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TNT AND 4-AMINO-2,6-DINITROTOLUENE INFLUENCE ON GERMINATION AND EARLY SEEDLING DEVELOPMENT OF TALL FESCUE

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Abstract

*Cost-effective and environmentally acceptable methods are needed to remediate munitions-contaminated soil. Some perennial grass species are tolerant of soil contaminants and may promote remediation because of their high water use and extensive fibrous root systems. The effects of 2,4,6-trinitrotoluene (TNT) and its reduction product, 4-amino-2,6-dinitrotoluene (4ADNT), on germination and early seedling development of tall fescue (*Festuca arundinacea *Schreb.) were determined. Tall fescue seeds were germinated in nutrient-free agar containing 0–60 mg TNT litre^{-1} or 0–15 mg 4ADNT litre^{-1}. Germination decreased linearly as TNT concentration increased but was not significantly affected by 4ADNT at these concentrations. Concentrations less than 30 mg TNT litre^{-1} or 7.5 mg 4ADNT litre^{-1} had little effect on seedling growth and development. Higher TNT or 4ADNT concentrations substantially delayed seedling development, caused abnormal radicle tissue development, and reduced secondary root and shoot growth. Seedling respiration rates decreased linearly with increasing TNT concentration. Experiments indicate that tall fescue may be grown in soils that maintain soil solution concentrations of 30 mg TNT litre^{-1} or less. Copyright © 1996 Elsevier Science Ltd*

Keywords: TNT, 4-amino-2,6-dinitrotoluene, germination, tall fescue, tolerance, remediation, respiration, xenobiotic.

INTRODUCTION

A serious environmental problem facing the US military and Environmental Protection Agency is the presence of contaminated soil at sites where munitions were formerly manufactured, stored, or demilitarized (Jenkins & Walsh, 1992). Incineration is the most commonly used remediation technology for munitions-contaminated soil. However, rising costs and public concern regarding incineration necessitate more cost-effective, environmentally benign alternatives. At some sites plants may be used to contain contaminants and may promote remediation in the rhizosphere (Cunningham & Berti, 1993). Perennial grasses may be particularly useful because they have extensive root systems, high water use, and are adaptable to a wide range of environments. Additionally, grasses can be rapidly established and may be tolerant of soil contaminants.

Toxicity thresholds for a contaminant and its degradates must be established to determine the remediation potential of a particular plant species. The explosive, 2,4,6-trinitrotoluene (TNT), causes DNA-frameshift mutations and disrupts normal cell division in biological systems (Won *et al.*, 1976). TNT concentrations between 2 and 50 mg litre^{-1} inhibited growth of gram-positive bacteria, fungi, actinomycetes, yeasts, and freshwater algae (Klausmeier *et al.*, 1973; Liu *et al.*, 1976; Smock *et al.*, 1976; Won *et al.*, 1976). TNT is readily reduced in both aerobic and anaerobic environments to initially produce 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT) (Isbister *et al.*, 1980; Kaplan, 1990; Schackman & Miller, 1991; Comfort *et al.*, 1995). These degradates are similar in structure to dinitroaniline herbicides and may have similar toxic effects in plants (Harvey *et al.*, 1990).

Schott and Worthley (1974) reported reduced growth of duckweed (*Lemna perpusilla* Torr.) when plants were exposed to 1 mg TNT litre^{-1} and concentrations greater than 5 mg TNT litre^{-1} resulted in death. TNT, 4ADNT, and 2ADNT significantly reduced growth of yellow nutsedge (*Cyperus esculentus* L.) in hydroponic culture (Palazzo & Leggett, 1986). Dry weights of leaves, roots, and rhizomes were significantly reduced in plants exposed to 5–20 mg TNT litre^{-1}. Uptake of TNT by the nutsedge was proportional to increases in TNT concentration. Although some of the TNT was translocated to shoots, TNT concentrations were higher in roots and rhizomes. Palazzo and Leggett (1983) demonstrated that TNT-contaminated effluent (pink water) reduced the growth of mixtures of tall fescue (*Festuca arundinacea* Schreb.), and ryegrass (*Lolium* L.) and alfalfa (*Medicago* L.), orchardgrass (*Dactylis glomerata* L.), and ryegrass. They suggested that the greater yield of the tall fescue–ryegrass mixture than the alfalfa–orchardgrass–ryegrass mixture

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indicated greater tolerance to TNT. Symptoms of injury to wild tomato (*Lycopersicon peruvianum* (L.) Mill.) and Jimsonweed (*Datura quercofolia* HBK) grown in a coarse-textured soil treated with 100–1000 mg TNT kg^{-1} included chlorosis, leaf and flower loss, while another Jimsonweed species (*Datura innoxia* Mill.) tolerated 1000 mg TNT kg^{-1} (Mueller *et al.*, 1993).

The former Nebraska Ordnance Plant near Mead, NE, is one of many sites targeted for remediation of munitions-contaminated soil. Tall fescue, as well as several other grass and legume species, are growing in contaminated soil at the Mead site. The responses of tall fescue to TNT need to be quantified to determine its tolerance threshold for remediation efforts. Our objective was to determine the effects of TNT and 4ADNT on germination and early seedling development of a turf-type tall fescue.

MATERIALS AND METHODS

Germination

Solutions of 0, 1.9, 3.75, 7.5, 15, 30, 45, and 60 mg TNT $litre^{-1}$ were prepared by dissolving technical grade TNT (US Biomedical Research and Development Laboratory, Fort Detrick, Frederick, MD) in deionized distilled H_2O. Solutions containing 0, 1.9, 3.75, 7.5, and 15 mg 4ADNT $litre^{-1}$ were prepared from analytical grade 4ADNT (Indian Head Division, Naval Surface Warfare Center, Indian Head, MD). These TNT and 4ADNT concentrations approximated those found in soil solution at the Nebraska Ordnance Plant (Comfort *et al.*, 1995). Nutrient-free solutions containing 2.8 g $litre^{-1}$ agar and TNT or 4ADNT were sterilized by autoclaving at 120°C and 0.124 MPa for 20 min. Approximately 25 ml of TNT or 4ADNT agar solution was poured into 10 cm diameter sterile plastic Petri dishes. Final solution concentrations of TNT and 4ADNT were confirmed by high performance liquid chromatography (Comfort *et al.*, 1995).

Tall fescue (cv. Rebel Jr.) seeds (Loftseed Co., Bound Brook, NJ) were surface disinfected by immersing in a 0.3% solution sodium hypochlorite in deionized distilled H_2O for 5 min, and rinsing with sterilized, deionized distilled H_2O. Thirty seeds were aseptically transferred to the surface of each agar plate. Dishes were placed in a controlled environment germinator maintained at 21°C and 100% relative humidity. Germinator lights were not used because TNT and 4ADNT may be photodegraded (Layton *et al.*, 1987). Newly emerged radicles, which constituted germination, and shoots were counted and recorded daily for 14 d. Germination rates were calculated by dividing the total number of radicles by the total number of days of the experiment (Maquire, 1962) and converted to percentages.

Germination experiments were conducted using four replicates of each TNT and 4ADNT concentration in a completely random design. TNT experiments were conducted three times and 4ADNT experiments were conducted twice.

Growth and respiration rate measurements

Petri dishes containing TNT and 4ADNT agar were similarly prepared for analysis of seedling development. Every 3 or 4 d, the number of germinated seed and number of seedlings with shoots were recorded. Also, lengths of the radicle, longest secondary root, and shoot of five randomly selected seedlings were measured and used to calculate growth rates. Seedling dry weights also were measured. Growth rates were calculated by dividing the growth measurement (length or weight) by the number of days after experiment initiation.

Seedling respiration was measured using the LI-COR 6200 photosynthesis system (LI-COR, Inc., Lincoln, NE), which utilizes an infrared gas analyzer to measure CO_2 gradients in a chamber. Twenty seedlings were placed in a mesh bag in a 1 litre chamber. CO_2 concentration gradient was measured for 1.5 min. Respiration rates were calculated by dividing CO_2 flux (mg CO_2 min^{-1}) by seedling dry weight (g).

Regression analysis was used to determine relationships between TNT or 4ADNT concentration and tall fescue germination, growth, or respiration. Germination data were tested for homogeneity among experiments using Hartley's F-maximum test (Ott, 1977), and the data were pooled.

RESULTS

Germination

Radicle emergence typically began 4 d after initiation (DAI) of each TNT and 4ADNT experiment. Shoot emergence began within 6 DAI. The effect of TNT concentration on seedling germination was reflected in mean cumulative germination percentage after 14 d of exposure (Fig. 1). Mean cumulative germination was

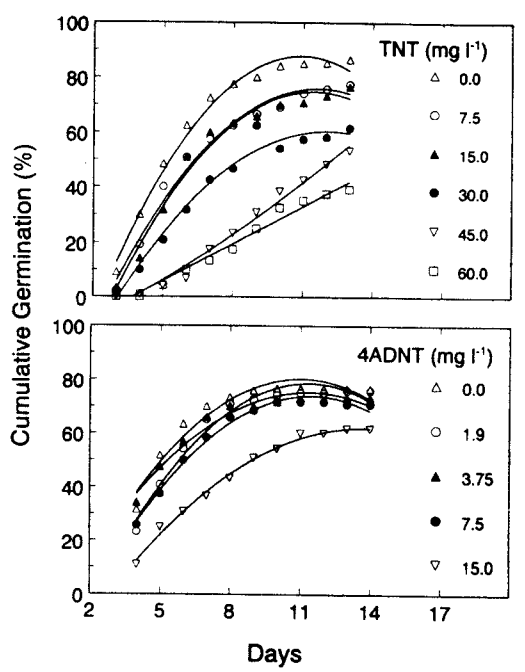

Fig. 1. Mean cumulative germination percent of tall fescue seed after 14 d exposure to TNT or 4ADNT concentration.

reduced with increasing TNT concentration, with substantial reductions at 45 and 60 mg TNT litre^{-1}. After 14 d exposure to TNT, germination percentage was significantly different among treatments; as TNT concentration increased, percent germination decreased linearly (Fig. 2).

The relationship between seed germination or mean cumulative germination and 4ADNT concentration was similar to that observed with TNT at concentrations of 30 mg TNT litre^{-1} or less. At 7.5 mg 4ADNT litre^{-1} or less, cumulative germination after 14 d of exposure was not substantially different (Fig. 1). However, a substantial reduction in germination percentage occurred at 15 mg 4ADNT litre^{-1} (Fig. 1). Germination percentage of seed exposed to 4ADNT (Fig. 3) was similar to that observed for TNT.

Seedling growth

Rates of root and shoot growth (length per day) decreased linearly with increasing TNT or 4ADNT concentrations (Figs 4 and 5). No clear relationship between TNT or 4ADNT concentration and seedling dry weight (seed, root, and shoot dry wt) was observed for the duration of the experiment (data not shown).

Shoots emerged in all treatments, but emergence and growth rates were affected by TNT or 4ADNT concentration. Shoot emergence at 30 mg TNT litre^{-1} or less and 7.5 mg 4ADNT litre^{-1} or less appeared similar to untreated seedlings; root emergence was rarely observed at 45 or 60 mg TNT litre^{-1}.

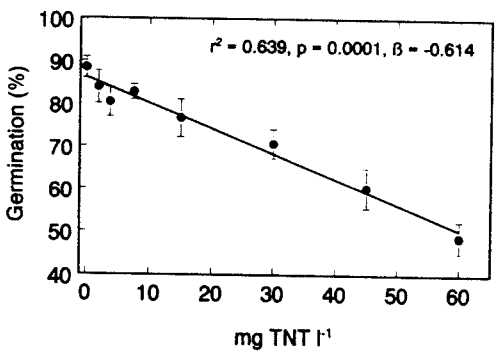

Fig. 2. Percent tall fescue germination at 14 d as affected by TNT concentration. The symbol β represents the slope of the fitted regression line. Bars on symbols indicate standard errors.

Root morphology

Roots of untreated seedlings emerged with a tuft of root hairs. Examination at 20-fold magnification revealed defined meristematic and elongation regions. Secondary roots were well developed, including root hairs (Plate 1). Seedlings exposed to 1.9 and 3.75 mg TNT litre^{-1} appeared similar to untreated seedlings.

At 7.5 mg TNT litre^{-1} growth of the primary roots was affected. Radicles were swollen with few of the root hairs normally associated with radicle emergence (Plate 2). Secondary roots were present but were thin without swollen tips and root hair development was incomplete. At 15 mg TNT litre^{-1} radicles were swollen, stunted, and no root hairs were observed. Cells appeared circular, and tissues of the radicles were undifferentiated. Secondary roots lacked root hairs and were typically small. Abnormal tissue development became increasingly apparent with increasing TNT concentration. At 45 and 60 mg TNT litre^{-1} radicles, if present, were very small and lacked secondary roots (Plate 3).

Necrosis was not observed on seedlings exposed to TNT; however, necrosis occurred on the meristematic region of radicles exposed to 7.5 and 15 mg 4ADNT litre^{-1} (Plate 4). Seedlings exposed to 1.9–15 mg 4ADNT litre^{-1} had swollen meristematic regions. Cells of seedlings exposed to 4ADNT appeared similar to cells exposed to TNT. Additionally, radicle tissues were undifferentiated and root hairs were absent.

Seedling respiration

Exposure to TNT affected cell respiration. The relationship between TNT exposure and respiration rate was linear (Fig. 6). Seedling respiration was significantly reduced at higher TNT concentrations, indicating decreased metabolic activity. Growth of seedlings exposed to 4ADNT was similar to those exposed to TNT, but respiration rates were not significantly reduced at concentrations of 4ADNT evaluated in the study.

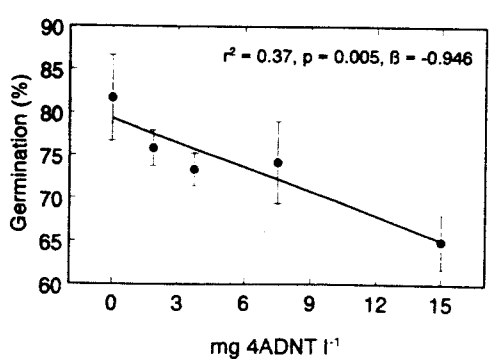

Fig. 3. Percent tall fescue germination at 14 d as affected by 4ADNT concentration. The symbol β represents the slope of the fitted regression line. Bars on symbols indicate standard errors.

DISCUSSION

TNT and 4ADNT can be toxic to tall fescue seedlings. Exposure to concentrations of 30 mg TNT litre^{-1} or greater and 15 mg 4ADNT litre^{-1} consistently reduced germination and inhibited seedling development. Phytotoxicity to seedlings increased with increasing concentrations of TNT. TNT and 4ADNT delayed and reduced tall fescue germination and reduced root and shoot growth. Our findings concur with previous studies on toxicity of TNT and its degradates to yellow nutsedge (Palazzo & Leggett, 1986; Pennington, 1988).

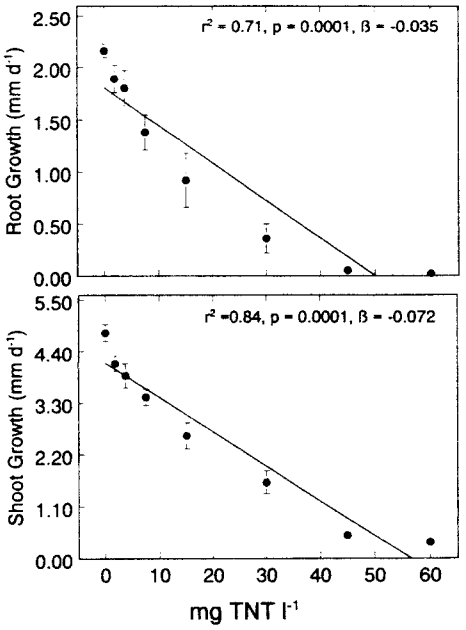

Fig. 4. Root and shoot growth rates of tall fescue as affected by TNT concentration. The symbol β represents the slope of the fitted regression line. Bars on symbols indicate standard errors; where absent, bars fall within symbols.

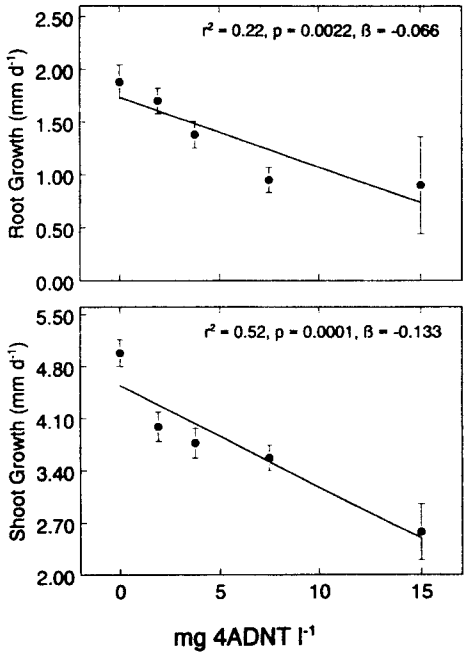

Fig. 5. Root and shoot growth rates of tall fescue as affected by 4ADNT concentration. The symbol β represents the slope of the fitted regression line. Bars on symbols indicate standard errors

Plate 1. Root of an untreated seedling at 9 DAI magnified 20-fold. The arrow indicates the primary root. Root hairs and secondary root are present.

Plate 2. Root of a seedling exposed to 7.5 mg TNT litre^{-1} at 9 DAI magnified 20-fold. The arrow indicates the swollen meristematic region of the primary root which lacks root hairs.

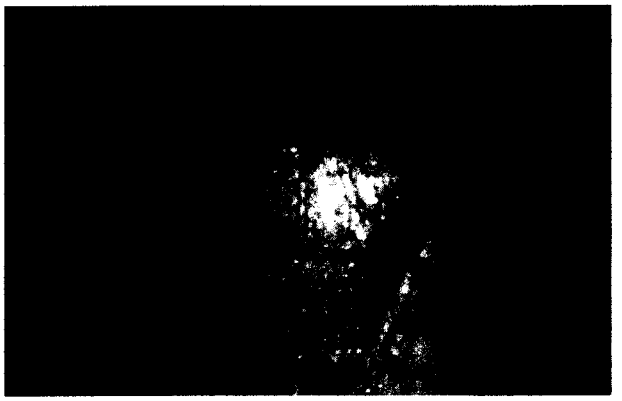

Plate 3. Root of a seedling exposed to 60 mg TNT litre^{-1} at 9 DAI magnified 20-fold. The arrow indicates the swollen meristematic region of the primary root which lacks both root hairs and a secondary root.

Plate 4. Root of a seedling exposed to 15 mg 4ADNT litre^{-1} at 9 DAI magnified 20-fold. The arrow indicates the swollen meristematic region of the primary root. Root hairs are absent; however, a secondary root is present.

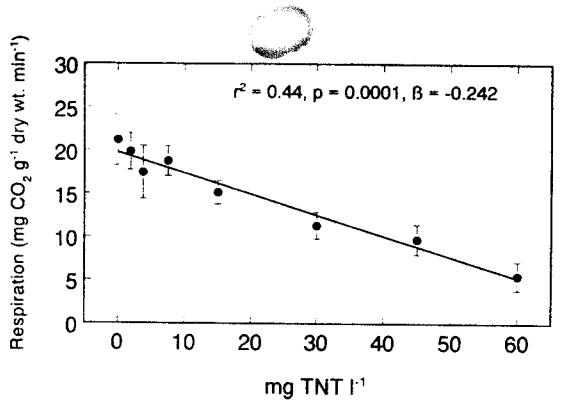

Fig. 6. Tall fescue seedling respiration rate as affected by TNT concentration at 9 d after experiment initiation. The symbol β represents the slope of the fitted regression line. Bars on symbols indicate standard error.

Although the modes of action for TNT and 4ADNT are not known, both compounds affected root and shoot growth rates. As the radicle begins to emerge from the seed, it comes into contact with the TNT or 4ADNT and may be absorbed into the cells. TNT and 4ADNT appear to affect tissue differentiation and respiration. This may be a direct effect on mitochondrial respiration or an indirect result of inhibition of cell growth and division.

Cell disruption also has been observed in plants after exposure to dinitroaniline herbicides and other mitotic disrupters, such as the herbicide dithiopyr (*S,S*-dimethyl 2-(difluoromethyl)-4-(2-methylpropyl)-6-(trifluoromethyl)-3,5-pyridinedicarbothioate) (Armbruster *et al.*, 1991). The effects of TNT and 4ADNT on root morphology appear similar to those of the dinitroaniline herbicides. Tall fescue seeds were germinated in 2.25 mg pendimethalin (*N*-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine) per litre were stunted and swollen, although root hairs developed on the primary root (concurrent study, data not shown). Meristematic cells were circular and disorganized. Respiration rates of seedlings exposed to pendimethalin were not significantly different from seedlings exposed to 60 mg TNT litre^{-1}. These observations are similar to those reported for bush bean (*Phaseolus vulgaris* L.) exposed to dinitroaniline herbicides and TNT (Harvey *et al.*, 1990).

Dinitroaniline herbicides reduce cell division, causing root tips to swell because of continued radial expansion (Parka & Soper, 1977; Strachan & Hess, 1983; Ahrens, 1994). These herbicides bind to microtubule ends and prevent polymerization which is necessary for mitotic spindle formation, determination of the cell division plane, and orientation of microfibril deposition in growing cells (Upadhyaya & Nooden, 1980; Strachan & Hess, 1983; Vaughn & Lehnen, 1991; Devine *et al.*, 1993). Normal cell division ceases and meristematic root cells become circular because of microfibril disorientation (Parka & Soper, 1977; Appleby & Valverde, 1989).

In summary, the results indicate poor growth of tall fescue seedlings when soil solution concentrations exceed 30 mg TNT litre^{-1}. Based on an adsorption isotherm conducted on a Sharpsburg silty clay loam (fine, montmorillonitic, mesic Typic Argiudoll) similar to soil at the former Nebraska Ordnance Plant site, 30 mg TNT litre^{-1} in solution is equivalent to approximately 100 mg of adsorbed TNT kg^{-1} (Comfort *et al.*, 1995). Therefore, tall fescue tolerance and, moreover, establishment may be limited to soils containing not more than 100 mg kg^{-1} of readily extractable TNT. The remediation goal set by the EPA for the Nebraska Ordnance Plant site is 17.2 mg TNT kg^{-1} soil. This goal is based on an 18 h sonication in acetonitrile, which removes available TNT from the soil (Comfort *et al.*, 1995). While most biological treatments (i.e. aerobic and anaerobic slurry treatments) can reduce extractable TNT to less than 100 mg kg^{-1}, the remediation goal of 17.2 mg TNT kg^{-1} is difficult to obtain. Consequently, a two-stage remediation treatment may be more practical in which an initial treatment process would be used to lower TNT concentration in highly contaminated soil to a range tolerated by plants. As the second stage, plants could be established to restrict further movement of TNT through the soil profile and to promote microbial degradation through the rhizosphere.

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