Nitroreductase Enzyme Mediated Phytodegradation of 2,4,6 Trinitrotoluene by Vetiver Grass

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ABSTRACT

2,4,6-Trinitrotoluene (TNT) is the most widely used explosive and a group C human-carcinogen. We are in the process of developing a phytoremediation technique to detoxify TNT using vetiver grass. In our earlier studies we found that vetiver grass, in the presence of urea used as a chaotropic agent, was highly effective in removing TNT from both soil and aqueous media. Presence of aminodinitrotoluenes in the TNT-treated-plant tissue suggested the role of nitroreductase (NR) enzyme in the metabolic pathway of TNT. The objective of the present study was to determine the saturation kinetics of the NR enzyme from vetiver grass as function of initial TNT concentrations, temperature and plant density. Saturation kinetics of NR was determined using two different approaches, using whole vetiver plants grown in different TNT containing nutrient solutions and using crude enzyme extract isolated from vetiver shoot. Results showed that the NR-activity was significantly ($p<0.001$) higher in both root and shoot tissues of the TNT-treated plants as compared to the control plants at all sampling intervals. Nitroreductase activity in the root was significantly ($p<0.0001$) enhanced by the increasing initial TNT concentrations. In contrast, while NR-activity in the shoot was significantly higher than that of the root, initial TNT treatments did not significantly influence the NR-activity. NR enzyme activity significantly ($p<0.001$) increased with increasing plant density. The NR activity increased with increasing temperature up to 35^0C. Further increase in temperature 45^0C resulted in minimal NR activity suggesting the denaturation of NR enzyme at higher temperature. The data obtained in this experiment is highly encouraging and will be used to optimize major parameters that influence the saturation kinetics of NR in vetiver grass.

INTRODUCTION

What is the importance of TNT transformation in plants?

Numerous studies have reported that different aquatic and terrestrial plants have successfully taken up TNT from hydroponic or soil medium and transformed it to similar metabolites (Hannink et al., 2002). As TNT does not contain the functional group required for conjugation, direct conjugation of TNT to plant macromolecules cannot be possible (Burken et al., 2000). Thus, as part of their detoxification mechanism plants must need to transform TNT to more hydrophilic metabolites having required functional groups for conjugation and transport, which is beneficial for phytoremediation as bound residues are presumably less bioavailable.

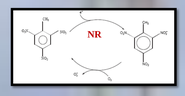
Why Nitroreductase?

The nitro group consists of two electronegative elements, Nitrogen and Oxygen. Oxygen is more electronegative than the nitrogen atom; hence, the N-O bond is polarized. The electronegative nitro groups remove the electrons from the aromatic ring of TNT, and thus the nucleus becomes electrophilic. Due to the higher electronegativity of Oxygen, partially positive charge of the nitrogen atom makes the nitro group easily reducible.

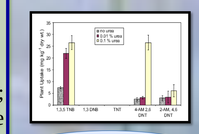
Our earlier phytoremediation studies using vetiver grass in both soil (Das et al., 2011) and water (Makris et al., 2007) systems reported presence of TNT-metabolites such as 2 amino dinitrotoluene and 4 amino dinitrotoluene in the root and shoot of vetiver grass suggesting a possible reduction of nitro group had taken place in vetiver root. This indicates a probable activity of a nitroreductase enzyme present in vetiver tissue which needs to be isolated and assayed to obtain the information on enzyme kinetics of the TNT detoxification pathway.

Need for optimizing the kinetic factors

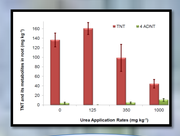
Vetiver grass has been shown to process detoxification systems resulting in TNT transformation, but precise nature of the activities of the enzymes responsible have yet to be identified. As evident from literature, three major factors influencing the saturation kinetics of TNT degrading enzymes are initial TNT load, plant density, and temperature. To design a successful plant based remedial system for TNT contaminated soil/water the factors influencing the kinetics of plant enzyme must be characterized.



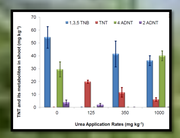
Nyanhongo et al., 2004



Makris at al, 2007



Das at al, 2011



Das at al, 2011

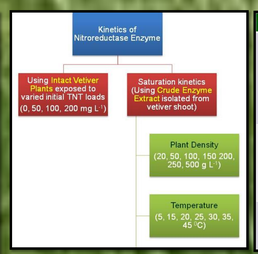
OBJECTIVES

Specific objectives of the current study were to

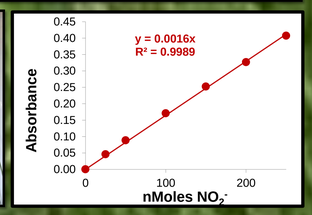
- *investigate the kinetics of NR enzyme activity in root and shoot tissues of vetiver grass as function of exposure to varied initial TNT concentrations, and*
- *determine the saturation kinetics of NR enzyme extracted from vetiver grass as functions of plant density and temperature.*

MATERIALS & METHOD

Experimental Conditions and Analyses		
Experimental Unit	Whole Vetiver plant in Nutrient Solution	Aqueous Phase Microcosm (10 mL crude enzyme extract + 10 mL Enzyme Assay + 5 mL of TNT solution)
TNT	0, 25, 50, 100, 200 mg L^{-1}	20 mg L^{-1}
Sampling Intervals	5, 10, 15, and 30 days	0,2,5,10,16,20,24, 32, 36, 40, 48 h
Temperature	25^0C	5,15,20,25,30,35,45^0C
Plant Density	50 g L^{-1}	20, 50,100,150, 200, 250, 500 g L^{-1}
Control	TNT free plant controls Plant Free TNT Amended Controls	TNT free enzyme extract Enzyme Extract with no TNT
Replicates	3	3
Analyses at each sampling interval	Residual TNT in solution (USEPA 8330), NR Enzyme activity (Harley, 1993),	
Statistical Analyses	Statistical Analyses were performed using the JMP. In version 8 (Sall et al., 2005). Q tests were performed on all data to eliminate the possible outliers at the 95% confidence intervals. Mean values were developed along with their standard deviations. A Tukey Kramer honest significant difference test was conducted to evaluate the significant differences along treatment means.	



Nitroreductase Enzyme Assay	
The extraction buffer	The extraction buffer (Nakagawa et al. 1985) consisted of 250 mM K-Phosphate buffer (pH 8) containing 1 mM phenylmethylsulfonyl fluoride, 5% isopropyl alcohol, 1 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM KNO_3, 20µM FAD and 126g of polyclar AT.
The Enzyme Assay	An assay buffer (Nakagawa et al. 1985) consisted of 25 mM potassium phosphate buffer (pH 7.5), and 100 µM NADH. Modification – No nitrate was added allowing TNT to be used as the only source of nitrate.
Analysis	Samples were analyzed in a microplate reader at 540 nM following the method described by Nakagawa et al. (1985) and Harley (1993).



CONCLUSIONS

- Nitroreductase, the major TNT degrading enzyme, was isolated from vetiver grass and characterized as functions of three initial factors such as initial TNT loads, plant density, and temperature.
- Nitrate reductase enzyme activity in both root and shoot tissues of vetiver grass significantly ($p<0.0001$) increased following varying levels of TNT exposures, suggesting a role for nitroreductase enzyme in TNT degradation in vetiver grass. Higher NR activity in the shoot suggests more TNT-degradation potential of shoot than that of root tissues of vetiver grass.
- NR activity increased with increasing plant density. The optimum temperatures for obtaining maximum NR activity lie between 30 to 35^0C.
- The results of the current study is highly encouraging and will help optimize the factors influencing phytodegradation of TNT and designing a successful plant based remediation system for TNT contaminated soil/water using vetiver grass.

References

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RESULTS & DISCUSSION

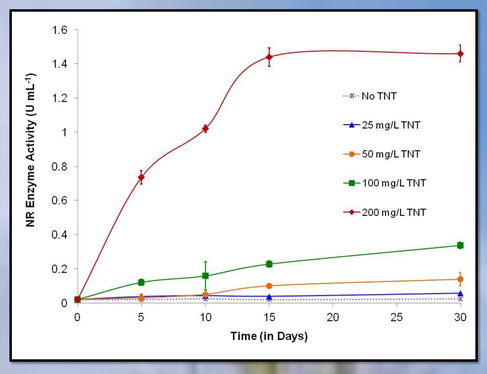


Figure 1. Kinetics of Nitroreductase enzyme activity in the root of vetiver grass following exposures to various concentrations of TNT. NR enzyme activity is expressed in U mL^{-1}. Data are expressed as mean (n=3) and one standard deviation.

- Kinetics of NR enzyme activity followed first order reactions in lower TNT concentrations (25, 50, and 100 mg L-1; R^2 = 0.98, 0.84, 0.94 respectively).
- Whereas, The increase in the enzyme activity at 200 ppm initial TNT concentration followed a second order reaction (R^2 = 0.92). The second order reaction rate constant was calculated using the slope ($(n-1)*k_n*A_0$) of the second order fit. The second order reaction rate constant (k_s) is found as 1.51 $U^{-1}mL\ h^{-1}$.

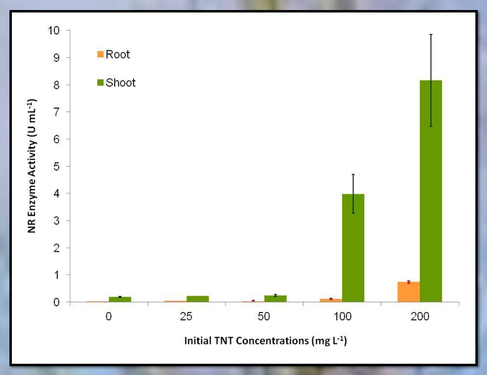


Figure 2. Relative NR activity in the root and shoot tissues of vetiver grass. Data are expressed as mean (n=3) and one standard deviation.

- NR activity is higher in shoot than that of root in all TNT treatments, suggesting more transformation of TNT in the shoot tissues of vetiver grass. This supports our current (data not shown) and previously reported results showing more TNT metabolites in shoot as compared to the root.

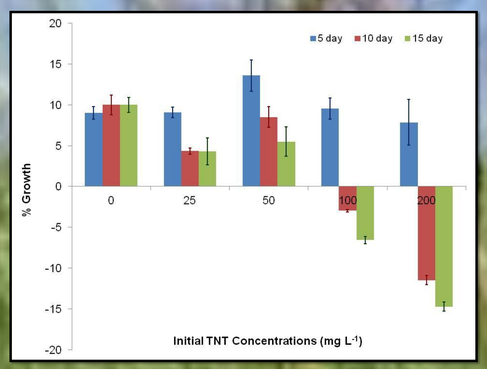
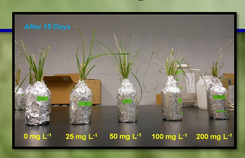


Figure 3. % Growth of vetiver grass following varying TNT exposures. Negative values express the reduction in biomass. Data are expressed as mean (n=3) and one standard deviation.

- Increasing concentrations of TNT showed negative effect on growth of vetiver grass.
- At lower concentrations (25 and 50 mg L^{-1}), no significant effect on growth was observed.
- In higher initial TNT loads (100 and 200 mg L^{-1}), biomass reduction was noted after 10 days.



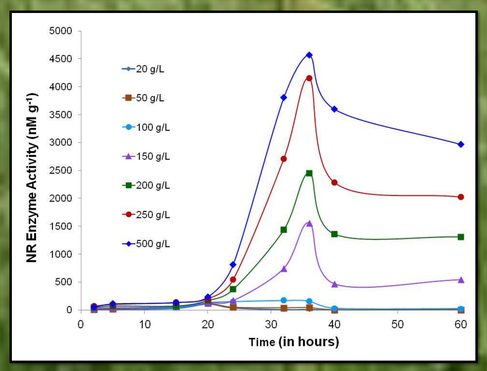
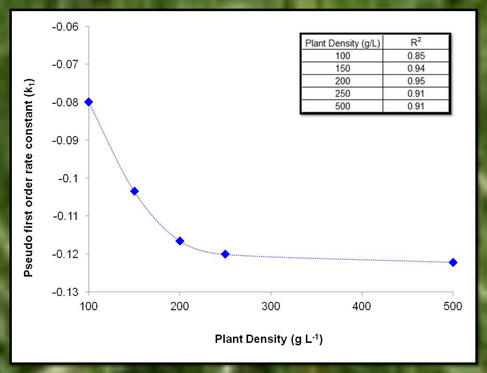


Figure 4. Saturation kinetics of NR (crude enzyme extract) as function of plant density, at constant temperature (30^0C) and TNT load (20 mg L^{-1}). Data are expressed as mean (n=3).

- NR enzyme activity significantly ($p<0.0001$) increased with increasing plant density.
- Initial kinetics were slow in all plant density, resulting minimal enzyme activity until 20 hrs.
- Optimum NR activity was observed between 20 to 40 hours in all densities tested.



Plant Density (g/L)	R^2
100	0.85
150	0.94
200	0.95
250	0.91
500	0.91

Figure 5. Pseudo first order rate constant (k_1) of NR enzyme activity as function of plant density (at constant temperature and TNT load).

- Kinetics of NR enzyme activity followed first order reactions at higher plant density (100,150, 200, 250, and 500 g L^{-1})
- Pseudo first order reaction rate constants (k_1) decreased with increasing plant density.

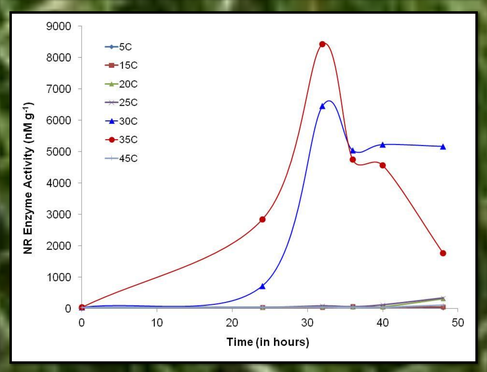


Figure 6. Saturation kinetics of NR (crude enzyme extract) as function of temperature, at constant plant density (250 g L^{-1}) and TNT load (20 mg L^{-1}). Data are expressed as mean (n=3).

- Temperature showed significant effects ($p<0.001$) on the kinetics of NR enzyme activity.
- NR activity increased with increasing temperatures up to 35^0C. Further increase in temperature denatured the enzyme, resulting in minimal activity at 45^0C.
- The optimum range of temperature is between 30 to 35^0C. Most consistent NR activity was observed at 30^0C.