morphology of callus cultures from short leaf, loblolly, Virginia, and white pine seedling explants was quite distinctive. Short leaf explants typically produced a friable callus which was uniformly cream in color but sporadically exhibited faint areas of green. Loblolly callus was tan and friable and never exhibited green areas. Unlike short leaf, loblolly tissue generally darkened to chocolate brown after extended periods without transfer. Virginia callus color was usually a bright mustard yellow bearing occasional areas of green. The tissue was watery with a pasty consistency. White pine callus was usually a mixture of colors, varying from light yellow to very dark green. The tissue growth tended to form rounded clumps of cells which were very watery but coherent. White pine callus also darkened considerably after extended periods without transfer.

Growth of Callus in Response to Paraquat

Short leaf. The growth response of short leaf callus explants cultured on medium containing paraquat is presented in Table V. Variation in callus response among lines is clear; however, mean dry weight values per paraquat concentration were calculated in order to obtain general trend information. These data seemed to indicate a trend toward increasing mean dry weight in callus grown at 10^{-8}M and 10^{-7}M paraquat over that of the controls. The tissue generally exhibited no significant

TABLE V

GROSS DRY WEIGHTS (MG) OF SHORT LEAF PINE CALLUS TISSUE AFTER 6 WEEKS' GROWTH ON PARAQUAT SUPPLEMENTED MEDIA

	Gross Dry Weights of Callus (mg) on Medium Containing Paraquat						
	Paraquat Concentrations						
Tissue Lines	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
SL-a	2.0	24.6	18.1	3.2	1.4	2.2	2.0
SL-b	62.2	48.7	94.4	72.2	5.7	8.5	7.0
SL-c	80.0	99.8	55.5	26.1	11.7	6.5	4.7
SL-d	4.3	115.6	33.2	10.0	3.3	3.1	2.9
SL-e	125.0	125.9	93.2	12.2	4.0	1.5	4.1
SL-f	74.3	113.2	67.8	7.7	5.8	2.4	3.2
SL-g	115.2	108.6	133.2	43.0	6.0	4.1	4.0
SL-h*	41.2	42.9	42.4	29.4	8.3	7.2	6.6
SL-i*	11.5	13.3	10.5	101.0	8.6	7.4	7.7
Mean	57.3	76.9	60.9	33.9	6.1	4.8	4.6
Standard deviation	46.1	43.9	40.2	33.2	3.1	2.6	1.9

*Mean value of clonal replicates.

growth over initial explant size at concentrations on 10^{-5}M paraquat and higher (Figure 1). Mean dry weight of initial explants was 4.1 mg with a standard deviation of 0.7 mg.

Loblolly. Table VI illustrates the response of 10 different loblolly clonal cell lines grown on paraquat supplemented media. Despite the variation among the lines mean dry weights were calculated in order to determine the general response trends for this species. As opposed to the trends in short leaf callus, there was no increase in mean dry weight over that of the controls on 10^{-8}M or 10^{-7}M paraquat. The point where there is no increase in dry weight over that of the initial explant occurred at a concentration of 10^{-5}M paraquat and higher. This lethal level appeared to be standard throughout the lines tested (Figure 2). The mean dry weight of the initial explant was 5.0 mg with a standard deviation of 0.5 mg.

Virginia. The individual growth responses of Virginia callus explants on media containing paraquat are presented in Table VII. Variation in response among the lines is quite obvious, but mean dry weight values were determined and are presented in Figure 3. This graph illustrates a general trend toward decreasing callus mean dry weight with increasing paraquat concentrations similar to that observed in loblolly callus. The mean dry weight of the initial explants was 4.5 mg with a standard deviation of 0.3 mg.

White. The growth response of several lines of white pine callus tissue grown on media supplemented with paraquat is summarized in

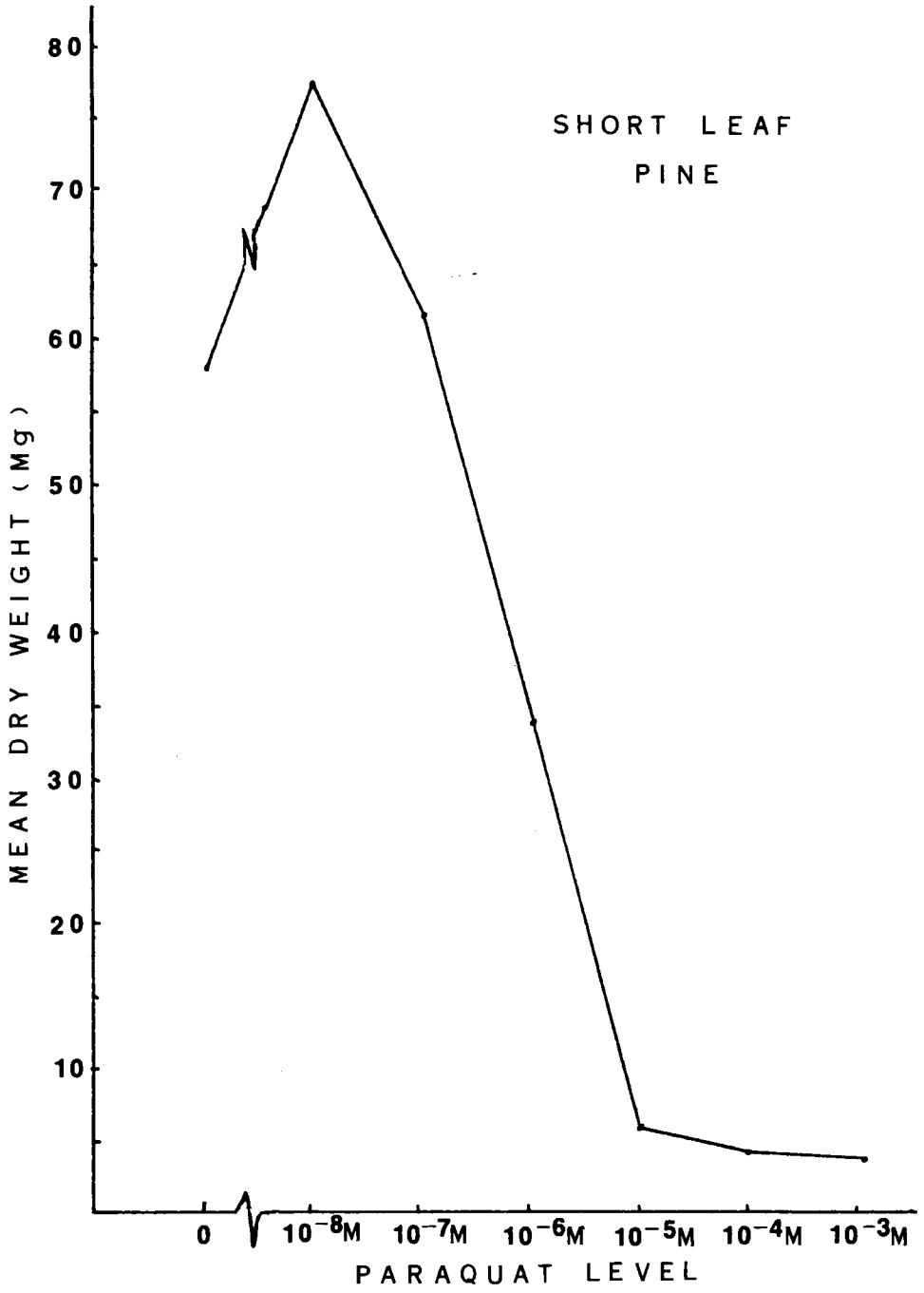

Figure 1. Response of short leaf pine callus toward paraquat supplemented media. Mean dry weight of initial explants was 4.7 mg with a standard deviation of 0.7 mg. Growth period was 6 weeks.

TABLE VI

GROSS DRY WEIGHTS (MG) OF LOBLOLLY CALLUS TISSUE AFTER 6 WEEKS' GROWTH ON PARAQUAT SUPPLEMENTED MEDIA

	Gross Dry Weights of Callus (mg) on Medium Containing Paraquat						
	Paraquat Concentrations						
Tissue Line	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Lob-a	84.2	55.7	83.8	44.7	6.4	3.1	5.1
Lob-b	37.4	87.6	64.6	11.2	7.4	8.2	6.2
Lob-c	71.9	48.5	55.3	9.9	5.5	7.6	9.3
Lob-d	103.4	77.5	72.5	50.3	6.4	2.8	4.2
Lob-e	38.1	15.3	10.0	10.0	8.1	11.9	8.4
Lob-f	103.2	108.6	88.5	8.0	5.5	4.6	3.0
Lob-g	34.6	77.8	6.9	7.0	4.6	3.9	5.0
Lob-h	107.4	54.3	28.8	9.2	4.5	3.3	3.0
Lob-i*	37.4	68.7	52.4	27.9	7.5	7.4	5.8
Lob-j*	97.9	68.0	60.8	24.9	7.0	6.4	5.9
Mean	71.6	66.2	52.4	16.3	6.3	5.9	5.6
Standard deviation	31.6	25.1	28.6	14.2	12.4	2.9	2.1

*Mean value of clonal replicates.

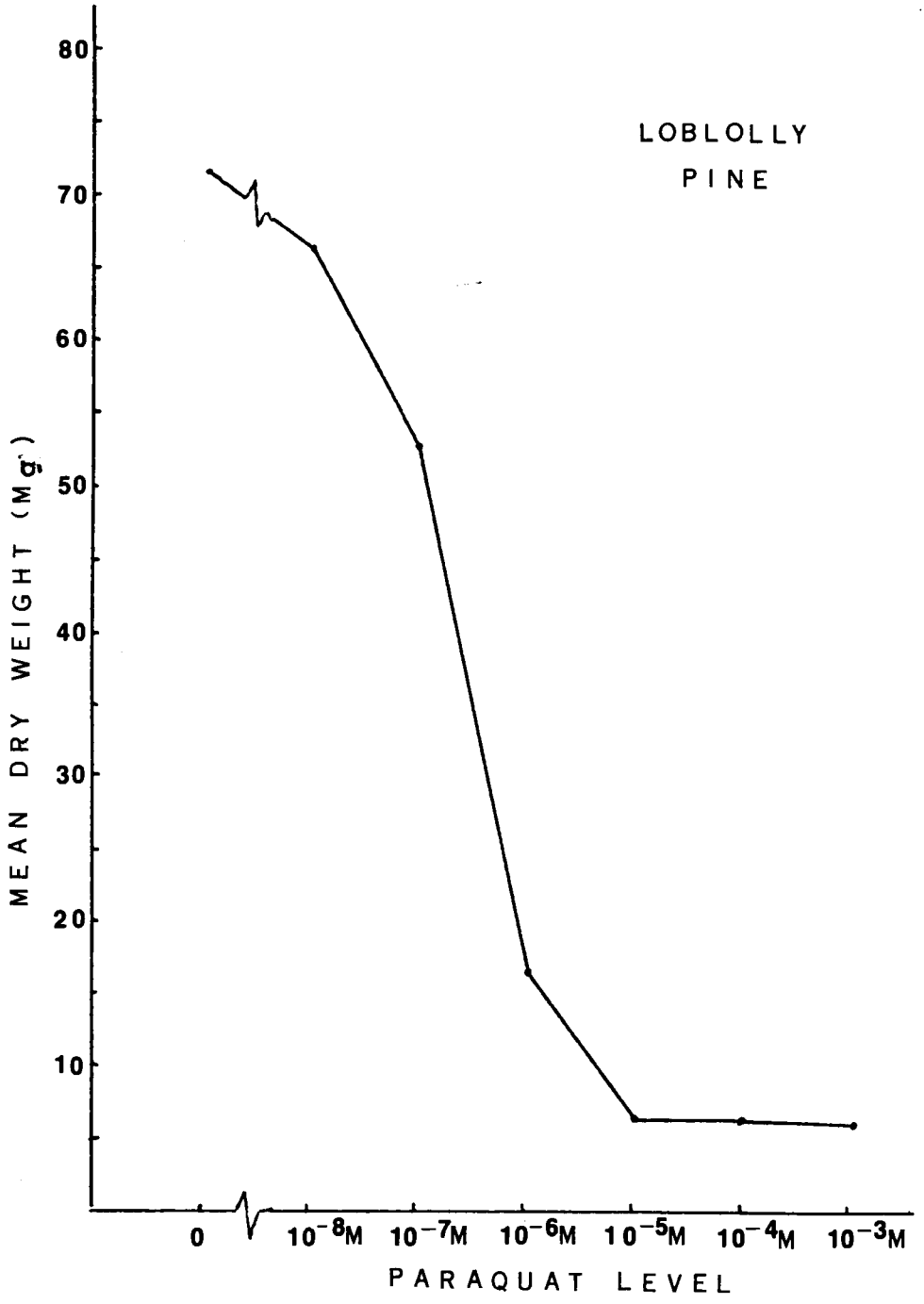

Figure 2. Response of loblolly pine callus toward paraquat supplemented media. Mean dry weight of initial explants was 5.0 mg with a standard deviation of 0.5 mg. Growth period was 6 weeks.

TABLE VII

GROSS DRY WEIGHTS (MG) OF VIRGINIA PINE CALLUS TISSUE AFTER 6 WEEKS' GROWTH ON PARAQUAT SUPPLEMENTED MEDIA

	Gross Dry Weights of Callus (mg) on Medium Containing Paraquat						
	Paraquat Concentrations						
Tissue Line	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Va-a	37.4	3.7	2.5	1.4	3.1	2.8	2.7
Va-b	60.0	58.6	66.5	10.0	6.8	6.7	8.0
Va-c	58.0	59.0	59.6	48.6	11.0	8.7	7.6
Va-d	30.0	20.0	9.5	6.0	7.0	5.2	5.5
Va-e	73.0	85.2	57.4	15.0	6.2	2.5	3.5
Va-f	123.4	124.2	78.0	8.9	5.0	3.6	5.0
Va-g	82.5	41.8	27.7	7.5	4.2	7.8	3.7
Va-h	55.7	64.5	60.0	48.3	8.1	3.7	4.5
Va-i	63.9	51.6	19.6	4.1	4.0	4.5	3.5
Va-j	45.0	45.9	45.2	2.7	2.9	6.9	7.0
Va-k	88.7	54.3	46.1	21.1	7.8	6.5	4.2
Va-l	31.4	26.3	11.8	5.9	5.7	5.7	4.2
Va-m*	72.7	67.5	25.2	5.0	4.3	4.8	3.8
Va-n*	104.2	34.0	33.1	6.5	7.1	7.8	5.2
Va-o*	73.2	77.5	59.1	35.6	8.5	5.3	3.2
Va-p*	43.9	26.3	11.8	6.2	5.8	5.7	4.4
Va-q*	72.8	86.8	71.6	6.8	10.5	10.0	5.5
Mean	65.6	54.5	40.3	14.1	6.4	5.8	4.8
Standard deviation	25.2	29.3	24.3	15.3	2.4	2.1	1.5

*Mean value of clonal replicates.

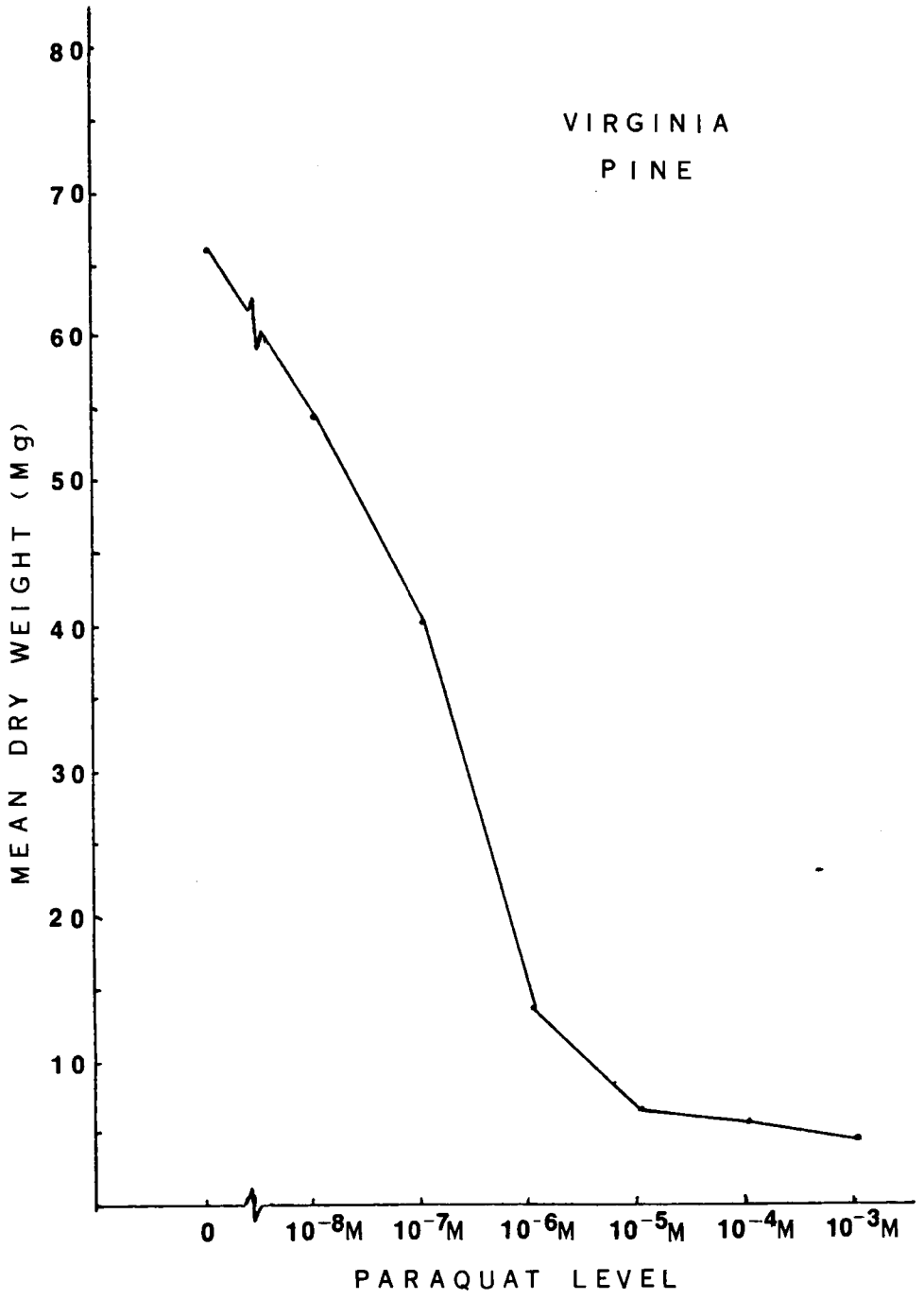

Figure 3. Response of Virginia pine callus toward paraquat supplemented media. Mean dry weight of initial explants was 4.5 mg with a standard deviation of 0.3 mg. Growth period was 6 weeks.

Table VIII. Individual variations among white pine growth response were similar to that found in the other three species tested; however, the mean dry weights of white pine callus exhibited trends quite unique to this species. Generally a slight increase in mean dry weight occurred at 10^{-8}M paraquat, but growth seemed to be severely inhibited at higher paraquat concentrations. The mean dry weight of the initial explant was 5.5 mg with a standard deviation of 1.2 mg (Figure 4).

Statistical analysis. A comparison of callus growth on medium containing paraquat was performed to determine if growth of clonal cell lines differed. Results indicated that in general the growth response of calli from the same clonal lines was not significantly different (Table IX). The single exception was a highly significant difference ($P < .01$) between clonal replicates of short leaf calli; however, the number of clonal replicates was limited for this species. Line SL-h was repeated only twice and one of the replicates was clearly aberrant. Line SL-i was replicated three times. With this limited amount of data, one aberrant line can affect the results obtained.

Analysis of growth response between different cell lines of each species was performed to determine the extent of initial genetic variation within species. ANOVA tests indicated that there were significant or highly significant differences between growth of calli on different levels of paraquat. Additionally, there were highly significant differences between different cell lines of each species with respect to growth on paraquat supplemented media (Table IX).

TABLE VIII

GROSS DRY WEIGHTS (MG) OF WHITE PINE CALLUS TISSUE AFTER 6 WEEKS' GROWTH ON PARAQUAT SUPPLEMENTED MEDIA

	Gross Dry Weights of Callus (mg) on Medium Containing Paraquat						
	Paraquat Concentrations						
Tissue Line	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
W-a	116.9	54.5	41.5	6.0	4.1	4.4	5.8
W-b	32.8	79.0	27.6	13.7	4.5	3.3	2.8
W-c	18.9	51.4	12.2	4.9	4.2	5.9	3.5
W-d	17.8	107.0	21.2	11.6	4.9	4.6	4.4
W-e	127.3	134.4	51.7	5.0	4.0	2.1	5.7
W-f	128.7	137.9	73.9	13.2	10.5	8.0	5.7
W-g	114.3	124.5	42.6	10.0	4.0	4.5	5.7
W-h	109.5	106.5	62.0	19.0	4.8	6.9	4.0
W-i	46.6	73.0	25.6	10.1	4.4	4.6	4.1
W-j	113.6	81.5	41.4	16.5	13.1	10.5	9.1
W-k	55.9	20.1	13.4	13.8	10.1	7.8	9.5
W-l*	113.6	81.5	40.8	17.7	14.1	11.2	9.1
W-m*	55.9	20.1	13.4	11.6	10.1	7.8	9.0
W-n*	38.6	33.7	6.9	7.3	6.8	5.9	5.9
W-o*	69.5	78.6	46.6	5.8	4.5	6.3	6.3
W-p*	19.3	14.5	10.2	8.6	6.8	7.1	7.2
Mean	73.7	74.9	33.2	10.9	6.9	6.3	6.1
Standard deviation	42.6	40.4	19.9	4.5	3.5	2.4	2.1

*Mean value of clonal replicates.

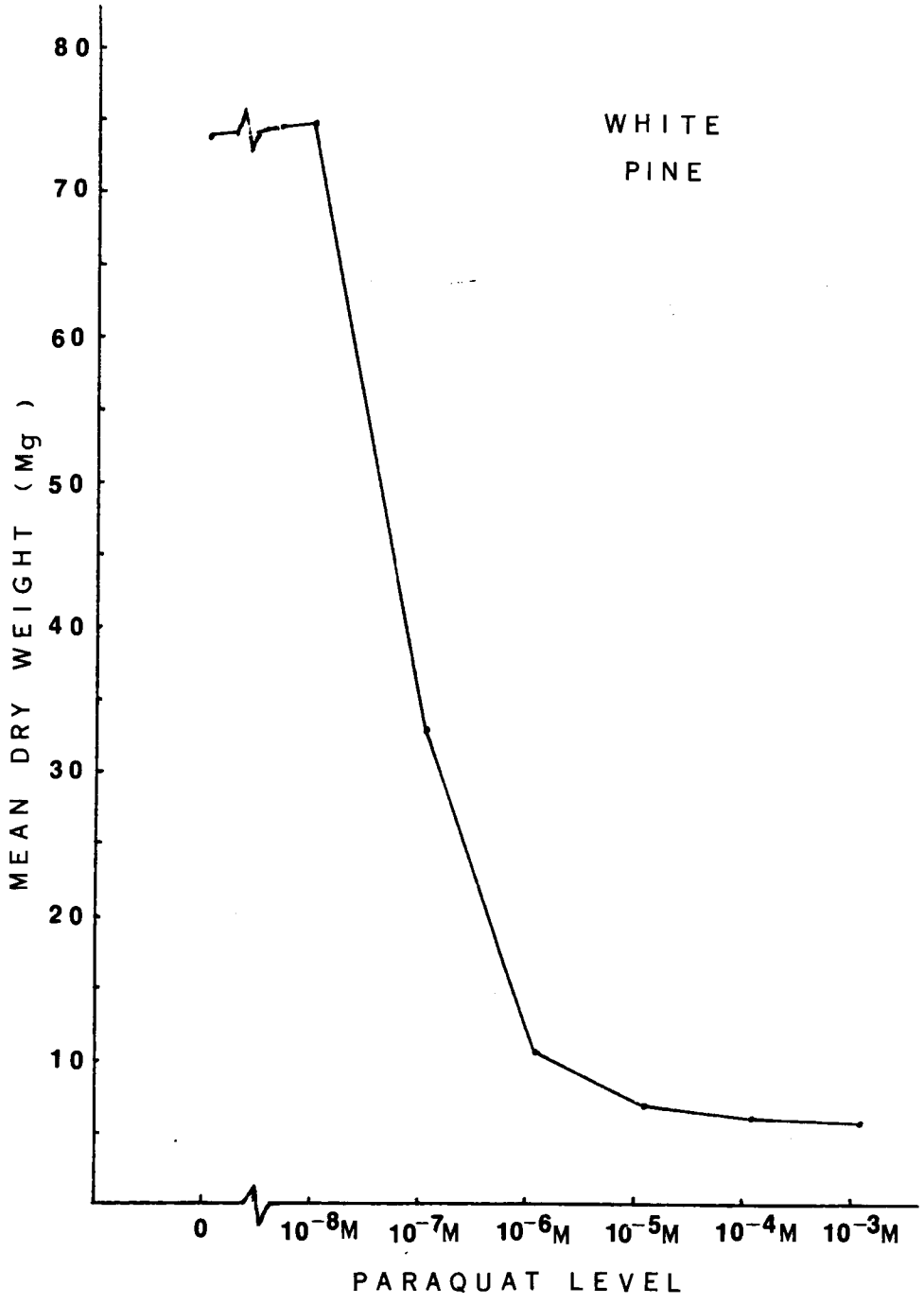

Figure 4. Response of white pine callus toward paraquat supplemented media. Mean dry weight of initial explants was 5.5 mg with a standard deviation of 1.2 mg. Growth period was 6 weeks.

TABLE IX

ANALYSIS OF VARIANCE OF GROWTH OF PINE CALLUS ON MEDIUM CONTAINING DIFFERENT LEVELS OF PARAQUAT

Species	Source of Variation	Probability
Short leaf	Between paraquat levels	<.01
	Between clonal lines	<.05
	Within clonal lines*	<.01
White	Between paraquat levels	<.01
	Between clonal lines	<.01
	Within clonal lines*	No significant difference
Virginia	Between paraquat levels	<.01
	Between clonal lines	<.01
	Within clonal lines*	No significant difference
Loblolly	Between paraquat levels	<.01
	Between clonal lines	<.05
	Within clonal lines*	No significant difference

*Differences between replicates within clonal lines were analyzed separately from other data.

Electrophoresis

Short leaf pine callus. Crude extracts of the soluble protein fraction from several short leaf callus lines were analyzed by polyacrylamide gel electrophoresis. SOD banding patterns of callus grown on media supplemented with 10^{-8}M or 10^{-6}M paraquat indicated no significant increase in band number over that of the controls. This electrophoretic survey of several cell lines indicated major banding pattern similarities throughout (Figure 5). Generally, four major SOD bands were present and occasionally indistinct minor bands appeared. The first major band of relatively slow electrophoretic mobility was a diffuse band, designated as band A, at a Rf of .24. The relative intensity of band A varied considerably among the lines surveyed. Band A was CN^- insensitive. A second major band appeared at an average Rf of .37 and was labeled band 1. Two additional bands of rapid mobility were located at Rf values of .51 and .61 and were designated as bands 2 and 3, respectively. Occasionally band 2 appeared to be of equal or greater intensity as band 1.

Spectrophotometric densitometer analysis of the gels was performed to obtain a relative quantitation of the SOD levels present and to substantiate the visual gel data. Densitometer scans confirmed the uniformity of banding patterns among the various short leaf lines (Figure 6). The four major bands were usually present and the variability in peak A was quite obvious. Areas of the scan peaks represented by the densitometer tracings were used to indicate specific changes in peak intensities due to paraquat application (Table X). The weights of the densitometer tracings were adjusted to account for the

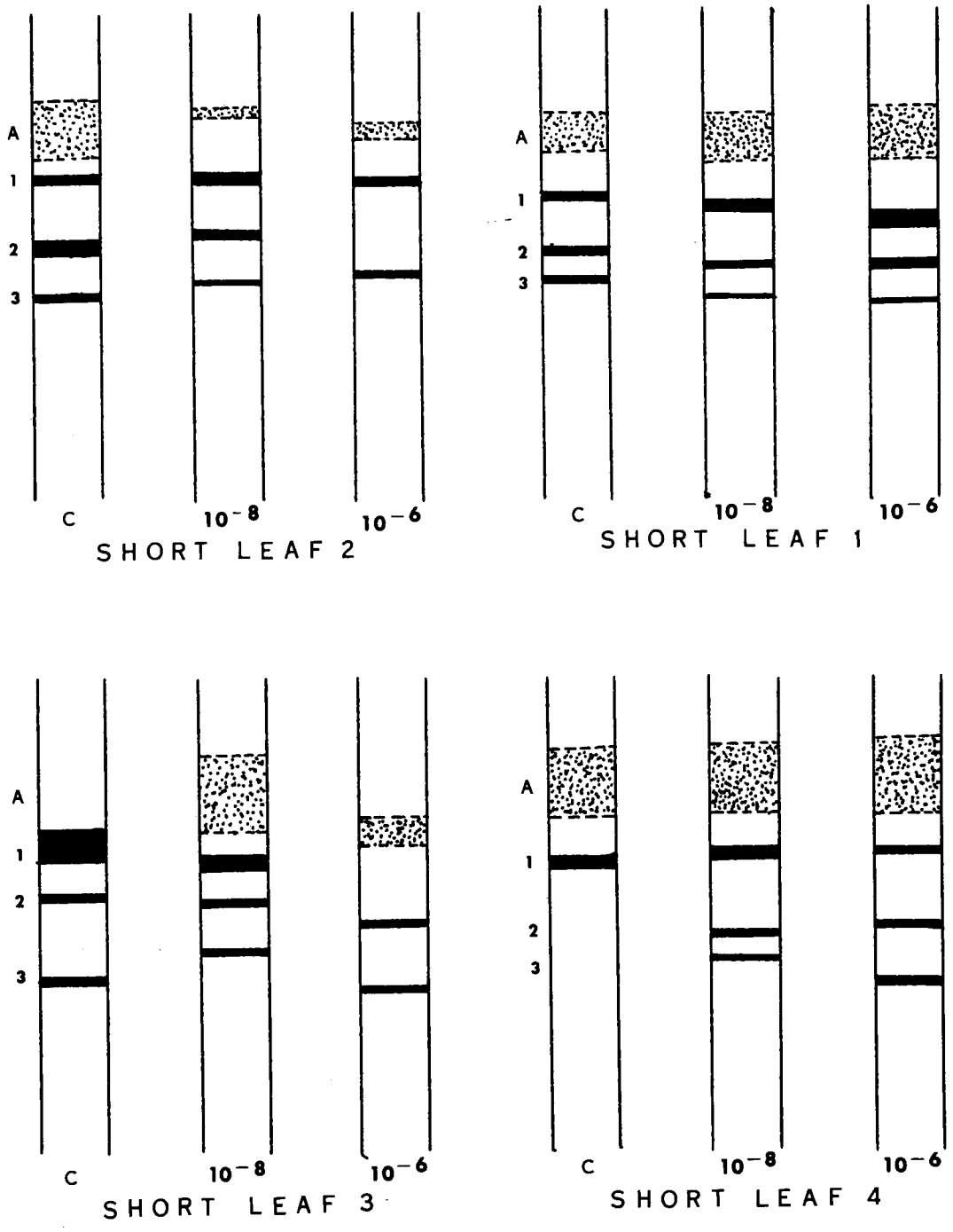

Figure 5. Polyacrylamide-gel electrophoresis of crude extracts obtained from representative lines of short leaf pine callus. Each line was grown on 0, 10-8M, and 10-6M paraquat supplemented media. The 200 µl of extract was applied to each gel and stained for SOD activity. Darkened bands on the figure represent achromatic regions of SOD activity on the actual gels.

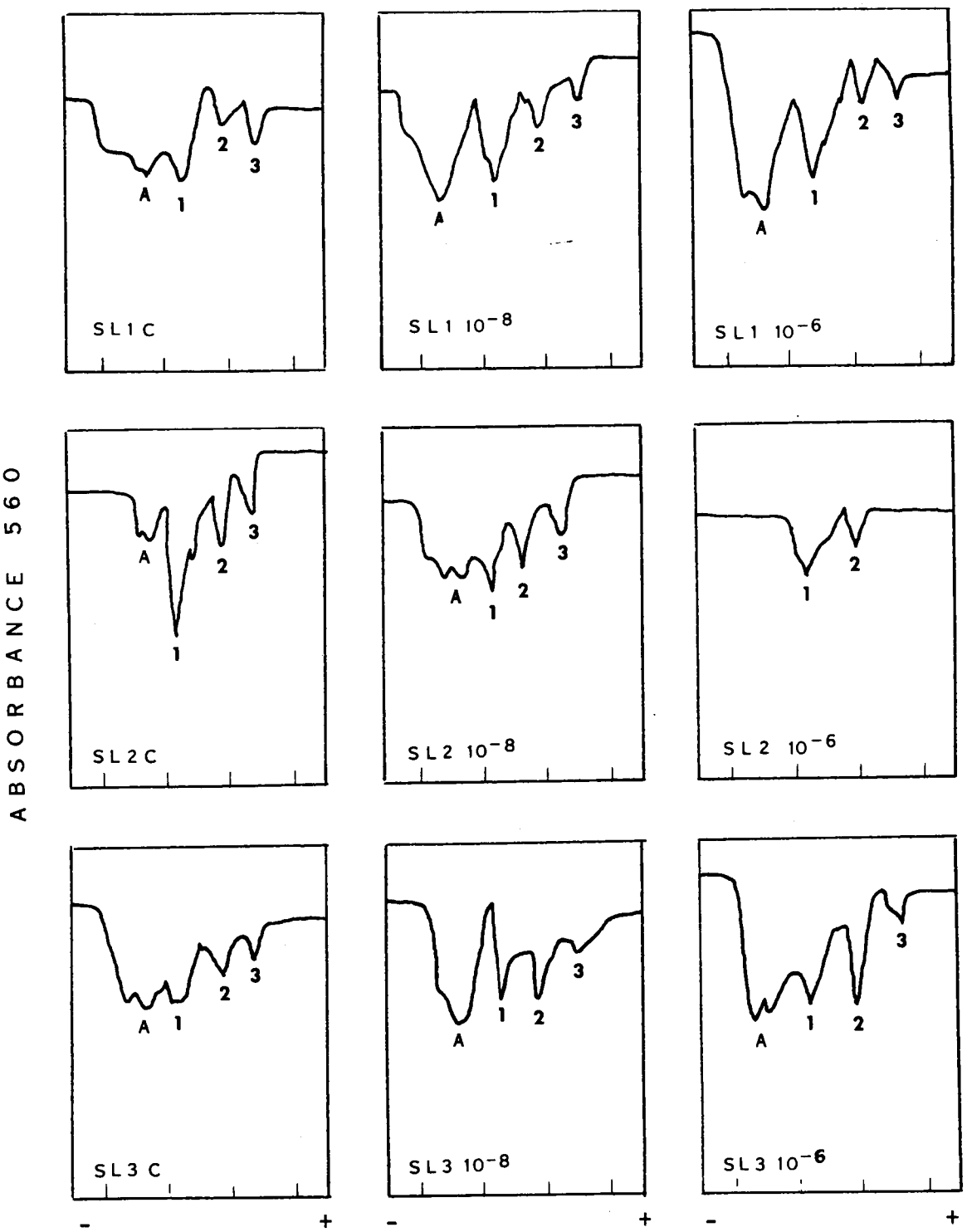

Figure 6. Densitometer tracings of representative gels from several lines of short leaf pine callus indicating superoxide dismutase activity. Peak number corresponds to that of bands labeled on gels in order of increasing mobility.

TABLE X

AREA* OF DENSITOMETER SCANS OF SOD FROM SEVERAL LINES OF SHORT LEAF PINE CALLUS

Tissue Line	Peak A	1	2	3	Total
SL 1					
0**	13.6	0.5	0.3	1.6	16.0
10^{-8}M	10.1	0.7	0.5	2.6	13.9
10^{-6}M	9.4	0.8	0.3	1.8	12.3
SL 2					
0	1.2	4.4	1.2	1.9	8.7
10^{-8}M	0.5	1.3	0.7	1.0	3.5
10^{-6}M	-	4.5	6.0	-	10.5
SL 3					
0	3.1	2.4	0.6	0.8	6.9
10^{-8}M	10.7	5.0	1.4	1.6	18.7
10^{-6}M	15.0	6.9	1.2	1.5	24.6
SL 4					
0	-	1.4	0.7	-	2.1
10^{-8}M	3.8	4.4	4.5	2.4	15.1
10^{-6}M	1.2	7.5	1.0	1.0	10.7
SL 5					
0	7.1	2.4	0.7	1.2	11.4
10^{-8}M	12.0	3.7	1.5	1.1	18.3
10^{-6}M	13.1	1.3	3.1	4.8	22.3

*Areas are represented by the (mg) weights of peaks, adjusted to 100 µg protein/gel.

**Concentration of paraquat in media.

variation in protein levels applied to the gels. Statistical analysis using a non-parametric sign test (Siegel, 1956) was performed after grouping data from callus grown on 10^{-8}M and 10^{-6}M paraquat. In short leaf callus cultures peaks A and 3 showed no significant changes in intensity due to paraquat treatment. Probability values for these bands were P=.377 for A and P=.254 for band 3. Over all bands 1 and 2 in short leaf callus increased in area when the tissue was grown on paraquat supplemented media as compared to SOD bands in callus grown in the absence of paraquat. Analysis of grouped data for peaks 1 and 2 yielded probability values of .172 and .02, respectively, indicating significant band area increases for band 2 in response to paraquat.

Loblolly pine callus. The electrophoretic survey of loblolly pine callus cultures indicated four major bands of SOD activity present in most gels (Figure 7). These bands corresponded quite closely to the Rf values of similar bands found in short leaf callus tissues. A wide and variable band with an average Rf value of .26 was present in most gels and was shown to be CN^- insensitive. This slowly moving band was labeled band A. Band 1, following band A at a Rf of .38 was present in all lines tested. A third major band appeared at an average Rf of .49 and subsequently was labeled band 2. Bands 3 and 4, of quite rapid mobility were located at Rf values of .59 and .67, respectively. These two bands, occasionally absent or masked by a darker bluish band of unknown origin, proved to be extremely variable from gel to gel. Similarly, band B, located between bands 1 and 2 at a Rf value of .45 was present in some lines but absent from others. Bands 1, 2, 3, 4 and

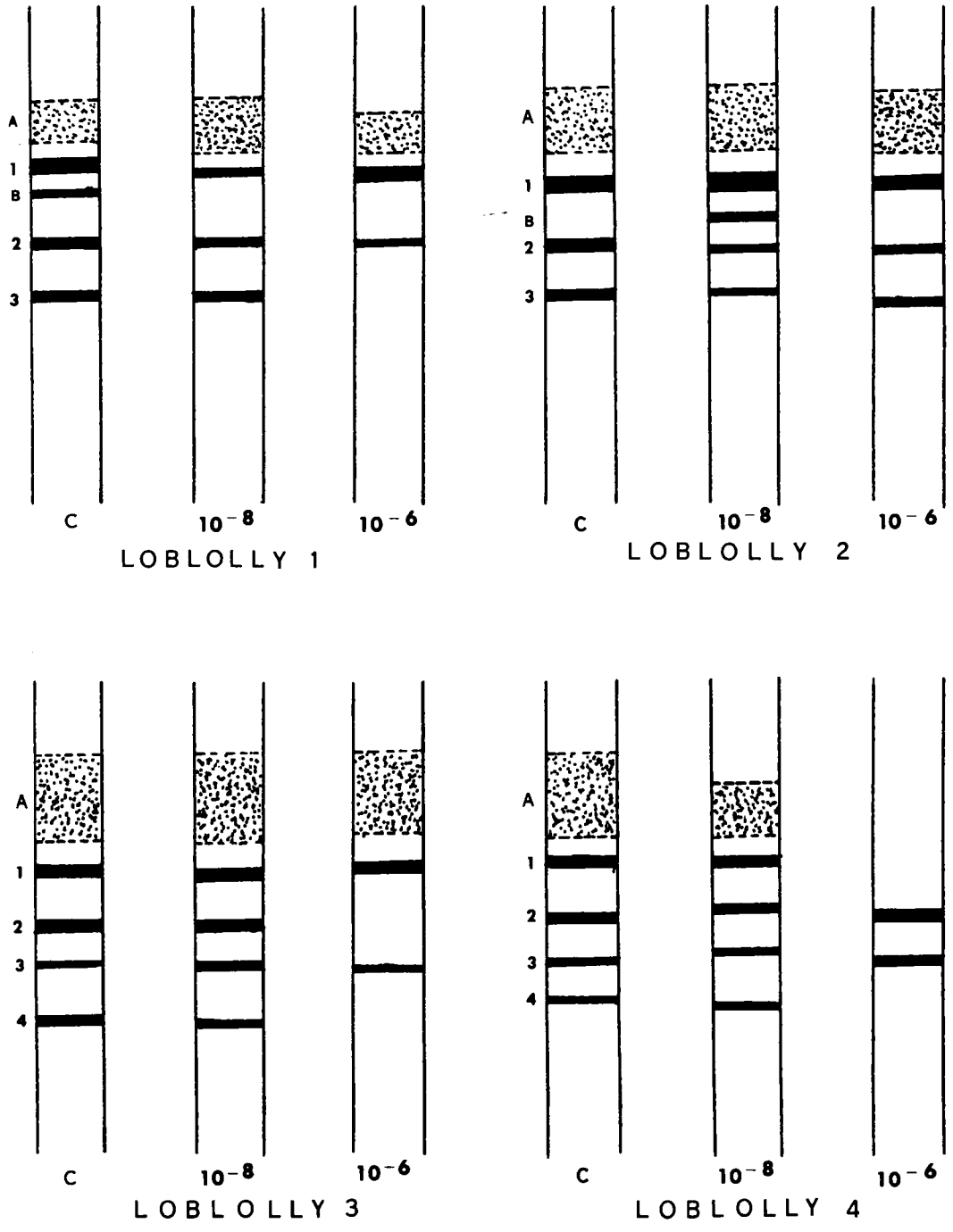

Figure 7. Polyacrylamide-gel electrophoresis of crude extracts obtained from representative lines of loblolly pine callus. Each line was grown on 0, 10^{-8}M, and 10^{-6}M paraquat supplemented media. The 200 µl of extract was applied to each gel and stained for SOD activity. Darkened bands on the figure represent achromatic regions of SOD activity on the actual gels.

B were all shown to be CN^- sensitive. Banding patterns of SOD from callus grown on $10^{-8}M$ or $10^{-6}M$ paraquat indicated no major increase in band number over that of the controls.

Densitometer scans substantiated the visual gel data and illustrated more clearly the variability of bands A, B, and 4 among the lines tested (Figure 8). Statistical analysis performed as described previously indicated no significant increases in any of the 6 bands present in loblolly callus in response to paraquat supplemented media (Table XI). Probability values for bands A, B, 1, 2, 3, and 4 were .656, .500, .377, .145, .344, and .623, respectively.

White pine callus. Electrophoretic banding patterns of white pine callus were quite simple and extremely consistent throughout the lines tested. One major, distinct band at an Rf of .49 was present in 100% of the callus extracts. This seemed to correlate with electrophoretic mobility with band number 2 in both loblolly and short leaf gels and was labeled accordingly. In addition to band 2, a light diffuse area similar in appearance to band A from loblolly and short leaf data was evident. A second variable band found in a few gels was located at a Rf of .75. This Rf corresponded to no other previously named band and was, therefore, assigned band number 5 (Figure 9). All bands except A were CN^- sensitive. Banding patterns of callus grown on media supplemented with $10^{-8}M$ or $10^{-6}M$ paraquat generally indicated no significant increase in band number over that of the controls.

Densitometer scans of the gels clearly illustrated the dominant peak 2 of Rf=.49 and its associated minor bands (Figure 10). Statistical

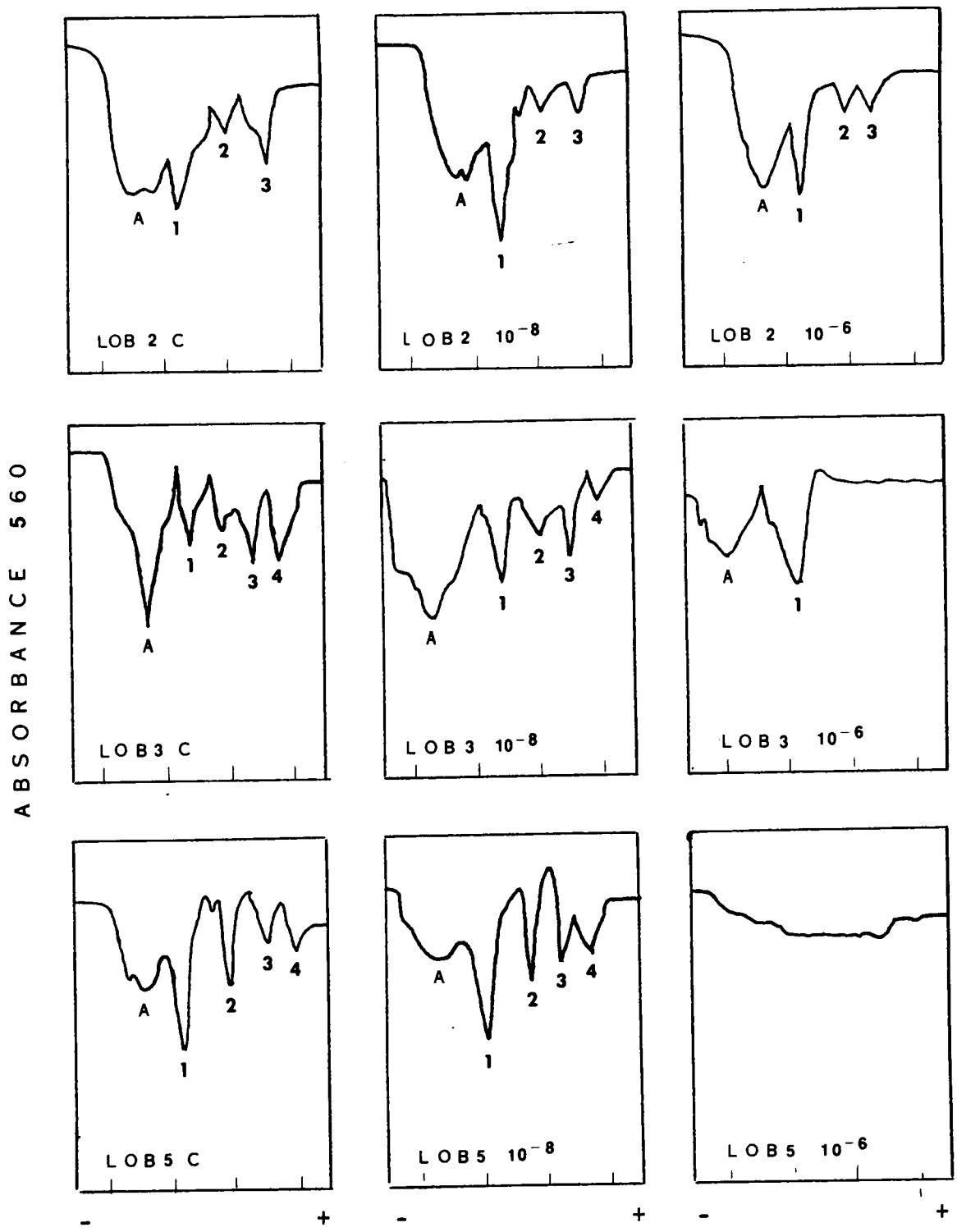

Figure 8. Densitometer tracings of representative gels from several lines of loblolly pine callus indicating superoxide dismutase activity. Peak number corresponds to that of bands labeled on gels in order of increasing mobility.

TABLE XI

AREA* OF DENSITOMETER SCANS OF SOD FROM SEVERAL LINES OF LOBLOLLY PINE CALLUS

Tissue Line	Peak A	B	1	2	3	4	Total
Lob 2							
0**	21.4	-	4.4	2.1	4.8	-	32.7
10^{-8}M	10.4	1.0	7.8	1.3	1.7	-	22.2
10^{-6}M	18.8	-	4.4	1.7	1.3	-	26.2
Lob 3							
0	9.0	-	1.9	0.8	0.8	1.3	13.8
10^{-8}M	15.5	-	1.6	1.2	1.5	1.0	20.8
10^{-6}M	16.8	-	5.7	-	-	-	22.5
Lob 5							
0	-	-	2.8	1.1	0.6	0.8	5.3
10^{-8}M	-	-	4.3	2.0	1.3	2.2	9.8
10^{-6}M	-	-	-	-	-	-	-
Lob 6							
0	16.3	-	2.5	4.8	-	-	23.6
10^{-8}M	16.6	-	1.0	1.4	-	-	19.0
10^{-6}M	11.2	-	1.2	-	-	-	12.4
Lob 7							
0	-	1.0	7.2	0.8	1.3	-	10.3
10^{-8}M	-	0.8	9.0	2.5	-	0.3	12.6
10^{-6}M	-	0.2	10.0	3.0	-	-	13.2

*Areas are represented by the (mg) weights of peaks, adjusted to 100 µg protein/gel.

**Concentration of paraquat in media.

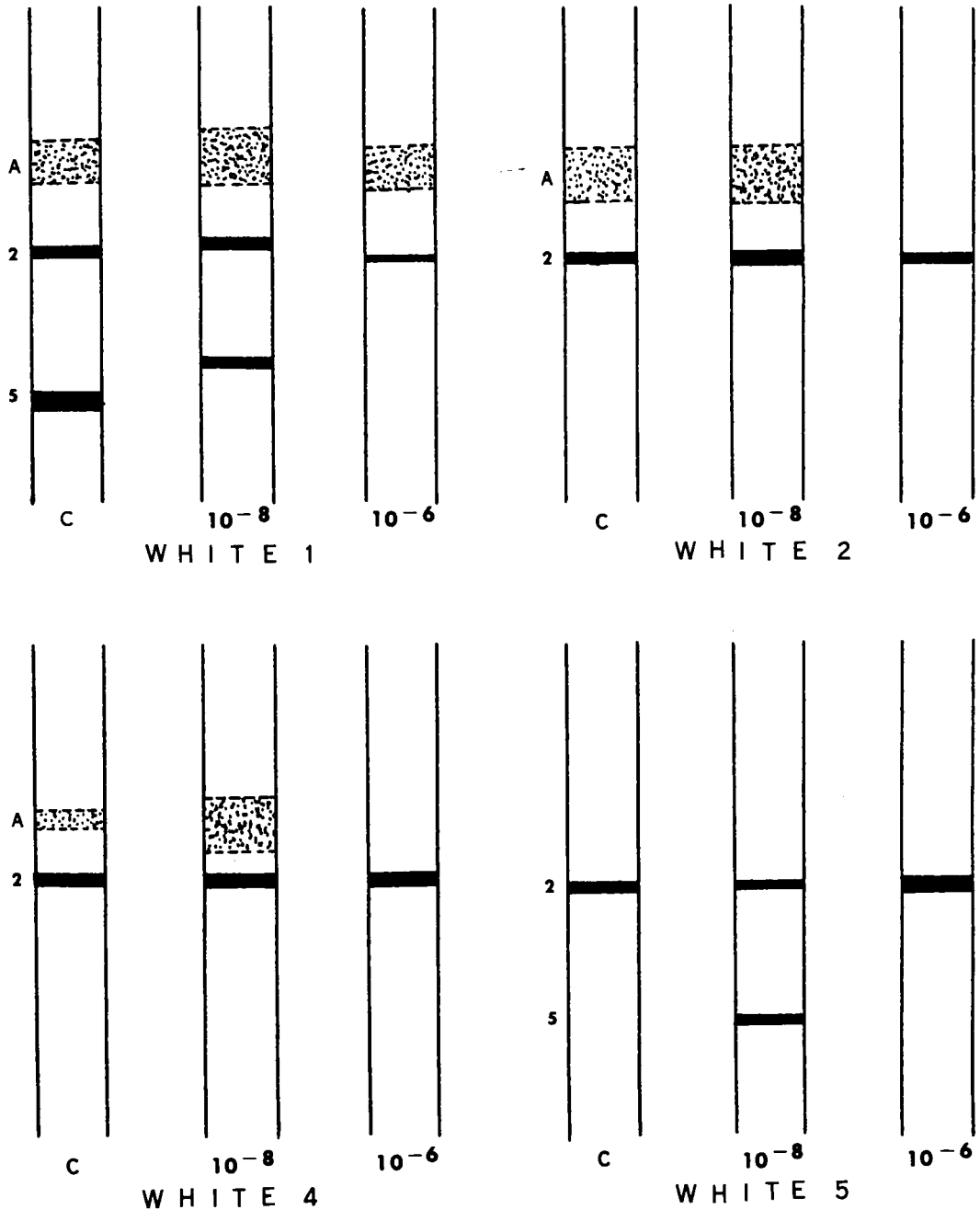

Figure 9. Polyacrylamide-gel electrophoresis of crude extracts obtained from representative lines of white pine callus. Each line was grown on 0, 10^{-8}M and 10^{-6}M paraquat supplemented media. The 200 µl of extract was applied to each gel and stained for SOD activity. Darkened bands on the figure represent achromatic regions of SOD activity on the actual gels.

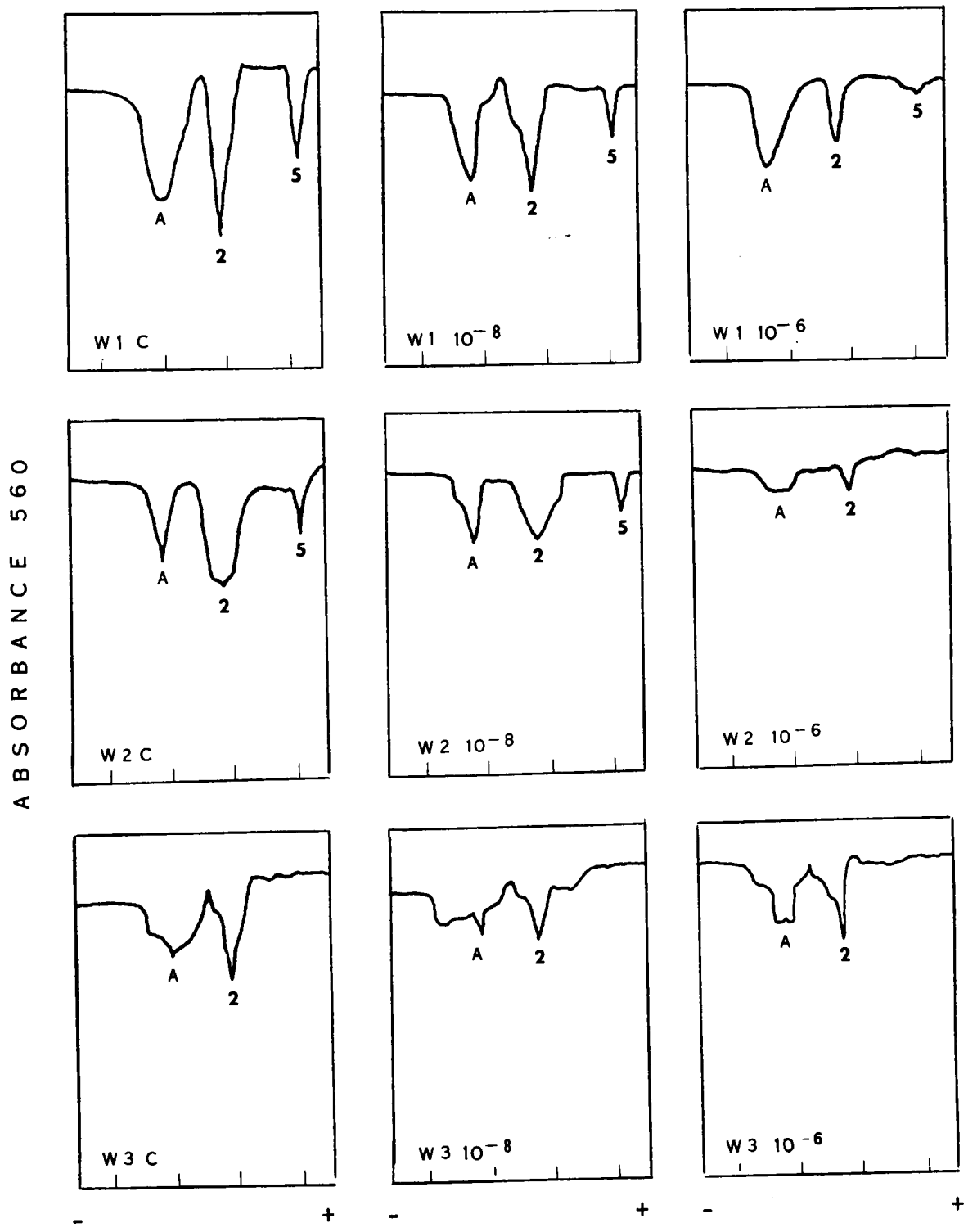

Figure 10. Densitometer tracings of representative gels from several lines of white pine callus indicating superoxide dismutase activity. Peak number corresponds to that of bands labeled on gels in order of increasing mobility.

analysis of the peak areas indicated no significant increases or decreases in relative areas when grown on paraquat supplemented media (Table XII). Probability values for bands A, 2, and 5 in white pine callus were .377, .623, and .656, respectively.

Virginia pine callus. Electrophoresis data from Virginia pine callus proved to be difficult to obtain and when obtained extremely variable among the lines tested. Most gels when stained for SOD activity showed no bands at all. The SOD bands from two lines of callus tissue which did resolve on gels presented some interesting results (Figure 11). One line expressed banding patterns similar to those found in white pine tissues. Only one major band at a Rf of .52, corresponding to band 2 in the three other species, was present. A diffuse minor band at Rf=.34 was similar to band A from the other species. The second line of Virginia callus which yielded gel data seemed to mimic loblolly SOD banding patterns. Generally four major SOD bands were present; band A at Rf=.27, band 1 at Rf=.40, band 2 at Rf=.52 and band 3 at Rf=.61. These values were in direct comparison with similar banding data from previously reported loblolly callus tissue. Minor bands were also correlated with band B at Rf=.46 and band 4 at Rf=.68. Similarly no change in band number was observed from callus grown on paraquat supplemented media.

Densitometer scans reflected the visual variability in Virginia pine callus (Figure 12). Due to this extreme variability within the lines tested and the paucity of data available, comparisons of the scan area were generally difficult; however, there was no apparent change in

TABLE XII

AREA* OF DENSITOMETER SCANS OF SOD FROM SEVERAL LINES OF WHITE PINE CALLUS

Tissue Line	Peak A	Peak 2	Peak 5	Total
W 1				
0**	20.6	16.1	7.7	44.4
10^{-8}M	18.6	11.7	4.8	35.1
10^{-6}M	7.7	5.0	0.4	13.1
W 2				
0	5.4	1.4	-	6.8
10^{-8}M	15.3	6.4	-	21.7
10^{-6}M	1.7	1.0	-	2.7
W 3				
0	12.3	8.9	-	21.2
10^{-8}M	4.2	3.7	-	7.9
10^{-6}M	26.3	17.9	-	43.2
W 4				
0	3.5	3.6	0.7	7.8
10^{-8}M	1.0	4.4	0.8	6.2
10^{-6}M	-	4.0	-	4.0
W 5				
0	-	11.1	-	11.1
10^{-8}M	3.8	6.8	6.1	16.7
10^{-6}M	4.6	11.8	5.9	21.3

*Areas are represented by the (mg) weights of peaks, adjusted to 100 µg protein/gel.

**Concentration of paraquat in media.

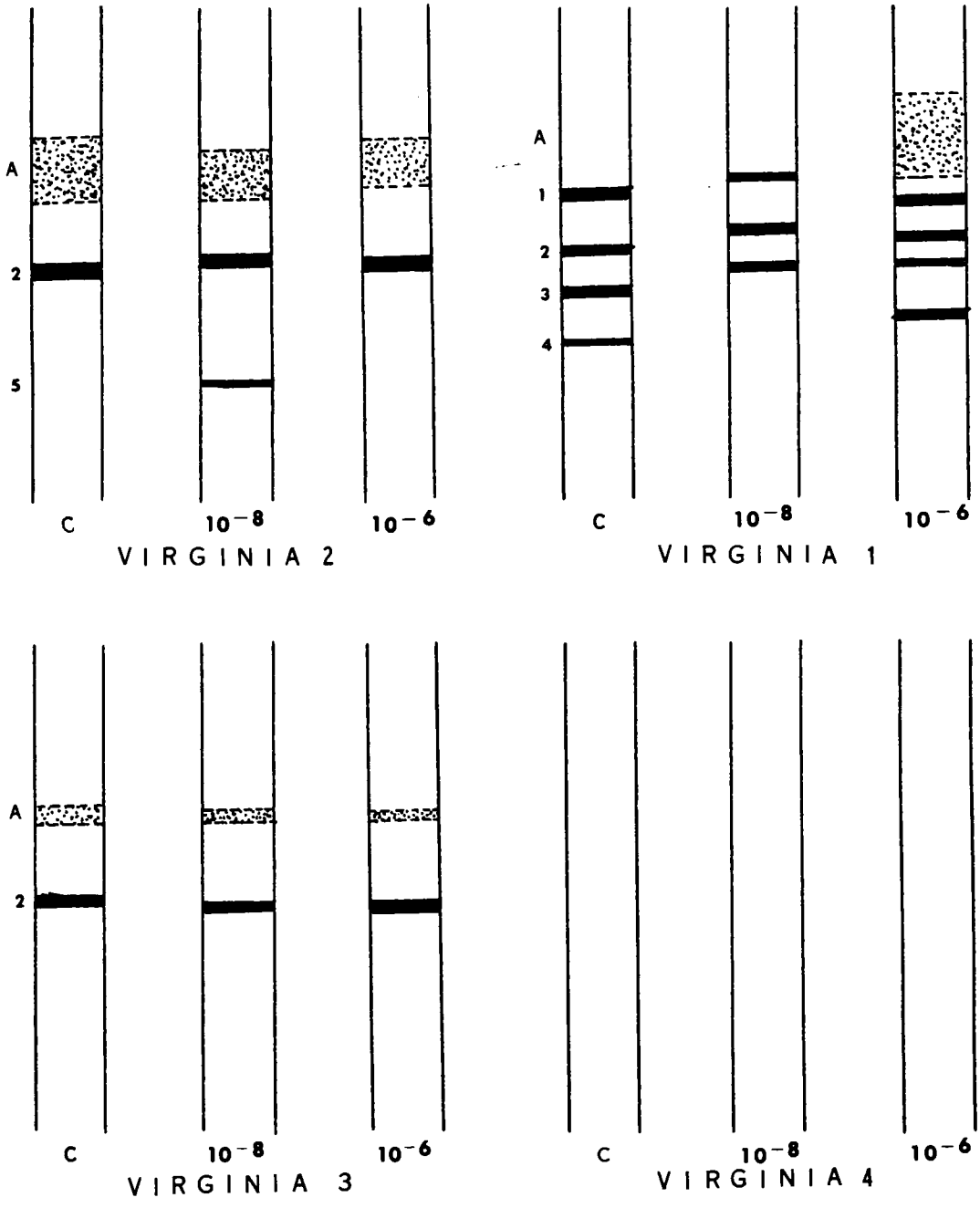

Figure 11. Polyacrylamide-gel electrophoresis of crude extracts obtained from representative lines of Virginia pine callus. Each line was grown on 0, 10^{-8}M, and 10^{-6}M paraquat supplemented media. The 200 µl of extract was applied to each gel and stained for SOD activity. Darkened bands on the figure represent achromatic regions of SOD activity on the actual gels.

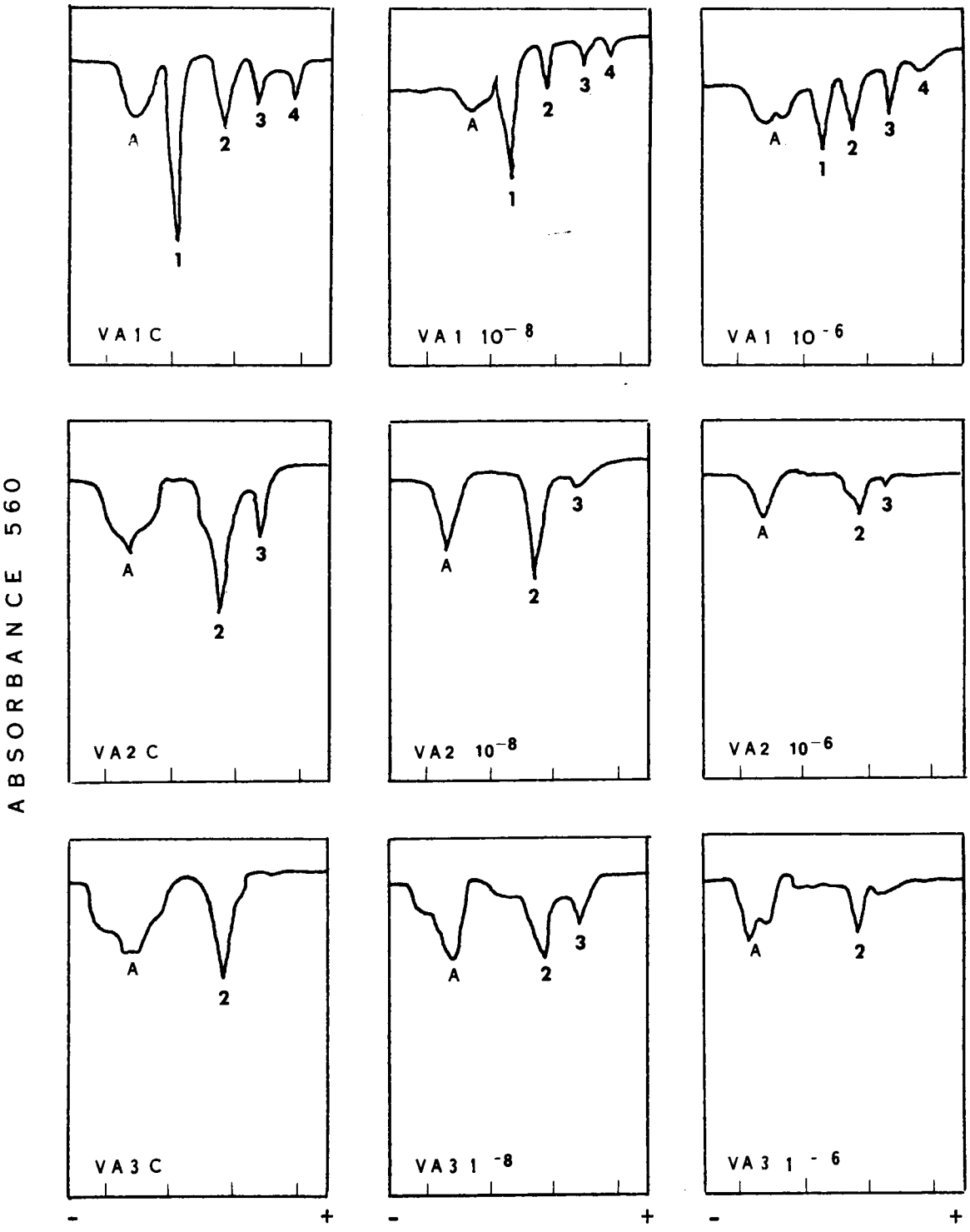

Figure 12. Densitometer tracings of representative gels from several lines of Virginia pine callus indicating superoxide dismutase activity. Peak number corresponds to that of bands labeled on gels in order of increasing mobility.

band intensity with exposure of the cell lines toward paraquat. Probability values for all of the peaks observed were .344 (Table XIII).

Total Protein and SOD Levels in Pine Callus Cultures Grown on Paraquat Supplemented Media

Total protein levels. Examination of the total soluble protein content (mg/gm fresh weight of callus) in calli grown on medium containing 0 or 10^{-8}M paraquat indicated that protein levels were generally higher on paraquat supplemented medium (Tables XIV-XVII). Of the 16 cell lines from 4 species, 12 cell lines showed increased protein levels when grown on medium containing paraquat as compared to protein levels of tissue grown on medium without paraquat. Statistical analysis by a non-parametric sign test (Siegel, 1956) indicated that this trend was significant (P=.038). Total soluble protein content in calli grown on medium containing 10^{-6}M paraquat was not significantly different from protein content grown on medium without paraquat. Of the 15 cell lines from 4 species tested, only 7 cell lines showed increased protein levels on medium containing 10^{-6}M paraquat whereas 8 showed decreases in these levels. Statistical analysis indicated that no trend, either towards increasing or decreasing levels of protein exists (P=.500) (Tables XIV-XVII).

SOD levels. Examination of SOD levels in calli grown on medium containing no paraquat or on medium containing paraquat at 10^{-8}M indicated that overall there was no difference. Of all the 16 lines from the 4 species, 9 showed increased levels of SOD when grown on medium

TABLE XIII

AREA* OF DENSITOMETER SCANS OF SOD FROM SEVERAL LINES OF VIRGINIA PINE CALLUS

Tissue Line	Peak A	1	2	3	4	Total
VA 1						
0**	2.9	7.7	2.0	1.1	2.0	15.7
10^{-8}M	2.1	4.7	1.6	3.0	1.1	12.5
10^{-6}M	4.0	11.2	2.4	1.9	1.0	20.5
VA 2						
0	10.0	-	8.1	2.8	-	20.9
10^{-8}M	11.5	-	15.0	0.8	-	27.3
10^{-6}M	7.6	-	6.0	0.4	-	13.4
VA 3						
0	21.8	-	3.8	-	-	25.6
10^{-8}M	8.7	-	8.0	4.2	-	20.9
10^{-6}M	11.8	-	5.6	0.6	-	18.0

*Areas are represented by the (mg) weights of peaks, adjusted to 100 µg protein/gel.

**Concentration of paraquat in media.

TABLE XIV

EFFECT OF PARAQUAT ON UNITS OF SOD ACTIVITY FROM SEVERAL LINES OF SHORT LEAF PINE CALLUS TISSUE

Tissue Line	Fresh Wt. (gm/Callus)	Protein (mg/gm Wet Wt.)	SOD Units/ mg Protein	SOD Units/ gm Wet Wt.
SL 1				
0*	0.79	0.85	47.4	40.3
10^{-8}M	0.63	2.16	5.7	12.5
10^{-6}M	0.66	0.91	76.9	69.9
SL 2				
0	0.57	3.42	6.2	21.1
10^{-8}M	0.78	2.78	8.2	22.7
10^{-6}M	0.58	1.28	8.1	10.4
SL 3				
0	1.42	1.32	13.9	18.4
10^{-8}M	0.91	1.66	17.3	28.5
10^{-6}M	1.09	1.38	30.4	41.9
SL 5				
0	1.28	2.58	20.4	52.6
10^{-8}M	1.68	2.54	20.8	52.8
10^{-6}M	1.09	2.68	19.5	52.3
SL 6				
0	0.67	1.45	55.4	80.3
10^{-8}M	0.96	1.41	95.6	134.8
10^{-6}M	0.75	0.60	68.9	41.3

*Concentration of paraquat in media.

TABLE XV

EFFECT OF PARAQUAT ON UNITS OF SOD ACTIVITY FROM SEVERAL LINES OF LOBLOLLY PINE CALLUS TISSUE

Tissue Line	Fresh Wt. (gm/Callus)	Protein (mg/gm Wet Wt.)	SOD Units/ mg Protein	SOD Units/ gm Wet Wt.
Lob 5				
0*	1.09	1.44	69.0	99.4
10^{-8}M	1.22	1.78	35.6	62.3
10^{-6}M	1.06	1.13	52.5	60.6
Lob 6				
0	1.12	1.61	29.3	47.1
10^{-8}M	0.79	2.75	9.7	26.6
10^{-6}M	0.84	1.88	8.7	16.4
Lob 7				
0	1.03	2.18	6.4	13.9
10^{-8}M	1.37	2.19	19.0	41.6
10^{-6}M	0.80	0.94	7.2	6.8
Lob 8				
0	0.69	1.08	6.2	6.7
10^{-8}M	1.21	1.12	36.4	40.8
10^{-6}M	0.95	0.87	35.2	30.6

*Concentration of paraquat in media.

TABLE XVI

EFFECT OF PARAQUAT ON UNITS OF SOD ACTIVITY FROM SEVERAL LINES OF VIRGINIA PINE CALLUS TISSUE

Tissue Line	Fresh Wt. (gm/Callus)	Protein (mg/gm Wet Wt.)	SOD Units/ mg Protein	SOD Units/ gm Wet Wt.
VA 3				
0*	2.26	0.66	33.4	22.2
10^{-8}M	1.91	0.79	37.9	29.8
10^{-6}M	1.88	0.99	28.6	28.5
VA 4				
0	1.15	1.57	60.4	94.5
10^{-8}M	1.08	1.80	12.2	22.1
10^{-6}M	0.96	1.93	34.3	66.3
VA 5				
0	1.10	0.68	22.6	15.4
10^{-8}M	0.82	2.29	24.6	56.3
10^{-6}M	0.79	0.95	14.3	13.6
VA 6				
0	2.09	1.40	25.2	35.3
10^{-8}M	0.90	1.00	5.2	5.2
10^{-6}M	0.73	0.82	13.8	11.3

*Concentration of paraquat in media.

TABLE XVII

EFFECT OF PARAQUAT ON UNITS OF SOD ACTIVITY FROM SEVERAL LINES OF WHITE PINE CALLUS TISSUE

Tissue Line	Fresh Wt. (gm/Callus)	Protein (mg/gm Wet Wt.)	SOD Units/ mg Protein	SOD Units/ gm Wet Wt.
W 2				
0*	1.06	1.84	88.4	162.7
10^{-8}M	1.16	2.13	63.0	134.2
10^{-6}M	1.09	1.79	78.5	140.5
W 3				
0	1.84	0.37	14.2	5.2
10^{-8}M	0.71	2.85	25.6	73.0
10^{-6}M	0.71	1.28	10.4	13.3
W 6				
0	1.37	1.97	31.2	61.5
10^{-8}M	1.69	2.49	26.4	65.7
10^{-6}M	-	-	-	-

*Concentration of paraquat in media.

containing paraquat as compared to SOD levels on medium without paraquat (Tables XIV-XVII). Statistical analysis by the non-parametric sign test indicated that the difference was not significant (P=.402). Similarly, there was no difference between SOD levels of calli grown on medium containing 10^{-6}M paraquat and medium without paraquat (P=.500). Examination of the data for individual species, however, indicated that for short leaf pine callus, there was a consistent increase in SOD levels in calli grown on both 10^{-8}M and 10^{-6}M paraquat (Table XIV). The probability value for this was .055 indicating a borderline response.

Correlations

Data from all four species were grouped and correlation coefficients were calculated to determine if there was any significant relationship between overall growth of callus, SOD levels (in units/mg protein), and protein levels (in mg/gm fresh weight of callus). The correlation between callus fresh weight and protein was -.1723, N=47. The correlation between callus fresh weight and SOD was .0393, N=47. The correlation between protein levels and SOD was .2226, N=47. None of these correlation coefficients are statistically significant; however, it should be noted that there is extensive interclonal variability in the results which might mask significant intraclonal correlations. In order to determine if significant intraclonal correlations exist, it would be necessary to repeat the experiment using replicates of one clonal line only.

CHAPTER IV

DISCUSSION

Although the mode of action of the herbicide paraquat is fairly well understood; its non-lethal effects on the metabolism of plant cells warrants considerable investigation. Numerous studies into the effect of paraquat on growth and cellular metabolic activities have been pursued through the use of plant cell and tissue culture.

In this study the response of short leaf, loblolly, Virginia and white pine callus cultured on paraquat supplemented media was observed. The response of the four species of pine callus toward paraquat treatment obviously differed from species to species (Figures 1-4, pages 27, 29, 31, and 34, respectively). Growth response of short leaf pine callus (Figure 1) indicated a mean dry weight increase over that of the controls at 10^{-8}M and 10^{-7}M paraquat, not observed in the other species. Similar increases have been observed in loblolly, Virginia and short leaf pine callus cultures when grown on medium supplemented with 10^{-8}M paraquat (Hughes and Caponetti, 1976). The data from this present study differ from that of the former in that loblolly and Virginia callus in this experiment, exhibited decreases in mean dry weights at 10^{-8}M, 10^{-7}M, and 10^{-6}M paraquat. Experiments of Hughes and Caponetti were carried out in the dark whereas the current study was performed in the light. This alteration may possibly account for the difference in reported growth response. In both the current experiments and those of Hughes and Caponetti, growth of white pine callus was considerably inhibited on medium containing paraquat.

Similar increases in growth have been observed in other studies involving cell cultures and paraquat treatment. Miller (1979) reported increases in growth of tobacco cell cultures at 10^{-8}M, 10^{-7}M, and 10^{-6}M paraquat over that of the controls. Results of Hughes (personal communication) show similar increases in soybean callus cultures.

Variations in growth response among different clonal cell lines of the same species were also quite evident (Tables V-VIII, pages 25, 28, 30, and 33, respectively). Statistical analysis indicated that these were highly significant differences which suggest a large degree of genetic variation existing in the initial seedling explants. Conversely, the growth response of calli from the same clonal lines was not significantly different. This data seems to reflect the fact that repeated subculturing of callus does not appear to affect its response toward paraquat treatment through genetic or epigenetic changes.

Electrophoresis indicated that extractable levels of SOD are present in pine callus cultures. Major differences do exist between SOD banding patterns in the four species tested (Figures 5, 7, 9, and 11, pages 37, 41, 45, and 49, respectively). These banding patterns in general did not change significantly in response to growth on paraquat supplemented media. No additional bands appeared and the band intensity changed very little in loblolly, Virginia, and white pine calli. Shortleaf cultures were the exception in that they exhibited the only significant increase in band area of any of the species tested (Table X, page 39).

All of the species exhibited multiple forms of SOD. Cu-Zn SODs are sensitive to cyanide, whereas manganese SODs are resistant to cyanide but sensitive to the Tsuchihashi precipitation. In all the gels, band A

was found to be resistant to cyanide and thus appeared to have Mn SOD like properties. All the other bands appeared to be isozymes of the Cu-Zn SOD.

Bands A and 2 were common to the gels of all four of the species tested. Bands 4 and B were found only in loblolly callus gels where as band 5 was seen in white pine cell lines and in one line of Virginia callus. Gels of short leaf, loblolly and one line of Virginia callus exhibited bands 1 and 3.

Quantitation of SOD levels in pine callus yielded variable results; however, the SOD values obtained were similar to values reported for other higher plants including corn, oats and peas (Giannopolitis and Ries, 1977b; Asada et al., 1977). SOD levels neither increased nor decreased significantly in calli of Virginia, loblolly and white pine cultured on medium containing low levels of paraquat, but short leaf SOD levels did show a significant increase when grown on medium containing paraquat. This increase was evident at both 10^{-8}M and 10^{-6}M paraquat. Since paraquat has been shown to induce SOD in E. coli, it is possible that this increase also represents induction; however, the inducible enzyme in E. coli was a Mn SOD while significant increases in pines were observed in band 2, a Cu-Zn SOD.

While interclonal variability was extremely high, there was an overall increase in growth of the callus cultures of short leaf as exhibited by data on growth response of calli on paraquat. A possible explanation for the observed increase in growth is that higher SOD levels may protect the cell from the toxic effects of paraquat and allow increased cell growth. An alternate possibility is that both

increased SOD levels and increased growth are the result of some third factor not identified by this study. The data from this study cannot substantiate either possibility.

Conversely, the extreme sensitivity exhibited by white pine callus towards paraquat supplemented media may be the result of its "non-inducibility" of SOD. Possibly its lower number of SOD isozymes may also be responsible for the high susceptibility toward paraquat.

In summary, this investigation revealed that callus cultures of short leaf, loblolly, white and Virginia pines exhibited changes in growth, total protein and some SOD levels when grown on paraquat supplemented media. Generally, dry weight, taken as an indication of growth, decreased in response to increasing paraquat concentrations in all the species tested except short leaf. Short leaf callus responded with an increasing mean dry weight when grown on 10^{-8}M and 10^{-7}M paraquat supplemented media. Practically all the calli from the four species tested responded with a mean increase in total protein levels when grown on 10^{-8}M paraquat. Only one species, shortleaf, responded with an associated increase in SOD levels as measured by gel electrophoresis and SOD assays.

These results may indicate that the increased levels of SOD in short leaf calli may be providing protection against the paraquat toxicity and thus allowing growth to continue as normal. Possibly this may explain the increase observed in growth in this species at 10^{-8}M and 10^{-6}M paraquat.

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APPENDIXES

APPENDIX A

MEDIUM

Composition of modified Knop's medium for culture of pine embryos (Nakosteen, 1978)

COMPONENT	AMOUNT
Knop's stock solution containing the following per liter	500ml/l
$Ca(NO_3)_2 \cdot 4H_2O$	0.5 g
KNO_3	0.125 g
$MgSO_4 \cdot 7H_2O$	0.125 g
KH_2PO_4	0.125 g
Nitsche's minor elements (B-5) containing the following per liter	0.5ml/l
H_2SO_4	0.5 ml
$MnCl_2 \cdot 4H_2O$	2.5 g
H_3BO_3	2.5 g
$ZnSO_4 \cdot 7H_2O$	0.05 g
$CoCl_2 \cdot 6H_2O$	0.03 g
$CuCl_2 \cdot 2H_2O$	0.015 g
$NaMoO_4 \cdot 2H_2O$	0.025 g
Ferric citrate containing the following per liter	0.4ml/l
$FeC_6H_5O_7 \cdot 5H_2O$	25 g
Sucrose	20 g/l
Agar	8 g/l

Medium was adjusted to pH 5.5 with the addition of 1 N NaOH before addition of agar.

TABLE XVIII

COMPOSITION OF BROWN'S GYMNOSPERM MEDIUM (BROWN AND LAWRENCE, 1968)

Stock Solution	Component	Concentrations of Stock (g/l)	Amount of Stock/Liter
A	NH_4NO_3	82.5	20 ml
B	KNO_3	95.0	20 ml
C	H_3BO_3	1.24	5 ml
	KH_2PO_4	34.0	
	KI	0.166	
	$Na_2MoO_4 \cdot 2H_2O$	0.050	
	$CoCl_2 \cdot 6H_2O$	0.005	
D	$CaCl_2 \cdot 2H_2O$	88.88	5 ml
E	$MgSO_4 \cdot 7H_2O$	74	5 ml
	$MnSO_4 \cdot H_2O$	4.46	
	$ZnSO_4 \cdot 7H_2O$	1.72	
	$CuSO_4 \cdot 5H_2O$	0.005	
F	Na_2EDTA	3.72	10 ml
	$FeSO_4 \cdot 7H_2O$	2.78	
Sucrose		-	20 g
Agar[a]		-	8 g
Organic Suppl.	Nicotinic Acid		0.5 mg
	Pyridoxin-HCl		0.1 mg

TABLE XVIII (Continued)

Stock Solution	Component	Concentration of Stock (g/l)	Amount of Stock/Liter
	Thiamin-HCl		0.1 mg
	Kinetin		0.5 mg
	2,4-D		5.0 mg
	Inositol		100.0 mg
	Asparagine		100.0 mg

[a]Stock solutions were added to approximately 900 ml of double distilled water. The pH was adjusted to 5.7-5.7 with 1N NaOH. Double distilled water was added to a total volume of 1 liter. Agar was added and the medium was sterilized by autoclaving.

APPENDIX B

PROTEIN AND ENZYME ASSAYS

Protein determination (Lowry et al., 1951) (modification according to J. Caponetti, personal communication)

1. Prepare Reagent A by mixing the following just prior to use:

   a. 100 ml of 4% Na_2CO_3

   b. 1 ml of 2% $CuSO_4 \cdot 5H_2O$

   c. 1 ml of 4% Na tartate

2. Prepare Reagent B by mixing the following:

   a. 1 part 2N Folin-Ciocalteau reagent

   b. 3 parts H_2O

3. Add 100 µl of sample and standards to separate 18 mm test tubes.

4. Add 6 ml of Reagent A (#1 above) to each tube, mix well, and let stand for 10 min.

5. Add 0.5 ml of Reagent B (#2 above) to each tube, mix well, and let stand for 30 min.

6. Read OD at 669 nm.

7. Simultaneously prepare a standard curve with bovine albumin and determine the protein concentration of the sample from the curve.

SOD assay (Giannopolitis and Ries, 1977)

1. Add reagents to a 3 ml cuvette in the following proportions:
   a. 1.3 μM riboflavin
   b. 13 mM methionine
   c. 63 μM NBT
   d. 0.05 M sodium carbonate (pH 10.2)
   e. H_2O or extract to bring final volume to 3 ml.
2. Place cuvettes beside a G.E. cool white fluorescent tube and initiate the reaction by turning on the light. Non-illuminated cuvettes served as blanks.
3. Illuminate for 1, 5, 7, 10, 12, and 15 minutes. Record change in absorbances at 560nm for each interval of illumination.
4. One unit of SOD activity is defined as the amount of enzyme required to reduce the rate of the given reaction by 50%.

APPENDIX C

GEL ELECTROPHORESIS PROCEDURES

Preparation of polyacrylamide gel electrophoresis tubes (B. J. Davis, 1964)

I. Tube preparation

1. Thoroughly wash glass tubes (12 x 2 cm) in soapy water.
2. Rinse the tubes in 95% ethanol and allow them to dry for 10 min.
3. Soak the tubes in a solution of 10% Photo-flo for 1 min. making sure all surfaces have been covered.
4. Remove the tubes from the Photo-flo and dry them for 15 to 30 min. in an oven at 110°C. Allow the tubes to cool and use immediately.

II. Separation gel stock solutions

1. Prepare stock solution 1 by combining the following and adjusting the final volume to 100 ml with double distilled H_2O.

   a. 48 ml of 1 N HCL
   b. 36.6 g Tris
   c. 0.23 ml of TEMED

2. Prepare stock solution 2 by mixing the following and adjusting the final volume to 100 ml with double distilled H_2O.

   a. 30 g acrylamide
   b. 0.8 g bis

3. Prepare stock solution 3 by mixing the following just prior to use.

   a. 0.14 g Ammonium Persulfate
   b. 100 ml of H_2O.

III. Spacer gel stock solutions

1. Prepare stock solution 4 by combining the following and bringing the final volume of stock to 100 ml with double distilled H_2O.

   a. 48 ml of 1 N HCl.
   b. 5.98 g Tris
   c. 0.46 ml TEMED

2. Prepare stock solution 5 in a final volume of 100 ml.

   a. 10 g of Acrylamide
   b. 2.5 g of Bis

3. Prepare stock solution 6 by mixing the following just prior to use.

   a. 4.0 mg of Riboflavin
   b. 100 ml of H_2O

4. Stock solution 7 is prepared by dissolving 40 g of sucrose in a final volume of 100 ml H_2O

IV. Pouring the tubes

1. Plug electrophoresis tubes with appropriate rubber stoppers.

2. Prepare separation gel mixture by mixing the following just before use:

   a. 1 part stock solution 1 (#II-1 above)
   b. 2 parts stock solution 2 (#II-2 above)
   c. 4 parts stock solution 3 (#II-3 above)
   d. 1 part H_2O

3. Place the separation gel mixture under vacuum for a few seconds to remove air.

4. Carefully fill each tube with enough mixture (about 2 ml) to give a column of about 10 cm.

5. Briefly agitate the filled tube with a vortex mixer to insure elimination of any trapped air bubbles.

6. Carefully layer a few drops of H_2O on top of the gel mixture to eliminate the meniscus.

7. Allow mixture to gel for 30 minutes.

8. Wick the H_2O off the gel's upper surface and prepare the spacer gel mixture by mixing the following just before use:

   a. 1 part stock solution 4 (#III-1 above)
   b. 2 parts stock solution 5 (#III-2 above)
   c. 1 part stock solution 6 (#III-3 above)
   d. 4 parts stock solution 7 (#III-4 above)

9. Add 0.5 ml of spacer gel mixture onto top of previous separation gel now hardened.

10. Carefully layer a few drops of H_2O on top of the gel mixture to eliminate the meniscus.

11. Illuminate the gels for 15 minutes with fluorescent light until the gel appears cloudy (polymerization of the spacer gel).

12. Gels may be used immediately or stored in the cold for up to three weeks provided the gel is protected from dehydration.

Gel running procedures

I. Electrode buffer solution

1. Prepare stock electrode buffer by combining the following and adjusting the final volume to 1000 ml.

   a. 6.0 g Tris (pH 8.3)
   b. 28.8 g Glycine

2. Dilute the stock to a 10% aqueous solution consisting of 100 ml stock in 900 ml H_2O.

II. Load previously prepared electrophoresis tubes into tank, removing rubber stoppers and wicking off any remaining layered H_2O.

III. Fill lower tank with buffer so that electrode is completely covered (600 ml). Tap the tubes to remove any air bubbles which may be trapped between the gel and the buffer solution.

IV. Load 100 µl-200 µl of the sample (containing 50-100 µg protein) which has been previously mixed with an equal volume of 40% sucrose solution onto the upper gel. Carefully layer buffer on top of sample and fill upper tank. Add a few crystals of brom phenol blue to the upper tank to serve as a front marker.

V. Attach (+) electrode (red) to lower tank and (-) electrode to the upper tank. Apply 1mA of current per tube for the first 5 minutes. Increase the current to 3mA per tube until the marker dye reaches the end of the tubes.

Stains used in detection of SOD on polyacrylamide gels

I. Negative staining technique (Edwards, Hopkinson, and Harris, 1978)

1. Prepare staining solution by combining the following prior to use:

   a. 10 mg of MTT tetrazolium
   b. 5 mg PMS
   c. 100 ml of 0.1 M Tris HCl, pH 8.0 buffer

2. Cover gels with staining solution and allow them to remain in the cold and dark for about 1 hour.

3. Remove the gels from the stain and illuminate them for 30 min. with 2 fluorescent lights.

4. Areas of SOD appear as light bands on a blue background.

II. Negative staining technique (Beauchamp and Fridovich, 1971)

1. Prepare staining solution 1 by combining the following before use:

   a. 20 mg NBT
   b. 40 ml of 0.036 M potassium phosphate buffer, pH 7.8

2. Place gels in solution 1 and allow them to remain for 20 min. in the dark.

3. Prepare staining solution 2 by combining the following before use:

   a. 0.8 mg riboflavin
   b. 0.12 ml TEMED
   c. 40 ml .036 M potassium phosphate buffer pH 7.8

4. Remove the gels from solution 1 and transfer them to staining solution 2. Allow them to remain in the second solution and in the dark for 15 min.

5. Expose the treated gels to 10 min. of fluorescent light.

6. Bands of SOD appear as light areas on a blue background.

VITA

Robert Michael Barni was born in East Lansing, Michigan on January 17, 1955. In 1959 he moved to St. Louis, Missouri where he began his elementary education. In 1967 he moved to Lexington, Tennessee where he concluded his elementary and secondary education and was graduated from Lexington High School in June 1973. The following September, he entered The University of Tennessee, Martin, and in May 1977 graduated cum laude with a Bachelor of Science degree in Biology.

In the fall of 1977 he entered The University of Tennessee, Knoxville and began study towards a Master's degree. In the fall of 1978 he received a Graduate Teaching Assistantship for two years from the Department of Botany. He received the Master of Science degree with a major in Botany in December 1980.