



Uptake and metabolism of four isomers of dinitrotoluene in *A.*
thaliana and their sequestration in feeding aphids: A
phytoremediation perspective.

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Abstract

Plants, being sessile, have wide variety of metabolic pathways to detoxify hazardous compounds therefore ensuring the success of phytoremediation of a wide variety of xenobiotics. One such a compound is dinitrotoluene (DNT) that is available as a mixture of six different isomers. Phytoremediation of DNTs is a cost effective, eco friendly and preferable approach compared to commercially available chemical and physical remediation systems. DNTs are toxic to plants at higher concentrations, however lower concentrations are tolerable. The present study considers the uptake of DNTs in the model plant *Arabidopsis thaliana* to observe the potential of phytoremediation and considers the metabolites during different phases of detoxification. Further it evaluates the possible propagation of DNT and its metabolites in ecosystem through a model system of plant-aphid interaction.

The first step in the study included establishment of the toxicity of four different available isomers of DNT (2,6-DNT, 2,3-DNT, 3,4-DNT and 2,4-DNT) in *A. thaliana*. This was achieved by root growth analysis in Phytagel (axenic) and hydroponic media (non axenic), root and shoot ratio in Phytagel and hydroponic media and finally a time course reduction in root length in hydroponic media. The results obtained from the study showed that all isomers of DNT have different toxicities (EC_{50} for 2,6-DNT, 2,4-DNT, 3,4-DNT and 2,3-DNT was 13.3 mg /L, 0.33 mg /L, 0.23 mg /L and 0.13 mg /L of Phytagel media). At lower concentrations these DNTs were tolerable but at higher concentration they induced biomass inhibition.

The uptake and metabolism of different DNT isomers was analysed by GC-MS in *A. thaliana* cultivated in Phytagel and hydroponic media amended with different concentrations of DNT isomers. The analysis of metabolites showed that the major metabolites of DNT were aminonitrotoluenes (ANTs), the concentration of which varied with the concentration of DNTs in the media (ranged from 0.54-5.2 μ g /g dry tissue wt. of *A. thaliana* for 2A6NT; 20.03 to 280.3 ng /g dry tissue wt. of *A. thaliana* for 2A4NT and 11.34 to 508.39 ng /g dry tissue wt. of *A. thaliana* for 4A2NT; 32.8 to 675.22 ng/g dry tissue wt. of *A. thaliana* for 2A3NT; 41.23 to 564.32 ng /g dry tissue wt. of *A. thaliana* for 3A4NT).

The qualitative and quantitative analysis of possible DNT based conjugates was carried out by LC-MS/MS analysis for *A. thaliana* cultivated in liquid MS media (axenic). The quantitative analysis of DNT based conjugates indicated ten significant conjugates with higher concentrations of *O*- and *C*- glucosides, mostly with hydroxylaminonitrotoluene. The abundant metabolites of DNT (aminonitrotoluene) have been observed to have lesser conjugation. The concentration of DNT based conjugates were observed to be indirectly related to the concentration of metabolites (increase with the decrease in the concentration of metabolites) and their concentrations were 10^2 times higher than aminonitrotoluenes. The concentration of conjugates varied with the type of isomer in the media.

The effect of DNT treatment to herbivore feeders was evaluated through analysis of survival, fecundity and growth of *Brevicoryne brassicae* (specialist) and *Myzus persicae* (generalist) aphids on DNT treated *A. thaliana* at different concentrations. The DNT based metabolites

were evaluated in the feeding aphid species to observe the possible propagation. Finally, the aphids were observed to tolerate lower concentrations of DNT treatments and their survival, fecundity and growth was highly compromised with high concentrations of DNT in the medium with *B. brassicae* showed higher sensitivity than *M. persicae* (in *M. persicae* LC₅₀ for 2,6-DNT, 2,4-DNT, 3,4-DNT and 2,3-DNT was 13.9 mg /L , 0.34 mg /L , 0.138 mg /L and 0.14 mg /L of Phytigel media and in *B. brassicae* LC₅₀ for 2,6-DNT , 2,4-DNT, 3,4-DNT and 2,3-DNT was 11.48 mg /L , 0.24 mg /L , 0.1 mg /L and 0.08 mg /L of Phytigel media). Both aminonitrotoluenes and glucosyl conjugates were present in both aphid species and their concentrations were directly related with the concentration of metabolites in the plants on which they were reared. GC-MS analysis showed that *B. brassicae* managed to accumulate more metabolites than *M. persicae*. LC-MS analysis showed that the type of conjugates varied with the type of isomer. Therefore this study shows that if Phytoremediation of DNTs is considered then plants convert the toxic DNTs to their polar (less toxic) reduced metabolites and glucosides which could potentially be transported to the feeding aphids.

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Chapter 1

Introduction and Literature review

1.1 General Introduction

Dinitrotoluenes (DNTs) are introduced into the environment through several anthropogenic activities. Large quantities of DNTs (global production is 1.6 million tons/ annum) (SIDS initial assessment profile, 2003) are produced as by-product of TNT manufacture (used as TNT precursor in the process of nitration) and degradation (Thorn et al., 2007, Rocheleau et al., 2010), toluene diisocyanate production in the manufacture of polyurethane foams, automobile air bags and certain dyes (Yoon et al., 2007; Yang et al., 2008; Dokken and Davis, 2011; Lent et al., 2012). There are no recent figures available for the production of DNTs through industrial processes; however, in the US alone production reached 124 Gkg in 1975. This release decreased by 1990 after environmental concerns but increased in 1999-2001 (EPA, Regulatory Determination Support Document for CCL 2, 2008). In the US alone, 0.8 million cubic metres of soil is contaminated with DNT as a by product of TNT (Rylott et al., 2011).

There is increasing concern regarding DNTs and environmental contamination and their potential bioaccumulation in the food chain with associated health risks (designated class-B carcinogen by EPA, 1999 Alaska Air toxics Emission Inventory and influence fertility on acute and moderate exposure) (Dokken and Davis 2011; Lent et al., 2012). This has led to extensive research to remediate these pollutants (SIDS initial assessment profile, 2009). Current remediation strategies including incineration (HSDB 1998), alkaline hydrolysis (Emmrich, 2001; Hill et al., 2010) and bioremediation (Nishino et al., 2000; Sangsoo et al., 2011; Hudcova et al., 2011) are expensive and could potentially alter the local beneficial

microbial population of the area (Zhu et al., 2001). Here phytoremediation, being affordable, inexpensive and highly applicable has been intensively researched to remediate the DNTs from the environmental resources (Riefler and Medina, 2006; Yoon et al., 2007; Tognetti et al., 2007; Dokken and Davis 2011).

Like other xenobiotics, DNTs follow common degradation, metabolism and transformation pathways within the plant via enzymatic reactions (Dosnon-Olette, 2011; Tognetti et al., 2007). As soon as DNTs gain entry into plant body they undergo Phase I, Phase II and Phase III reaction of metabolism (Yoon et al., 2007; Subramanian et al., 2006). These reactions occur as a result of the plant's effort to reduce the toxicity of DNT. Like TNT, the DNT aromatic ring is electron deficient due to the electron with-drawing nature of nitro- groups and undergoes reduction during Phase I of DNT metabolism (Dokken and Davis, 2011). The reduced DNT (aminotoluenes) then undergoes conjugation to glucose, glutathione or malonate (Hannink et al., 2002; Nepovim et al., 2004). This results in Phase II conjugation step. The polarity of DNTs is increased gradually from Phase I to Phase II reactions, so that they can be transported more efficiently and they compartmentalized in the vacuole during Phase III step (Darwish et al., 2010). The Phase I and II modification is necessary to limit the availability of DNT within plants, so that they are no longer toxic (Coleman et al., 1997). It has been shown to depend upon the functional group attached to the DNT metabolite which signals for compartmentalization of the resulting conjugated compound into the vacuole or it binds to biopolymers in plant cell wall (Hannink et al., 2002).

The potential remediation of DNTs depends upon the tolerance of the plant to abiotic stress (Sun et al., 2011). Therefore it is necessary to measure the optimum concentration of DNT that plants can remediate effectively (Pavlostathis et al., 1998). This determinant, however, has been observed to vary with the position of nitro group attached to the benzene aromatic ring (Nishino et al., 1999; Chua and Pumera, 2011). Therefore, all the positional isomers of

DNTs have different effects on the plants tolerance (Yoon et al., 2007; Esteve-Nunez et al., 2001; Chua and Pumera, 2011). The position of nitro group determines the electron distribution in the aromatic ring therefore exposing certain sites for relatively easy transformation via nitroreductases (Tognetti et al., 2007). This is a major factor that results in the variation of toxicities of different isomers of the same compound (Chen et al., 2007).

The transformation products of DNTs were investigated due to the concern over their bioaccumulation in the plant and the food chain (Rocheleau et al., 2010). It is thus necessary to determine the endpoints of phytoremediation that are environmentally safe so that they do not have a negative impact on the biotic health of the ecosystem (Cojocar and Macoveanu, 2011).

Studies have revealed the bioavailability of xenobiotics in plants (Jeong et al., 2012; Alsayed et al., 2008). This fact may concern the bioavailability of DNTs in the food chain and therefore aphids feeding on them. The exposure of aphids to the plant contaminants also vary with their diet. The specialists have less choice to avoid contamination if a particular crop species becomes host for xenobiotics, however, generalists can avoid contaminant exposure by foraging on various other non-contaminated plant species (Torregrossa et al., 2011).

To understand the phytoremediation of DNTs it is necessary to understand the whole phenomenon and the processes involved. Following details, therefore, provide an overview of phytoremediation and its applications.

1.2 Phytoremediation:

The elevated levels of anthropogenic activities have led to the introduction of toxic and persistent compounds into the environmental resources. The common prevalent contaminants

include polychlorinated hydrocarbons (PCBs) (Ueda et al., 2005; Anyasi and Atagana, 2011), halogenated hydrocarbons (Megharaj et al., 2011), polycyclic aromatic hydrocarbons (PAHs), hazardous chloroaromatics (Antizar-ladislao and Galil, 2010) pesticides, metals and several salts (Donner et al., 2012; Kubiak et al., 2012; Zheng et al., 2012). Phytoremediation as the term suggests and encompasses techniques that utilize plants and associated microorganisms to remediate contaminated matrices such as water, soil, aquifers, and tailings through absorption, transformation and containment (Seth, 2012; El Mehdawi and Pilon-Smits, 2012). Although, the use of plants for removal of contaminants from the soil was first suggested in 1948 when an Italian researcher reported nickel hyperaccumulation in the Italian serpentine plant *Alyssum bertolonii* and then in 1950s the plants were used to remove radionuclide from the contaminated soils but the term remediation was not used until the 1980s (Massa et al., 2010). The rapid expansion in phytoremediation and phytotechnologies only began in the last few decades (Mench et al., 2010). Solar energy has driven this natural option and has several advantages over the commercially available chemical and physical remediation techniques. It is publicly acceptable, utilizes low cost and setup expenses, prevents soil erosion and therefore keeps the contaminants confined. Groundwater pollutants are effectively and permanently remediated without compromising water quality (Dundek et al., 2011).

Several green house trials are carried out before large scale phytoremediation of the compounds. These trials provided an insight into phytoremediation and have shown the processes are an accumulation of several sub-processes. Phytoremediation is a generic term of two vast phenomena: phytoremediation and rhizoremediation (Massa et al., 2010; Dundek et al., 2011). The rhizoremediation as the name indicates is the degradation of contaminants in the rhizosphere area through synergistic interaction of plant based microbes (fungi and

bacteria) and phytoremediation is the plant based in situ remediation of contaminants from the contaminated matrices.

Rhizoremediation involves roots, root exudates, microorganisms in the rhizosphere zone (Dundek et al., 2011). This remediation is very beneficial in the degradation of certain organic compounds (PAHs) which cannot be easily absorbed by the plants (Slater et al., 2011). The microbes associated with the plants include mycorrhizal fungi, endophytic bacteria and root colonising microbes. Ectomycorrhizal and endomycorrhizal strains of fungi and microbe play an important role in promoting nutrient and stress tolerance in plants through higher nutrient intake and water interception (Sun et al., 2011; Slater et al., 2011). Additionally they help in providing certain hormones, buffering contaminants accumulation and increase the sorption of trace elements through the production of glycoproteins. Several activities of the associated microbes include the production of O-antigen of lipopolysaccharide, thiamine and biotin synthesis and amino acids synthesis (Garg and Chandel, 2010). Among the beneficial ectomycorrhizal fungi include *Paxillus involutus*, *Populus nigra*, *Hebeloma crustuliniforme*, *Salix viminalis* and *Hebeloma salix* arbuscular micorrhizal fungi (such as *Glomus intradices* and *Glomus claroideum*) enhance the Zn uptake in *Solanum nigrum* (Marques et al., 2007). Endophytic bacteria have a symbiotic relation with the plants in which they take shelter and obtained food. In return they fix atmospheric nitrogen and assist in atmospheric nitrogen fixation. Excessive ethylene production can limit the plant growth, here the plant growth promoting rhizobacteria and endophytic bacteria metabolize 1-aminocyclopropane-1-carboxylate (Divya and Kumar, 2011).

Once the contaminants are degraded through rhizoremediation, they are absorbed by the roots and enter the xylem pathway of the vascular tissues (Dokken and Davis, 2011). Plants break down the organic pollutants or stabilize the inorganic metal contaminants. Ions from the

solution enters the cortex into the apoplast and then to symplast. Once inside, a contaminate can be stored in the roots, stems and leaves. This selectivity is based on the nature of the contaminant. It is then converted to less harmful products and sometimes released into air through transpiration (Edwards et al., 2011). Several plant based enzymes help in degradation and sequestration of the absorbed contaminants in the plant. Phytoremediation over a two year period decreased the polyhalogenated compounds (PHC) concentration by 42% using *Lolium annual* and 50% decrease of PHC was observed after 21 months by using *Stenotaphrum secundatum* compared to non-vegetated area. *Festuca arundinacea* enhanced the biodegradation of polyaromatic hydrocarbons, fluorine, pyrene, fluoranthene and chrysene in a contaminated aged creosote soil relative to the non-vegetated soil. In a trial of 60 days, about 90% of trinitrotoluene was removed by *Zea mays* (Sun et al., 2011).

Each part of the plant along with the detoxification enzymes involved play a role in Phytoremediation. In broader terms Phytoremediation and Rhizoremediation includes phytoaccumulation, phytodegradation, phytostabilization, phytovolatilization, rhizodegradation and rhizofiltration (Table 1.1).

Table 1.1: Different aspects of phytoremediation

Phytoremediation process	Mechanism	Reference(s)
Phytoaccumulation	Uptake and translocation of compounds from contaminated matrix via roots to shoots	Laurette et al., 2012
Phytodegradation	Transformation or breakdown of absorbed contaminants in plants (shoots)	Kagalkar et al., 2011

	or foliar)	
Phytostabilisation	Immobilization of contaminants through absorption, adsorption and accumulation in the roots and root zone. The mobility of contaminant is usually limited.	Moreno-Jimenez et al., 2012
Phytovolatilization	Release or transpiration of absorbed and modified contaminants through leaves. Usually volatile compounds undergo this release.	Edwards et al., 2011
Rhizodegradation (also called phytostimulation)	Plant-assisted microbial degradation of the contaminants. The microbes in the rhizosphere area of roots degrade the contaminants and plant assist by loosening and aerating the soil.	Lu et al., 2011
Rhizofiltration	Adsorption or precipitation of contaminants on the roots. They segregate all the contaminants from the water.	Mahmood, 2010

The nature of contaminants to be remediated is very important in determining the dominance of one process over the other. For example, volatile organic compounds with low molecular weight can be extracted from the contaminated matrix (Phytoextraction) and transported

across the plant cell membranes, degraded (Phytodegradation) and either get stored in the plants (Phytoaccumulation) or transported out through leaves by evapo-transpiration (phytovolatilisation) (Kagalkar et al., 2011; Dixit et al., 2011). The non volatile organic and inorganic compounds either precipitate out of roots and just accumulate on the root surface (rhizofiltration) or get transformed by plant based microbes (bacteria or fungi) (rhizodegradation). On entering the plant body (through phytoextraction) these compounds are degraded within the plant cell (phytodegradation) and are rendered non-toxic through plant based enzymatic activities. The stable compounds are usually are retained within the plant biomass (phytoaccumulation) and could only be remediated through incineration (Table 1.2).

1.2.1 Factors affecting Phytoremediation:

Most of the time plants are exposed to pollutants via absorption by roots and foliage and the main reported pathways of entrance are through aerosol deposition on canopies, uptake through the root cells and molecular diffusion through cuticular membrane. In the case of phytoremediation of contaminated matrices, the efficiency varies with the nature of the contaminated matrix. Here there are several factors which influence their bioavailability (labile pools) (Mzoughi and Chouba, 2012; Zhang et al., 2012). The contaminants are subject to ion exchange, redox processes and bonding factors and are under the influence of temperature, water, biota and carbon (C) contents of the soil. These processes divide the contaminants into bound (not available) and dissolved portion (bioavailable) of the contaminants (Ramdine et al., 2012). Usually the two states (mobile and bound) are in equilibrium with one another, however, the variations in the quantities are highly likely due to pH, hydrology, weathering, mineral contents, oxygen, organic matter quantity resulting in oxidation-reduction, adsorption-desorption and complexation-dissociation.

Table 1.2: Studied aspects of Phytoremediation in different plants.

Application	Pollutant	Medium	Plant	Reference(s)
Phytoextraction/ Rhizodegradation	Petroleum and hydrocarbons	Soil and groundwater	Alfalfa, poplar, juniper, fescue	Hamdi et al., 2012
Phytostabilisation	Heavy metals	Soil	Hybrid poplar, grasses	Sekabira et al., 2011; Moreno-Jimenez et al., 2012
Rhizofiltration	Radionuclides	Groundwater	Sunflowers	Lee and Yang, 2010
Phytodegradation	Radionuclides	Soil	Indian mustard, cabbage	Fuhrmann et al., 2003
Phytodegradation	Explosive waste	Groundwater	Duckweed, parrot feather, <i>Arabidopsis thaliana</i>	Perez-de-Mora et al., 2011; Hannink et al., 2001
Phytodegradation	Nitrates	Groundwater	Hybride poplar	Dixit et al., 2011

The most important part in the contamination is provided by the mobile and biologically active forms of the contaminants as it provides the toxicological stand points and ecological status of the contaminants. Another factor to influence is the contaminants location (Marmiroli et al., 2011). The efficiency of Phytoremediation is highly dependent of the nature of contaminant. Organic and inorganic compounds following different mechanisms of phytoremediation and are found in different compartments in plants (Figure 1.1) (Edwards et al., 2011).

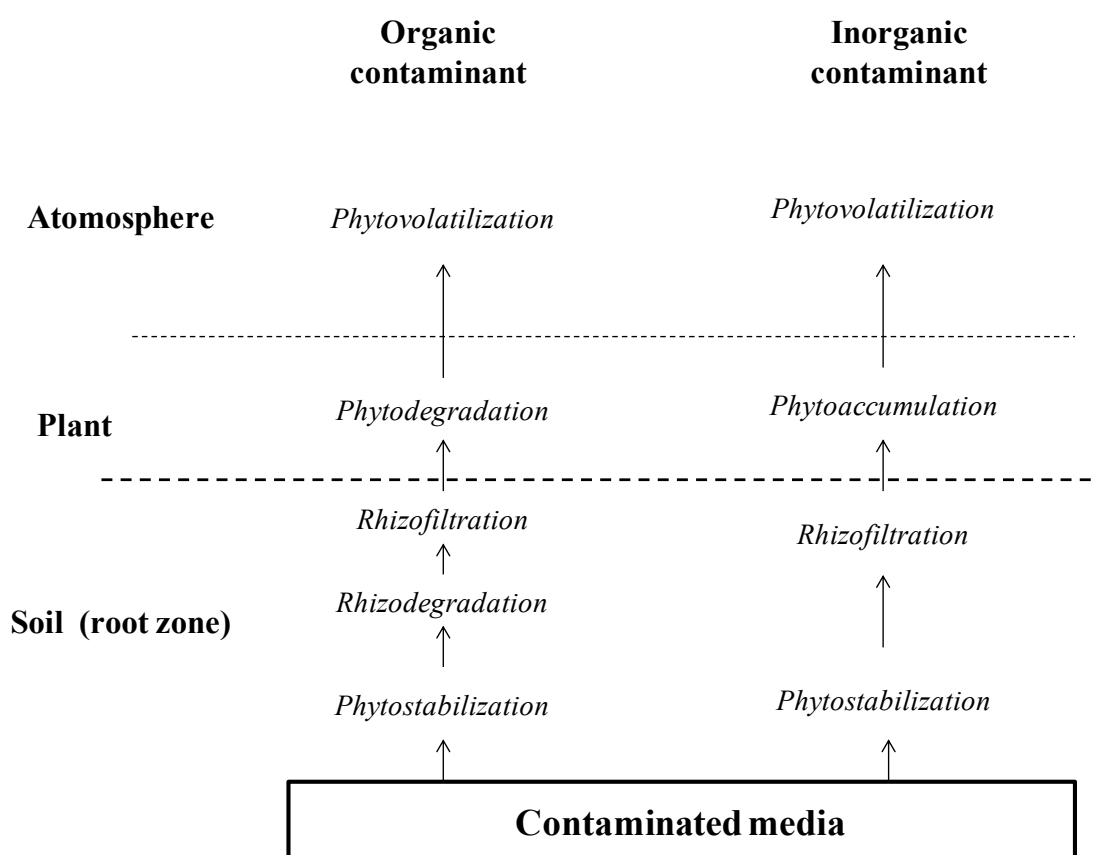


Figure 1.1: Different degradation processes of organic and inorganic compounds.

1.2.2 Phytoremediation of inorganic contaminants

Plants act like vacuum cleaners for heavy metal pollutants in the contaminated matrix. They hyperaccumulate metals and fix them within leaf cells. However, the efficiency of phytoremediation highly varies with the plant species and type of metal (Zhang et al., 2012). *Thlaspi spp.*, a weedy member of cabbage and broccoli family has been shown to be very effective for phytoremediation of zinc and cadmium since it can tolerate very high levels (30,000 ppm zinc and 1,500 ppm cadmium) of these metals compared to the other plant species (toxic at 1,000 ppm of zinc and 20-50 ppm of cadmium) (Drava et al., 2012). The hyperaccumulation of metals in plants is dependent on the genes which contribute towards the hyperaccumulation trait. The proteins that transport the metals from the roots to plant cells are under the control of these genes. Every metal follows a different root of entry in plants (Table 1.3). Translocation of contaminants from roots to shoot occurred only for cadmium when *Brassica juncea* was allowed to phytoremediate both cadmium and lead under similar soil conditions (Vatehova et al., 2012).

The mobility of metals can be improved through the additive chemicals (surfactants, chelating agents, organic acids and co-solvents) in the media. The bioavailability of uranium has been shown to be enhanced by the addition of citric acid. Surfactants have also been reported to enhance desorption of certain metals from the soil (Chen et al., 2011). Non-ionic surfactants Triton X-100 and Tween 80 have been reported to enhance the accumulation of cadmium and lead from engine oil contaminated sandy soil by *Brassica juncea* (Zhang et al., 2011).

Table 1.3: Some examples of Phytoremediation of inorganic contaminants.

Plants	Inorganic /radionuclide contaminant	Mechanism	References
<i>Salvia splendens, Tagetes patula, Impatiens walleriana, Verbena bipinnatifida</i>	Cadmium	Suitable plants can be used to remove heavy metal contaminants from soil	Vatehova et al., 2012
<i>Populus spp.</i>	Mercury, Selenium	Extraction through roots to remove the contaminants	Raskin et al., 2000
<i>Eichhornia spp., Salvinia spp.</i>	Copper	Addition of Ethylenediaminetetraacetic acid (EDTA) enhances the capacity of plant to absorb copper	Dave et al., 2010
<i>Eichhornia spp., Salvinia sp., Pistia spp., Azolla spp., Lemna spp.</i>	Lead	By using EDTA the capacity of plant to take up metals can be increased but pathway of uptake remains same	Dipu et al., 2012
<i>Eleusine indica</i>	Copper, Cadmium, Cobalt, Lead	By using EDTA the capacity of plant to take up metals can be increased	Manab et al., 2008
<i>Vetiver grass</i>	Copper, Cadmium, Lead, Selenium, Nickel, mercury, Zinc		Danh et al., 2011
<i>A. thaliana</i>	mercury	Mercury was removed by changing it to volatile form	Park et al., 2012

<i>Nicotiana tabacum</i>	Cadmium	Metallothionein assisted in accumulation of metal in the plant roots	Krystofova et al 2012
<i>Nicotiana tabacum</i>	Mercury	Transgenic tobacco plant was found to be more efficient in removal of mercury	Ruiz et al., 2011
<i>Lespedeza chinensis</i> , <i>Lespedeza davidii</i>	Lead	Lead is transported through apoplastic and symplastic pathways independent of EDTA presence	Zheng et al., 2012
<i>Chara globularis</i> , <i>Hydrodictyon reticulatum</i>	Lead	Both species effectively phyto remediate Lead at concentration less than 40 mg /L which normally exists in most of the polluted water bodies.	Gao and Yan, 2012
<i>Brassica napus</i>	Uranium	Direct correlation of uranium speciation in the environment and its uptake	Laurette et al., 2012
<i>Prunus persica</i> (peach), <i>Pyrus spp.</i> (pear)	Selenium	Higher selenium accumulation occur in leaves and fruits.	Pezzarossa et al., 2012
<i>Atriplex hortensis</i> var. <i>purpurea</i> , <i>Atriplex hortensis</i> var. <i>rubra</i> and <i>Atriplex rosea</i>	Copper, Lead, Nickel, Zinc	Phytostabilization was mostly seen among the plants	Kachout et al., 2012

1.2.3 Phytoremediation of organic contaminants:

Organic pollutants have raised concerns because of their potential interactions with the biological processes of plants and animals. The most important class are the persistent organic pollutants (POPs) of the potential for biomagnification in the food chain. Unlike inorganic pollutants they can be metabolised by the living organisms (Zhu et al., 2012; Longhua et al., 2012). Through the multifarious activities of plants organic pollutants can be immobilized, volatilized, transformed and stored in the cells. Like inorganic pollutants, the phytoremediation of organo-chemicals varies with the plant species, pollutant type and root system (Table 1.4). Poplar (*Populus spp.*) or willow (*Salix spp.*) are extensively used for remediation of several organic pollutants due to their high transpiration rates and extensive root spread. Biodegradation of PAHs has been shown to be enhanced through the assistance of non-ionic surfactants (Kim et al., 2001).

Table 1.4: Some examples of Phytoremediation of organic contaminants.

Plant	Organic contaminants	Mechanism	Reference
<i>Arabidopsis thaliana</i>	Trichloroethylene	1020 transcript were observed to be up-regulated to contribute in transformation, conjugation and compartmentalization of compound in plants	Zhu et al., 2012
<i>Arabidopsis thaliana</i>	Dinitrotoluenes (DNT)	Phytotoxicity and phytoremediation of 2,6-DNT and 2,4-DNT	Yoon et al., 2006 and 2007
<i>Salix spp.</i>	Organochlorine pesticides (DDT /DDE)	Addition of carboxylic acids enhance the translocation of compounds in plants	Mitton et al., 2012
<i>Sedum</i>	Polychlorinated bi phenyls	Intercropped with <i>M.</i>	Wu et al., 2012

<i>plumbizincicola</i>		<i>sativa</i> promoted 25.2% of soil PCB degradation	
<i>Cucurbita pepo</i>	Persistent Organic pollutants	Bioconcentration factors were calculated for 20 polychlorinated dibenzo-p-dioxins/dibenzofurans and 14 polyhalogenated biphenyls	Bordas et al., 2011
<i>Nicotiana tobacum</i>	Anthracene	Transgenic plants degraded anthracene to naphthalene derivatives where wild type could not process	Dixit et al., 2011
Wheat, maize	Phenanthrene, anthracene	In roots and branches polychlorinated biphenyl (PCB) was degraded to anthrone, hydroxyanthraquinone and anthraquinone	Du et al., 2011
<i>Cucurbita pepo ssp</i>	polychlorinated biphenyl (PCB)	Number of chlorines in molecules effected the movement of PCB in plant	Greenwood et al., 2011
<i>Triticum aestivum</i>	Phenanthrene	Root cell walls provide better partitioning of phenanthrene	Li et al., 2012
<i>Hypnum cupressiforme</i>	Phenanthrene	The morphological and physiological features of plant control transport and storage mechanism of phenanthrene	Keyte et al., 2009
<i>Spathiphyllum clevelandii</i> , <i>Epipremnum aureum</i>	Aldehydes, Ketones	C3-C6 aldehydes and C4-C6 ketones were taken up but C3 ketone was not taken up. These are	Tani et l., 2009

		metabolized in leaf and translocated through petiole	
<i>Q. myrsinaefolia</i>	Methacrolein, Methyl Vinyl Ketone	Methacrolein uptake rate is more than methyl vinyl ketone	Tani et al., 2010
<i>Panicum virgatum</i>	Phenanthrene	After hydrolyzation the capacity of root tissues for phenanthrene increased.	Chen et al., 2009
<i>Canada goldenrod</i>	polychlorinated biphenyl	Study revealed that PCB is also accumulated along stem length and plant and shoot accumulation increases with time	Ficko et al., 2011
<i>Leucaena leucocephala</i>	Ethylene dibromide, trichloroethylene	Tropical trees also have capacity to remove organic pollutants	Doty et al., 2003
<i>Oryza sativa</i>	Pentachlorophenol	The phytoremediation of PCP by rhizosphere in wetland and rainfed have different results.	Hayat et al., 2011
Potatoes	Polyphenol oxidase (PPO)	Raw potato can degrade pentachlorophenol by dechlorination and ring-opening	Hou et al., 2011
<i>Kandelia candel</i> (L.) Druce	Phenanthrene	Direct uptake of phenanthrene was slow but it was accelerated by introducing mangrove rhizosphere	Lu et al., 2011
<i>Pteris vittata</i> L	Phenanthrene	<i>Pteris vittata</i> L removed both phenanthrene and arsenic same time	Sun et al., 2011

<i>Lactuca sativa</i> L, <i>Spathiphyllum</i> spp	triclosan (TCS), hydrocinnamic acid (HCA), tonalide (TON), ibuprofen (IBF), naproxen (NPX), and clofibric acid (CFA)	Compounds having carboxylic groups were taken up more efficiently than others	Calderón- Preciado et al., 2012
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Like other organic compounds, the phytoremediation of DNTs is preferred over available incineration and chemical remediation methods (Dokken and Davis, 2011). The present study focuses on the phytoextraction and phytoremediation of DNTs in a model plant *A. thaliana*. The sequenced genome and enhanced tolerance has made *A. thaliana* a preferable choice in toxicity studies.

To understand the phytoremediation of DNTs it is important to understand the chemical and physical properties of these compounds and various other available remediation options. This would help in understanding the underlying principle mechanism of degradation.

1.3 Chemical and physical properties of DNT

DNTs (Figure 1.2) are orange-yellow semi-volatile compounds. Technical grade DNT is a mixture of six isomers with approximate proportions of 76.5% of 2,4-DNT, 18.8% of 2,6-DNT, 4.7% of minor isomers (1.54% of 2,3-DNT, 0.69% 2,5-DNT, 0.04% 3,5-DNT and 2.43% 3,4-DNT) (Burrows et al., 1989; Bayer, 2003; Lent et al., 2012).

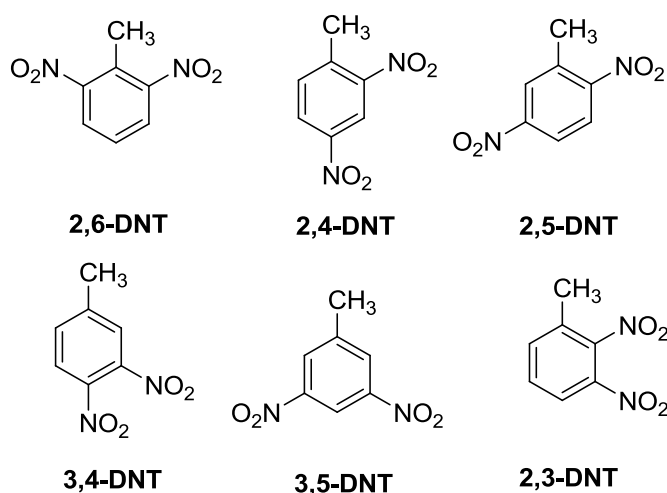


Figure 1.2: Different isomers of DNT. 2,6-dinitrotoluene (2,6-DNT), 2,4-dinitrotoluene (2,4-DNT), 2,3-dinitrotoluene (2,3-DNT), 3,4-dinitrotoluene (3,4-DNT) and 2,5-dinitrotoluene (2,5-DNT).

All of the isomers have varied physicochemical properties (Table 1.5) that determine their interactions with environmental components (Kawai et al., 1987).

Table 1.5: Chemical and physical properties of four isomers of DNT.

Properties	2,4-DNT	2,6-DNT	2,3-DNT	3,4-DNT
Boiling Point	300 °C	285 °C	250 °C	351
Melting point	71 °C	66 °C	60 °C	54-57 °C
Molecular weight	182.14 g /mol	182.14 g /mol	182.14 g /mol	182.14 g /mol
Log Koc	2.45	2.31	NA	2.2
Log Kow	1.98	2.10	2.0	NA
Water solubility	270 mg /L at 22 °C	180 mg /L at 22 °C	260 mg /L at 22 °C	Insoluble in water (values are not available)

*NA = not any

**Log Kow = octanol water partition coefficient.

***Log Koc = distribution coefficient normalized to the organic carbon content of a sorbent.

(Data taken from-Wolfram alpha (archieved on 2012), Sigma Aldrich, UK; Booth, 2003; Bayer 2003; Hansch and Hoekman, 1995 and Budavari, 1996).

1.3.1 Sources and introduction of DNTs in the environment

DNTs do not occur naturally, but are synthesized through the reaction of toluene with a mixture of nitric and sulfuric acids (Booth, 2003). Large amounts of DNTs are produced industrially to be used for further processes. The most important is the use of DNTs for toluene diisocyanate manufacture for polyurethane foam (Table 1.6).

Table 1.6: Total amount of DNT produced globally (SRI, 2002; SIDS Initial Assessment Profile, 2003).

Region	TDI capacities (1000 t/a)	Estimated DNT capacity (% of total 1.6 Milo. t)
Western Europe	488	31
North America	684	44
Eastern Europe	43	3
Japan	211	14
Korea	106	7
Other	20	1
Total	1552	100

TDI= toluene diisocyanate
Millo. t= million tonnes

1.3.2 Release of DNTs into the environment and remediation

DNTs are released from the production units to water bodies, soil and air. In the environment DNTs are not easily biodegradable, unless they are taken up by plants source or metabolised by bacteria (Tognetti et al., 2007; Hudcova et al., 2011).

Once released into water the only way to effectively remove DNTs from water bodies is through photolysis or phytoremediation through aquatic flora (Mihás et al., 2007; Chen and

Huang, 2011). The photolytic half-life for 2,4-DNT in river, bay and pond waters were 2.7, 9.6 and 3.7 hr respectively. The estimated half life of 2,4-DNT in the Rhine River was calculated to be 1.7 days (Chemical fact sheet, Spectrum laboratory, 1999). A surface water half-life of 1 day was calculated for 2,4-DNT for direct photolysis under latitude 40° N radiative conditions with 2,4-DNT degraded through photochemically produced hydroxyl (OH) radicals (Chemical fact sheet, Spectrum laboratory, 1999; Mihas et al., 2007). The soil organic carbon-water partitioning coefficient (K_{oc}) value ($K_{oc} = 123$) calculated with an equation for nitrobenzene and experimental bioconcentration factor ($BCF = 204$) showed less sorption potential and bioconcentration of 2,4-DNT onto the organic phase of sediments in water (Lang et al., 1997; Conder et al., 2004). According to Henry's law significantly low potential volatilization ($0.0094 \text{ Pa m}^3 / \text{mol}$ at 25°C) of 2,4-DNT was observed from the surface water (Chemical fact sheet, Spectrum laboratories). Several other methods to remediate DNTs released in water bodies include chemical reduction (Patapas et al., 2007), thermal decomposition (Minier et al., 1991), zero-valent iron (Fan et al., 2010), electrochemical reduction (Doppaplapudi et al., 2003) and ozonolysis (Andreev et al., 2004). Zero-valent iron ($\text{Fe}(0)$) has been employed in DNT degradation as an electron source for the reduction process (Fan et al., 2010) and was observed to be very effective only when ion composition (Cl^- , NO_3^- , NO_2^-) of waste water was controlled. To achieve complete reduction of DNTs in wastewater (as a step to reduce the toxicity) a two-step removal of 2,4-DNT and 2,6-DNT from water using zero-valent iron reduction coupled with peroxidase-catalyzed polymerization was suggested (Patapas et al., 2007). The resulting compounds at the end of the reaction were diaminotoluenes (DAT) achieved through enzymatic activity used in second step of the process. For the reduction reaction by zero-valent iron the pH values were maintained between 5.4 and 5.2 for optimal activity of enzymes (*Arthromyces ramosus* peroxidase and soybean peroxidase). Although effective this method (using zero-valent iron

for DNT degradation) was sensitive in terms of enzyme sensitivity towards hydrogen peroxide concentrations and pH value requirements (Patapas et al., 2007; Fan et al., 2010). Another effective remediation approach was the use of subcritical water (Li et al., 1993), has been used to extract fluids of several nitroaromatic compounds from water. The solubility of several organic compounds (such as DNTs, TNTs) is increased at high temperatures and water acts as a good solvent for several organic compounds at higher temperatures. However, the recovery of nitrotoluenes cannot be achieved effectively at less than 200 °C (Li et al., 1993).

Once released into soil there are several chemical (alkaline hydrolysis, solvent extraction) (Chen et al., 2011), biological (bacterial and plant consortia) (Wang et al., 2011) and physical (land excavation and incineration, composting, soil slurry reactors) methods researched so far to remove DNTs from the soil. Alkaline hydrolysis of 2,4-DNT in soils through lime treatment has been shown to be effective in remediating dinitrotoluene affected soils (Emmrich et al., 2001). The lowest activation energy barriers and energetically favourable pathway was the formation of anionic Meisenheimer complex. Soon after this a Janovsky complex is formed which is represented by a dimer of two 2,4-DNTs and is associated with a high activation energy. Another popular method for remediation of DNT form contaminated soils is the use of soil slurry reactors (Cassidy et al., 2009; Daprato et al., 2005). The reactors are filled with slurry, cosubstrate (e.g starch, glucose etc.) and nutrients are added. The mechanical aeration and mixing is done to optimize electron acceptors and mass transfer of nutrients. This technology uses anoxic incubation and avoids a polymerization reaction. The co-substrate provides electrons for reduction and removes oxygen (Esteve-Nunez et al., 2001). The process is utilized to mineralize DNTs and irreversible binding to the soil. Zhang et al (2001) utilized this approach by introduction of mineralizing bacteria into a bioslurry reactor. This approach enabled rapid processing of 2,4-DNT degradation (Diaz et al., 2011).

Of these available methods, the most researched are biological methods to remediate DNTs. The bioremediation is carried out through the use of aerobic and anaerobic microbes, fungus and plants (Nishino et al., 2000; Hughes et al., 1999). Biological methods are less costly, environmentally feasible and more practical compared to the other available methods. The details of all methods are shown in Table 1.7.

Table 1.7: Common methods of DNT remediation from contaminated soil.

Type of remediation	Method	Reference
Chemical remediation	Alkaline hydrolysis, Solvent extraction, sorption on K-smectite clay	Roberts et al., 2007; Hill et al., 2010; Chen et al., 2005
Physical remediation	Land excavation, Soil bioslurry reactors, incineration, composting land farming	Zhang et al., 2001; Diaz et al., 2011; Pennington et al., 2003
Bioremediation	Anaerobic bacteria, aerobic bacteria, fungus and plants	Valli et al., 1991; Nishino et al., 2000; Spain et al., 1999; Hughes et al., 1999; Yoon et al., 2007

1.4 Microbial transformation of DNT

In bacteria and fungi, DNTs can undergo both reduction and oxidation, however, in plants they are more susceptible to reduction (Hudcova et al., 2011). In case of bacteria, DNTs have been shown to undergo both aerobic and anaerobic degradation. Aerobic degradation is initiated by deoxygenation of the DNT molecule which is achieved through an initial oxygen

reaction (Hudcova et al., 2011). This results in the formation of nitrite and 4-methyl-5-nitrocatechol (4M5NC) (Yang et al., 2009). *Burkholderia cepacia* strain JS850 and *Hydrogenophaga palleronii* strain JS863 use DNTs as the sole carbon and nitrogen source (Nishino et al., 2000). The conversion of 2,4-DNT to 4-methyl-5-nitrocatechol (4M5NC) accompanied by release of nitrite during the dioxygenation reaction has been shown due to the activity of *B. cepacia* and *H. pelleronii*. The degradation product then undergoes an extradiol ring cleavage yielding 2,4-dihydroxy-5-methyl-6-oxohexa-2,4-dienoic acid (DHMOHA) (Figure 1.3).

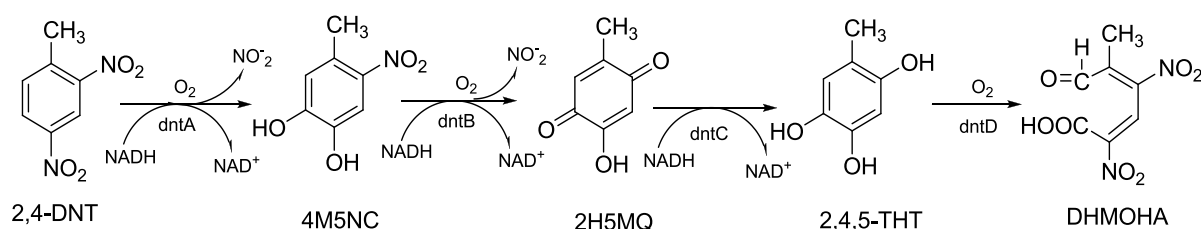


Figure 1.3: Catabolic pathway for 2,4-DNT degradation in *Burkholderia* sp. strain DNT. 2,4-DNT pathway intermediates are 4M5NC (4-methyl-5-nitrocatechol), 2H5MQ (2-hydroxy-5-methylquinone), 2,4,5-THT (2,4,5-trihydroxytoluene) and DHMOHA (2,4-dihydroxy-5-methyl-6-oxohexa-2,4-dienoic acid) (Nishino et al., 2000).

The degradation of DNTs has been studied in the strict anaerobic bacteria *Clostridium acetobutylicum* (Hughes et al., 1999). This anaerobic pathway for degradation following rapid reduction usually takes place at low redox potential. The initial reduction of DNT produced aminohydroxy compounds that lead to the formulation of amines (Figure 1.4). It was observed that the reduction of amines continued until diaminotoulene was produced.

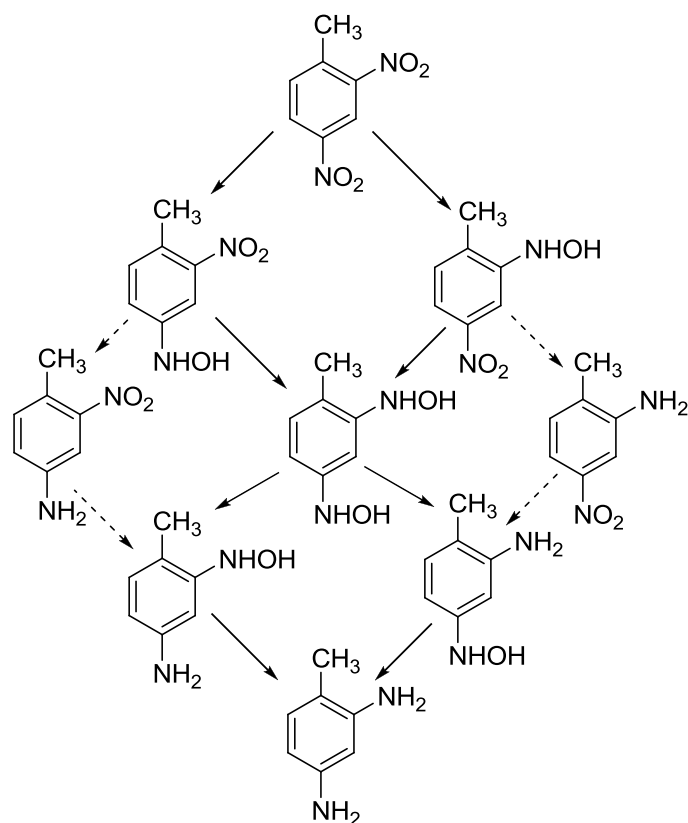


Figure 1.4: Anaerobic transformation of 2,4-DNT in cell culture and cell extracts of *Clostridium acetobutylicum* (Hughes et al., 1999).

The details of DNT degradation through different microorganism species is given in the Table 1.8.

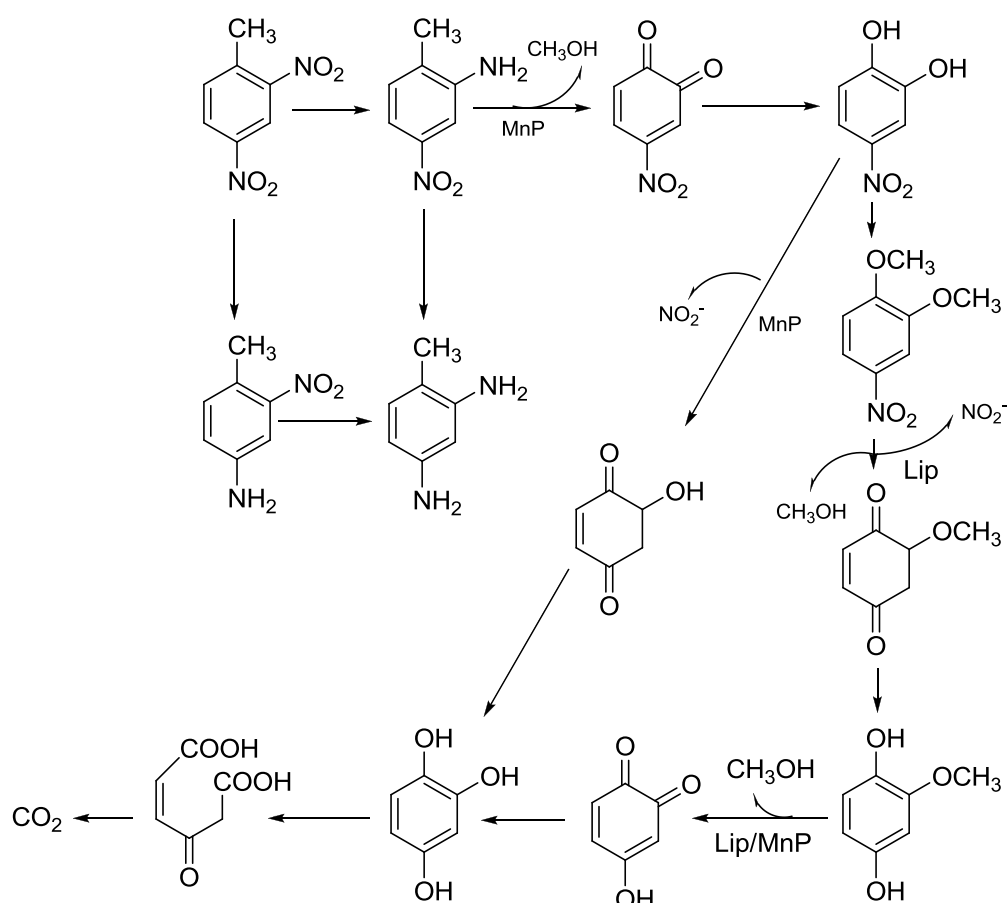
Table 1.8: Bacteria studied in degradation of DNTs.

Microorganism	Metabolism	Reference (s)
<i>Bacillus cereus</i> NDT4	Metabolism rate was 0.21 mg /L 2,4-DNT per day was studied	Hudcova et al., 2011
<i>Pseudomonas fluorescens</i> NDT2	Metabolism rate was studied and rate established was 0.66 mg /L 2,4-DNT per day for	Hudcova et al., 2011

	complete mineralization.	
<i>Pseudomonas</i> sp.	Metabolism rate was 328 mg /L of DNT per day.	Johnson and Spain, 2003; Haidour and Ramos, 1996
<i>Pseudomonas putida</i> NDT1	Metabolism rate was 0.67 mg /L 2,4-DNT per day through complete mineralization.	Hudcova et al., 2011
<i>Achromobacter</i> sp. NDT3	Metabolism rate was 0.31 mg /L 2,4-DNT per day through complete mineralization.	Hudcova et al., 2011
<i>Sinorhizobium meliloti</i> USDA 1936 pJS1	Genetically modified species had metabolism rate of DNTs at 10 mg /L per day.	Dutta et al., 2003
<i>Alcaligenes</i> sp. JS867	Oxygen limited conditions effectively worked for complete degradation of DNTs. Rates were not considered in this study.	Smets and Mueller, 2001
<i>Pseudomonas aeruginosa</i>	Reduction and acylation was achieved however incomplete mineralization observed. Rates were not	Noguera et al., 1996

	considered.	
<i>Pseudomonas fluorescens</i>	Wild strain worked less efficient however engineered strain worked efficiently for complete reduction. Rates were not considered.	Monti et al., 2005
<i>Variovorax paradoxus</i> VM685	Metabolism rate of 15.2 mg /L 2,4-DNT per day through complete mineralization.	Snellinx et al., 2003
<i>Pseudomonas marginalis</i> VM683	Metabolism rate was 28.8 mg /L 2,4-DNT per day through complete mineralization.	Snellinx et al., 2003
<i>Pseudomonas</i> sp. VM908	Metabolism rate was 15.2 mg /L 2,4-DNT per day through complete mineralization.	Snellinx et al., 2003
<i>Burkholderia cepacia</i> R34	Study of deoxygenase enzyme to degrade DNTs effectively	Johnson and Spain, 2003
<i>Pseudoxanthomonas</i> sp. JA40	Complete mineralization of DNTs was observed	Cheng, 2007
<i>Clostridium acetobutylicum</i>	Anaerobic transformation of DNTs	Hughes et al., 1999

Initial studies for the degradation of DNTs by fungi have been reported by Valli et al (1991). A lignin-degrading fungus *Phanerochaete chrysosporium* was studied for degradation of 2,4-DNT and the mineralization pathway elucidated by examining fungal metabolites in crude intracellular cell extracts. The pathway was initiated by reduction of DNT to 2-amino-4-nitrotoluene (2A4NT). This product was oxidised by manganese peroxidase (MnP) to yield 4-nitro-1,2-benzoquinone and methanol (Figure 1.5).



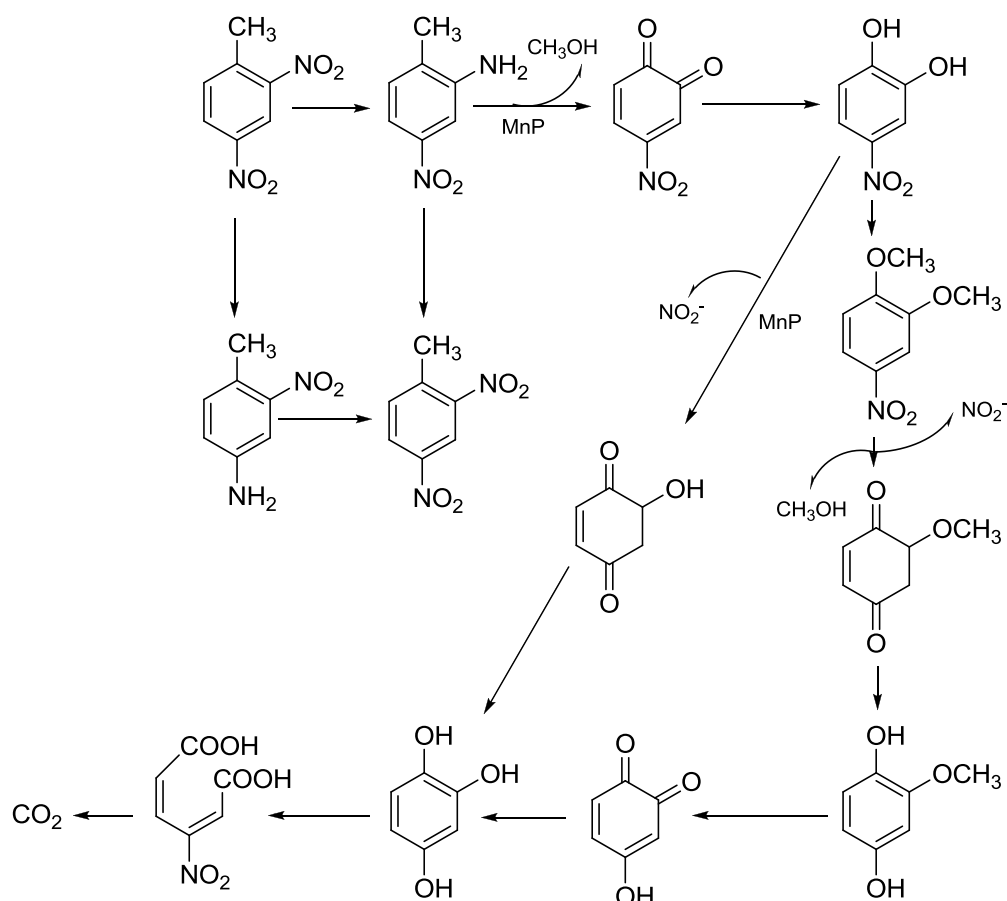


Figure 1.5: Transformation of 2,4-DNT in *P. chrysosporium* (Valli et al., 1992)

Nitrate release through fungal degradation of 2,4-DNT over 10 days has been shown under lignolytic and non-lignolytic conditions when *Phanerochaete chrysosporium*, *Keuhneromyces mutabilis*, *Hypholoma fasciculare* and *Phlebia radiata* were allowed to grow on 25 ppm of 2,4-DNT (Perkins et al., 2003). Improved denitrification was observed compared to the soil without and fungal assistance.

1.5 Transformation of DNTs in plants:

In plants, DNTs are susceptible to reduction, which proceeds through reduction of a nitrogroup (never reported to be isolated in plants) to nitroso group and hydroxylamino groups with amines are the final product of reduction (Yoon et al., 2007, Rocheleau et al., 2010). Aromatic amines develop electrophilic sites (centres with low electron density) and

are very reactive resulting in the formation of covalent adducts with a variety of biological molecules (French et al., 2001; Stenuit and Agathos, 2010).

Like TNT and other dinitrophenols, the aromatic rings of DNTs become electron deficient due to electron withdrawing property of nitro-groups and are highly likely to be attacked by nucleophiles (Rieger and Knackmuss, 1999; Stenuit and Agathos, 2010; Chua and Pumera, 2011). These highly electronegative nitro-groups remove the electron from the aromatic ring of DNT and make the nucleus electrophilic. The oxygen of the nitro group is more electronegative than nitrogen and therefore the N-O bond becomes polarized. Therefore, the partial positive charge and high electronegativity makes the nitro group highly susceptible to reduction (French et al., 2001; Symons and Bruce, 2006).

Plants do not use DNTs as a source of nutrients or energy, rather they mobilise, metabolise and distribute DNTs to ultimately neutralize the hazardous affect of these toxic substances (Hannink et al., 2002; Becanova et al., 2010). In general xenobiotics are metabolised in the plant in an effort to compartmentalise them away from the active biochemical machinery of the plant so that there is no interference in the plant development. This detoxification metabolism can be divided into three phases. The phase I reaction occurs to modify a foreign compound into a reactive form through enzymatic activity (Coleman et al., 1997; Huber et al., 2009; Dixit et al., 2011). This step does not occur if the compound already has a reactive functional group (French et al., 2001). The molecules then undergo phase II reaction. This is essentially a conjugation step during which the modified molecules are converted to a more polar compound. This enhances the mobility of molecules so that they can be metabolised further (Sandermann, 1992; Dixit et al., 2011). Plants lack an effective excretion (phytovolatalization) step to completely get rid of all the absorbed contaminants and that is only assisted by heavy evaporation and depends upon the volatility of the contaminant absorbed (Seeger et al., 2011; Edwards et al., 2011). However, the molecules produced

during phase II reactions can be compartmentalized in the vacuole or bind to the lignin of plant cell wall. This compartmentalization or sequestration is referred as phase III reaction of xenobiotic metabolism (Dixit et al., 2011).

1.5.1 Phase I reaction:

The phase I reaction of the DNTs is initiated by the transformation of the compounds to more reactive forms through introduction of functional groups such hydroxyl (-OH) or amino (-NH₂) via a series of enzymatic reactions (Esteve-Nunez et al., 2001). DNT can undergo both reductive and oxidative transformations, however only reduction has been reported in plants (Yoon et al., 2007; Tognetti et al., 2007; Su et al., 2011; Ye at al., 2011).

The reduction of DNTs occurs as a series of double electron transfers, which yield nitroso, hydroxylamino (never isolated in plants) and amino derivatives of DNT molecules (Figure 1.6) (Yoon et al., 2007). In plant cells, the aromatic ring of DNT becomes electron-deficient due to electron-withdrawing nature of the nitro-groups and is likely to be attacked by nucleophiles and resist any electrophilic attack (French et al., 2001; Becanova et al., 2010).

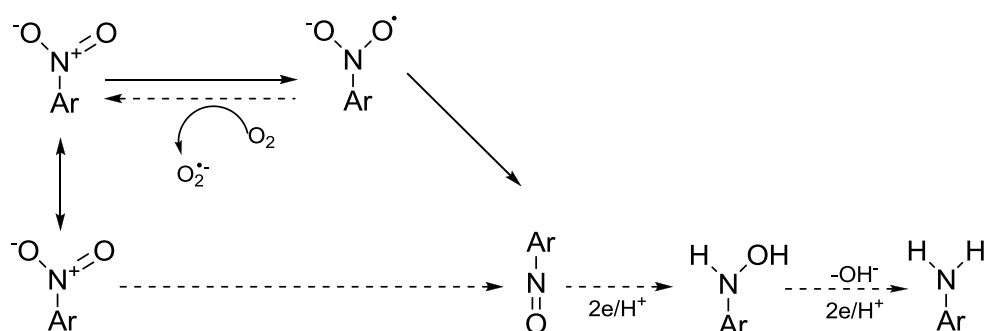


Figure 1.6: Reduction of nitrogroups in nitroaromatics. The initial reduction can be achieved through single electron transfer (solid line) or double electron transfer (dashed line). The single electron can yield superoxide radical and parent nitroaromatic compound through futile cycle (dotted line). The double electron transfer can yield hydroxylamine leading to the formation of nitroamines (Esteve-Nunez et al., 2001).

The resistance towards electrophilic attack creates an active site on the DNT molecules and increases their polarity which facilitates the phase I reaction. In certain cases the electron deficient ring, being susceptible to attack by a nucleophile, forms a Meisenheimer complex. These are negatively charged and results in the loss of nitro group from the aromatic ring (Bunel, 1982). Meisenheimer complex formation (Figure 1.7) has been reported by Hill et al., (2010) in case of 2,4-DNT degradation through alkaline hydrolysis. This complex has also been observed by Beynon et al., (2009) for TNT degradation in *A. thaliana* and also speculated to be formed in plants during DNTs degradation.

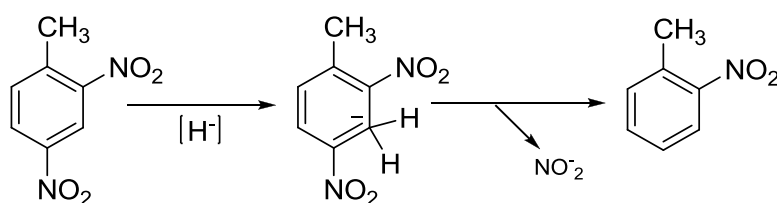


Figure 1.7: Meisenheimer complex may lead to the loss of nitro group from aromatic ring of DNT (Hill et al., 2010).

These amino-nitrotoluenes are the most observed transformed products of DNT metabolism recovered from plants (Figure 1.8).

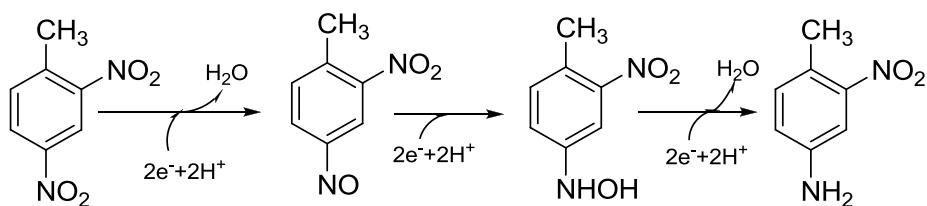


Figure 1.8: General reduction reaction of 2,4-DNT to aminonitrotoluene (Oliveira et al., 2010).

The hydroxylamino compounds are never isolated from plants after DNT exposure, which may be due to their rapid dissociation or reactivity towards other metabolites. This process is oxygen independent and is carried out by oxygen insensitive (type I) nitroreductases (Esteve-

Nunez et al., 2001). Nitroreductases catalyze pyridine nucleotides-dependent reduction of nitroaromatics. The two electrons from NAD(P)H are sequentially transferred to nitro groups of the nitroaromatic compound in the presence or absence of oxygen (Figure 1.9). The two electron transfer is carried out via a ping-pong, bi-bi kinetic mechanism. The nitroso intermediate is so reactive that second two-electron reaction occurs at a much faster rate than the first two-electron reaction (Oliveira et al., 2010).

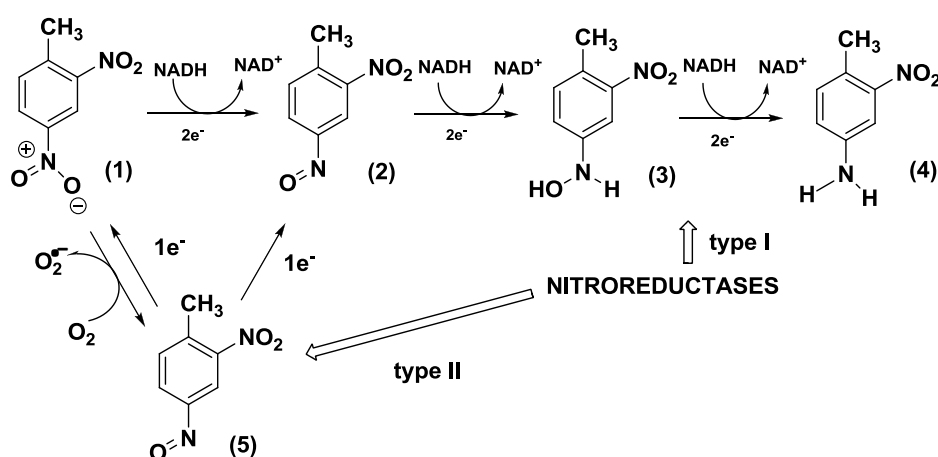


Figure 1.9: Reduction following two and single electron transfer. Two electron transfer is assisted by type I nitroreductases and results in the formation of (2) hydroxylamino (3) intermediate compound (4) amino compound. Single electron transfer is assisted by type II nitroreductases and form (5) superoxide anion leading to a futile redox cycle and then generate the amino intermediates (Oliveira et al., 2010).

There are several other enzymes involved in two electron reduction of DNTs, such as azoreductase (Lui et al., 2007), cytochrome b₅ reductase (Johnson et al., 2000) and hydrogenases (Johnson et al., 2002). Certain other enzymes have also been studied in the reduction of hydroxyl-dinitrotoluenes (intermediate in TNT degradation) and are likely to influence DNT metabolism in plants. These are aldehyde oxidase (Bryant et al., 1991), dihydrophilic amide dehydrogenase, CO dehydrogenase and xanthine oxidase (Decad et al.,

1984; Chapman et al., 1994; Esteve-Nunez et al., 2001; Liu et al., 2007). If the reduction of nitro-groups of DNTs proceeds through a single electron transfer then they form a nitro-anion radical which leads to the formation of a nitroso group or superoxide anion in the presence of oxygen. This can cause oxidative stress through the production of superoxides leading to a futile redox cycle (Oliveira et al., 2010; Nishino et al., 2000).

These transformations play an important role in determining the interactions of the compounds (Hudcova et al., 2011). The nitroso or hydroxylamino compounds formed are responsible for the toxicity of DNT and other nitroaromatics. Since they become more hydrophilic after phase I transformation therefore, the likelihood of their interactions with most the cellular organelles increases (Whong and Edwards, 1984). The nitroso or hydroxylamino products, formed after reduction of nitroaromatics, form covalent adducts with biological molecules such as proteins and are likely to cause mutagenesis or cytotoxicity to the host cells (French et al., 2001; Symons and Bruce, 2007). This toxicity is reduced by the complete reduction of nitro groups (Hannink et al., 2002). Several studies have reported the reduction and isolation of aminotoluenes from DNT treated plants (Van Aken, 2001; Doken and Davis 2011). The formation of 2A6NT confirmed through NMR and UV chromatograms from the plants treated with 2,6-DNT has also been shown (Figure 1.10) (Yoon et al., 2006 and 2007).

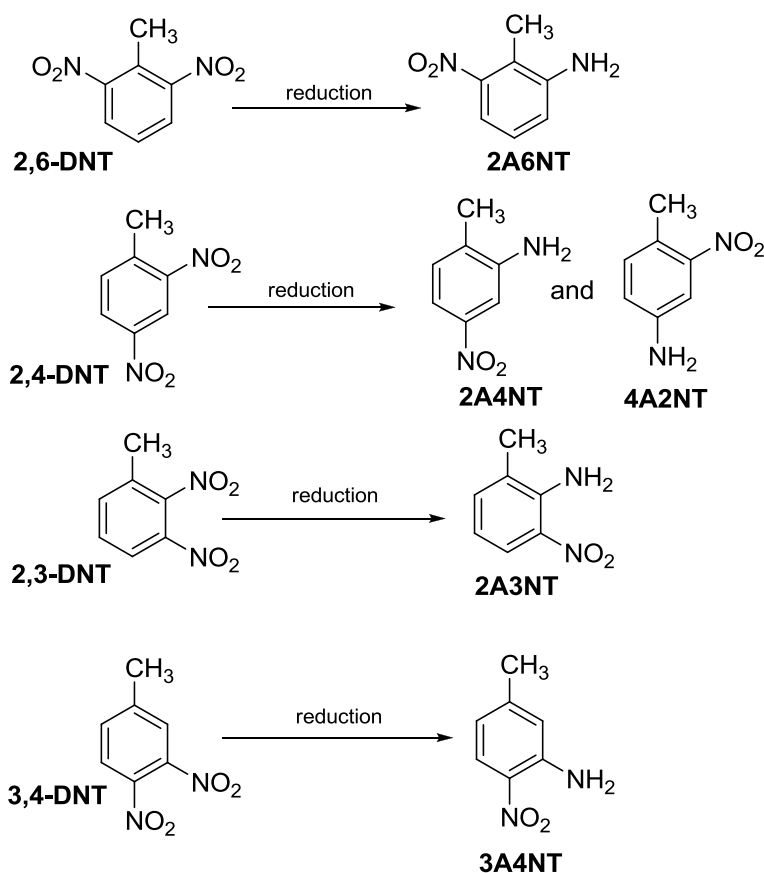


Figure 1.10: Different isomers and their respective reduced products. 2-amino-6-nitrotoluene (2A6NT), 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT), 2-amino-3-nitrotoluene (2A3NT) and 3-amino-4-nitrotoluene (3A4NT).

In nature the reduction of first nitro group in nitroaromatics such as DNTs is very rapid due to the electron withdrawing nature of nitro groups that make the aromatic ring electron deficient. The formation of amino group reduces this deficiency and a low redox potential (-200 mV) under anoxic environment is required to convert the rest of the nitro groups to their respective amines to get a diaminotoluene (never isolated for plants but found in bacterial degradation of DNTs) (Esteve-Nunez et al., 2001). The nitro-group with the greatest negative charge localisation is likely to be protonated more readily. This determines the preference of reduction of one nitro-group attached to the same aromatic ring over the other, and therefore

determines the toxicity difference in different isomers of same compound (Barrows et al., 1996).

The reduction rate of nitroaromatics is reduced with increasing concentrations in the media (Yoon et al., 2007, Pavlostathis et al., 1998). In the case of DNT according to Yoon et al., 2007, the rate of metabolism was observed to decrease with increasing concentrations of 2,6-DNT in growth media. This indicates a threshold concentration of DNTs where plant growth is optimal and the metabolism of DNT to reduced products is at a maximum.

The product of the reduction then enters the phase II step of metabolism in which the reduced compounds get conjugated to more polar molecules such as glucose or glutathione that also enables their compartmentalization (Gandia-Herrero et al., 2008; Khater et al., 2012).

1.5.2 Phase II reaction:

Following the transformation phase I reaction, the polar DNT molecules enter phase II of metabolism. During this phase, the polar DNT metabolite undergoes conjugation in the cytosol of the plant cell (Coleman et al., 1997). The reaction takes place with a polar molecule of which the most common are glucose, glutathione, and malonate (Hannink et al., 2002). This reaction limits the movement of molecules by confining it to some organelles (such as in vacuole or to the walls of lignin) and does not allow them to interact with the cellular processes. DNT conjugation has only been investigated in *A. thaliana* (Yoon et al., 2007) where it has been shown that wild type *A. thaliana* on exposure to 2,6-DNT that glutathione *S*-transferases (*gst*) is induced 4.7 fold compared to a control. This indicates the conjugation of glutathione to the reduced products of 2,6-DNT. Due to the lack of information on observed conjugates for reduced products of 2,6-DNT, the chemistry involved was based on the glutathione conjugation of other nitroaromatics.

The initial research to report conjugation was conducted by Harvey et al., 1990 on TNT metabolites. According to this study highly polar compounds were investigated in bush beans exposed to the ^{14}C -TNT. Acid-hydrolysis of plant material revealed higher molecular weight compounds which were partly comprised of 4-amino-2,6-dinitrotoluene (4ADNT) and 2-amino-4,6-dinitrotoluene (2ADNT) extracted from the roots and shoots with 80% of radiolabel fixed in the biomass. In another study (Thompson et al., 1998) similar results were reported with the identification of a polar metabolite associated with 2ADNT and 4ADNT when plants grown hydroponically were treated with TNT.

The identification of these polar metabolites (glucosidic conjugates) (Bhadra et al., 1999) showed four conjugates (two with spectra similar to 2-ADNT and two similar to 4-ADNT) in the axenic root culture of *Catharanthus roseus*. Enzyme hydrolysis of these conjugates produced monoamino DNTs. The mass spectrum showed that there was a six carbon compound that conjugates to the amino group of the monoamino DNTs (reduced product of TNT). Various enzymes involved in the conjugation process are namely, the glutathione, malonyl and glucosyl transferases. Glycosidic conjugation (Figure 1.11) usually occurs at the hydroxyl, sulfhydryl, amino and carboxyl groups of the transformed nitroaromatics however, the malonate conjugation occurs predominantly to hydroxyl and amino groups (Reinhold et al., 2011). Glutathione, however, has been observed to conjugate at electrophilic sites in the molecule which often occurs by displacing a nitro group.

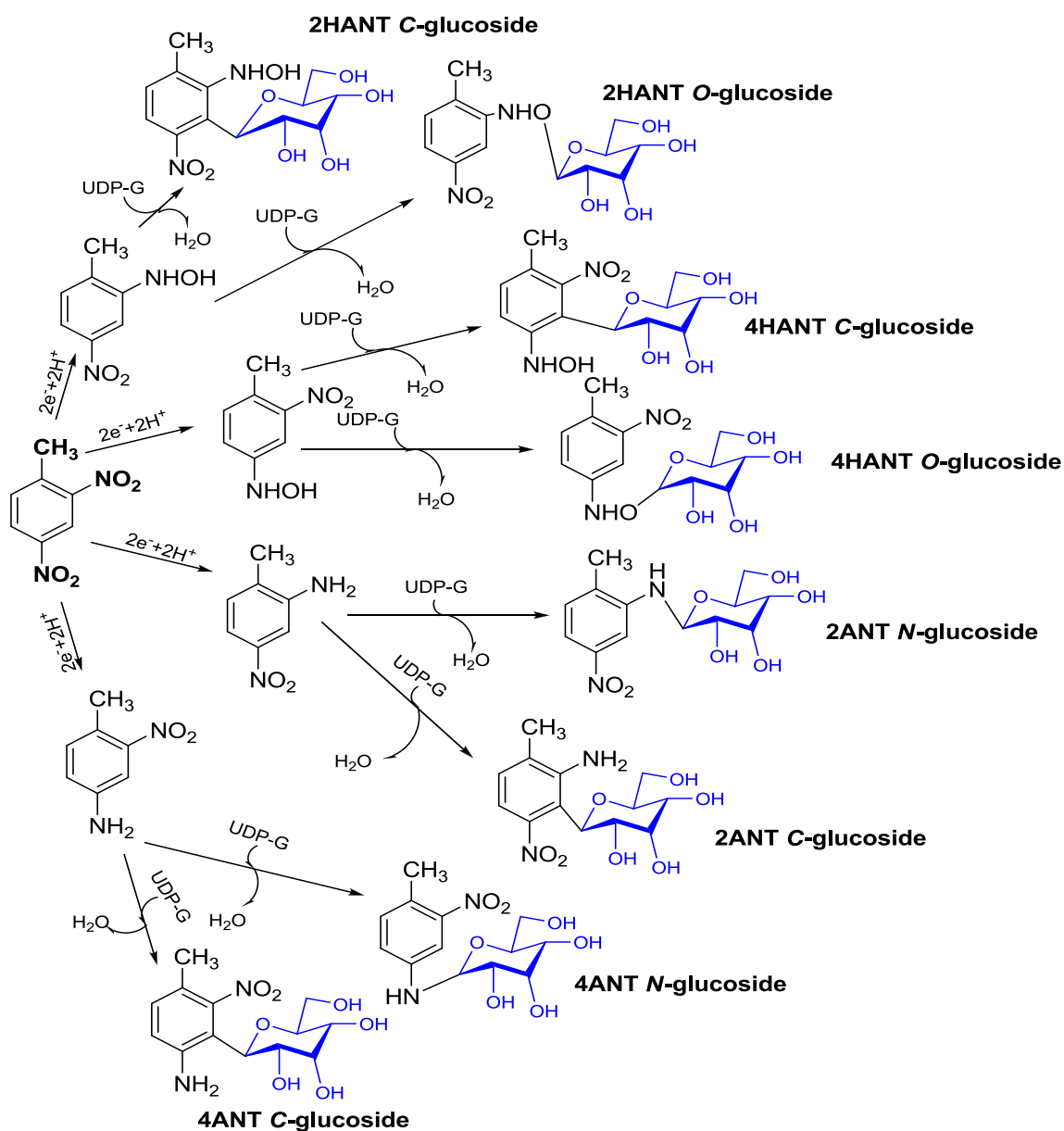


Figure 1.11: Possible conjugates of 2,4-DNT with UDP-glucose assisted by glucosyltransferases based on the analogy with TNT and other nitroaromatic glycosylation pathway (adapted from Gandia-Herrero et al., 2008).

The conjugated molecules (phase II) enter into phase III step, which is important since their solubility determines their compartmentalization in the plant cell. Generally xenobiotics conjugate to glucosides, glutathione, malonic acid (Figure 1.12) or amino acids they result in soluble conjugates that can be stored in the vacuole of the plant cell.

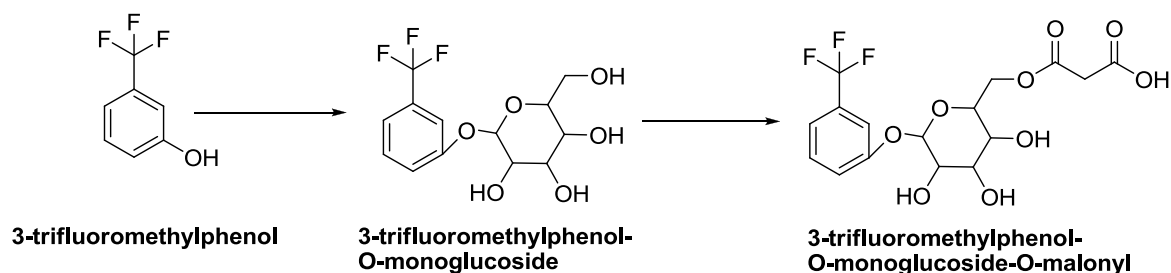


Figure: 1.12: Glucosidic and malonated conjugation during phytometabolism of 3-trifluoromethylphenol (3-TFMP) by *Lemna minor* (Reinhold et al., 2011).

The conjugation reaction with different molecules is assisted by isoforms of glutathione-*S*-transferases (Figure 1.13), malonyl-transferases and glucosyl-transferases (Schroder and Collins, 2006). These conjugates sometimes get incorporated into the plant cell wall (phase III) after conjugation with cell wall biopolymers. According to a study (Burken et al., 2000) the wetland plants have been shown to excrete these Phase II conjugated compounds (TNT glycosides) of TNT outside the plant body into the surrounding water. This incorporation of the conjugated DNTs leads to Phase III reaction in DNT metabolism in plants.

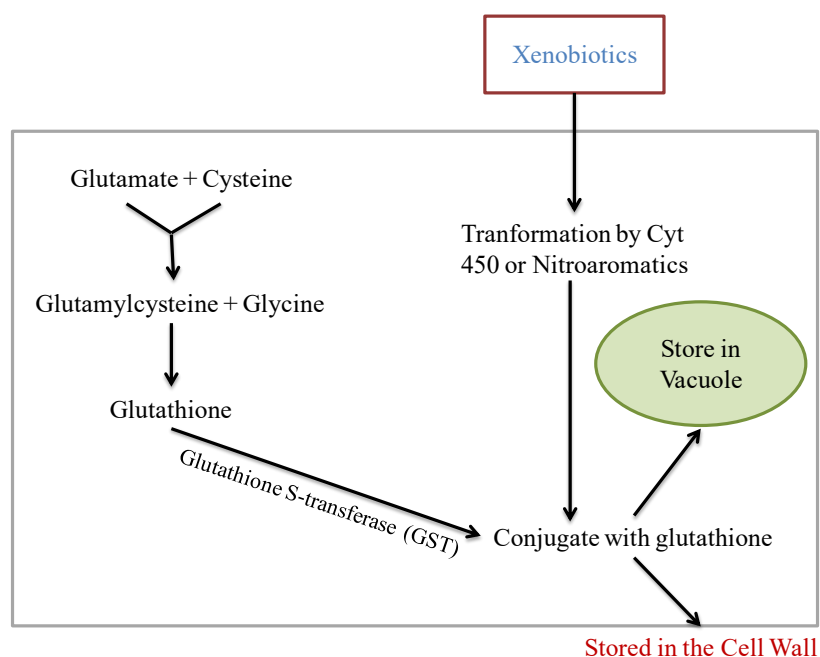


Figure 1.13: Glutathione mediated xenobiotic metabolism in plant cells (Yoon et al., 2007).

1.2.7.3 Phase III reaction:

Research has revealed nitroaromatic conjugates bind to the cell wall of the plant cells (Coleman et al., 1997; Hannink et al., 2002; French et al., 2001). Again there is lack of information regarding the conjugation of DNT metabolites with the cell wall and this conjugation is just speculative.

The functional group of conjugates of the transformed DNTs determine the compartmentalization of the compounds. It has been shown that 6-*O*-malonyl- β -D-glucosides are stored in the vacuole. It has been suggested that the addition of a malonyl group to the transformed DNTs provides a signal for the storage of such compounds into the vacuole (Hannink et al., 2002). There are certain conjugates that signal the conjugation of compounds to the plant cell wall. It was (Sens et al., 1998) was observed ^{14}C was partitioned equally in the cytoplasm and cell wall after treatment of *Phaseolus vulgaris* with ^{14}C TNT. Similar results were (Scheidemann et al., 1998) observed for herbaceous plants treated with TNT and analysis of cell wall fractions through polar and non polar solvents.

Phenanthrene an aromatic pollutant, which is a semi volatile organic compound like DNT was found to accumulate in the cell wall, vacuole and cytoplasm of *Hypnum cupressiforme* after 12 days of treatment and later migrated across the cell wall and membranes to adjacent cells (Keyte et al., 2009).

The plant cell wall has unique structure and acts as sink for different type of pollutants. For example the cork oak tree (*Quercus suber*) bark has hydrophobic biopolymers (suberin and lignin) and hydrophilic polysaccharides (hemicelluloses and cellulose), which offer sink for wide variety of pollutants for conjugation (Olivella et al., 2010). The conjugates covalently get incorporated into the cell wall are difficult to extract but can be identified through fluorescence microscopy. However, there are certain staining and microscopic methods such

as the use of two-photon excitation microscopy (TPEM) coupled with plant autofluorescence reported in a study (Wild et al., 2004) to track anthracene (a polycyclic aromatic hydrocarbon) in *Zea mays*.

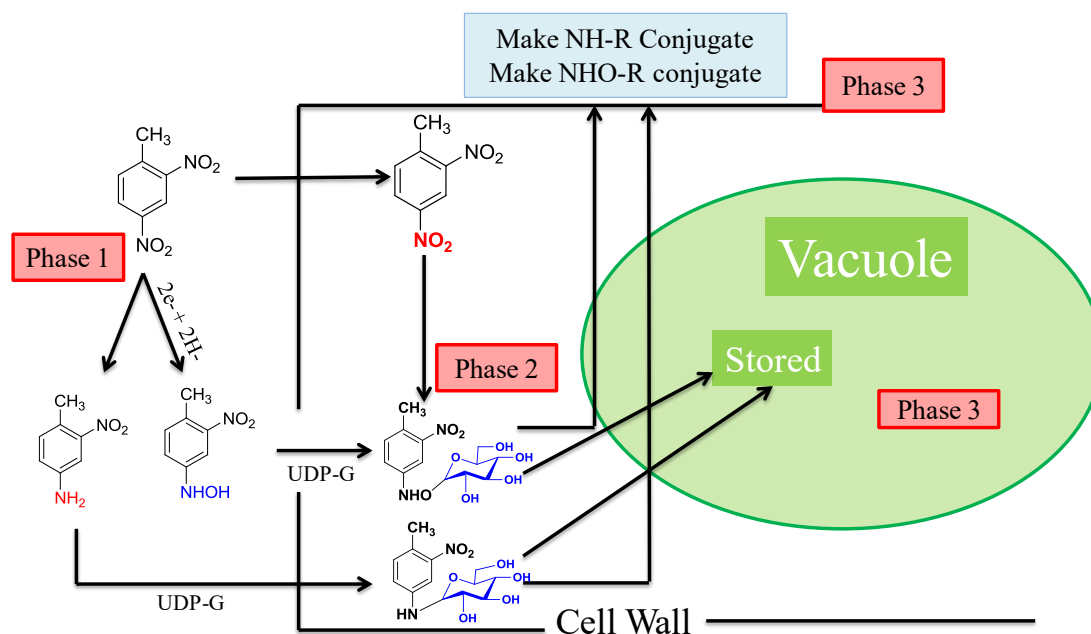


Figure 1.14: Fate of DNTs in plant cells. Three phases of transformation in DNT metabolism hypothesized from the available literature about xenobiotic transformation.

Table 1.9: Various studies showing transformed products of DNT isomers studied in different media.

Plant type	DNTs concentrations and isomers	Condition	Metabolites	Reference
<i>A. thaliana</i>	2,6-DNT upto 100 mg /L	Sterile hydroponic and sterile Phytigel media	2A6NT unknown polar metabolites studied through UV spectra	Yoon et al., 2007
<i>A. thaliana</i>	2,4-DNT upto 80 mg /L	Sterile Phytigel media	2A4NT unknown polar metabolites studied through UV spectra	Yoon et al., 2006
<i>Cyperus esculentus</i> (Yellow nutsedge)	2,4-DNT in upto 0-8 mg /L	Non sterile hydroponic solution	2A4NT	Reifler et al., 2006
<i>Medicago Sativum</i> (alfalfa)	2,6-DNT and 2,4-DNT upto 100 mg /L	Soil, non-sterile	2A6NT and 2A4NT	Schneider et al., 1996
<i>Triticum aestivum</i> (wheat)	2,6-DNT	Xylem uptake studies	2A6NT	Sui and Liang et al., 2011

<i>Medicago sativum</i> (alfalfa)	2,6-DNT and 2,4-DNT	Soil, non-sterile	2A4NT and 2A6NT	Rocheleau et al., 2010
<i>Echinochloa crusgalli</i> (barn yard grass)	2,6-DNT and 2,4-DNT	Soil, non-sterile	2A4NT and 2A6NT	Rocheleau et al., 2010
<i>Lolium perenne</i> (perennial rye grass)	2,6-DNT and 2,4-DNT	Soil, non-sterile	2A4NT and 2A6NT	Rocheleau et al., 2010
<i>Nicotiana tobaccum</i> (Tobacco)	2,4-DNT	Sterile hydroponic	Reduced efficiently to 2A4NT as it was genetically modified	Tognetti et al., 2007

1.6 Phytotoxicity of DNTs:

DNTs are toxic to plants if their concentration exceeds a threshold limit. The remediation efficiency of a particular plant therefore depends upon its tolerance to the specific concentration of DNT in the plant growth media. The phytotoxicity mechanism of all xenobiotics usually appears to be the same in all plants through initiation of chlorosis (gradual loss of green pigment due to competition in availability of non-haem iron) (Pavolstathis et al., 1998). The reduced products of DNT could have a similar mechanism to dinitroaniline herbicides. The toxicity of 2,6-DNT in axenic culture with a median EC_{50} root toxicity was determined to be 16 mg /L (Yoon et al., 2007). The results indicated that recovery of 2,6-DNT and its metabolites [using U-C-14 2,6-DNT] was over 87% and less than 2% accounted for DNT mineralization after 14 days of exposure.

The DNTs in aqueous media have been shown to be very toxic to duckweeds (Roberts et al., 2007) which reduced after the treatment of media with K-smectite clay. The reason for DNTs toxicity was that in aqueous media they are more available to the duckweeds compared to dry media where minerals do not bind easily. This can be reduced through sorption by K-smectite clay.

The phytotoxicity of 2,4-DNT and 2,6-DNT towards *Medicago sativa* L., *Echinochloa crusgalli* L. Beauv. and *Lolium perenne* L. grown in Sassafras sandy loam soil has been investigated (Rocheleau et al., 2010) where plants were grown on 13 week old weathered soil amended with 2,6-DNT and 2,4-DNT through the activity of drying, wetting and exposure to sunlight. The toxicity of 2,4-DNT thus obtained was compared with the plants grown in freshly 2,4-DNT and 2,6-DNT amended soils. It was found that DNTs were more toxic for the plants in freshly amended soils with EC_{20} values ranging from 3 to 20 mg / kg of soil and toxicity of DNTs was observed to reduced as age in the media increases.

The toxicity of DNTs also varies with the plant species as well as clay, organic matter and nutrient contents. In another study (Rocheleau et al., 2006) the phytotoxicity of 2,4-DNT towards *Medicago sativa* L., *Echinochloa crusgalli* L. Beauv. and *Lolium perenne* L. using four different natural soils with varying organic matter, pH and clay contents was investigated. It was found that median toxicity of 2,4-DNT ranged from 8 to 229 mg /kg and was highly variable for both the plant and soil type. The highest toxicity of 2,4-DNT was observed for sandy loam soils with toxicity ranging from 8 to 44 mg /kg, however plants can tolerate these concentrations in clay loam soils with the median toxicities determined to be higher than 40 mg /kg. Among the different species studied the *Lolium perenne* L. showed least resistance to 2,4-DNT even in weathered soils. This shows that the species used for phytoremediation also has a great impact on the tolerance to different concentrations of DNTs.

To show the phytotoxicity of DNTs to be species dependent (Picka and Friedl 2004), 2,4-DNT toxicities to a monocotyledon, wheat (*Triticum aestivum* L.), and three dicotyledons, lentil (*Lens culinaris* Med.), mustard (*Sinapis alba* L.) and lettuce (*Lactuca sativa* L.) were investigated. It was observed that lettuce is most sensitive amongst the tested plants while wheat was the most resistant.

1.7 Bioaccumulation in the food chain:

One possible issue with phytoremediation is that the uptake of xenobiotics by plants can create another problem in that herbivorous feeders will potentially accumulate these compounds thus impacting on the food chain. The herbivores usually do not discriminate in the polluted and unpolluted food sources. Therefore, if a pollutant is long-lived, there is a high chance of its transport through the ecosystem with the concentration increasing at each trophic level. The bioaccumulation of 2,4-DNT has been shown in common carp (*Cyprinus*

carpio) and more than 10 fresh water species in Songhua River of North China having low concentrations of 2,4-DNT (0.88 µg /L of River water) (Xu and Jing, 2012). Fish were shown to have 2,4-DNT concentrations of <0.22 and up to 0.95 mg /kg wet weight. This led to increased ethoxyresorufin-*O*-deethylase (EROD) activity to increase by 0.39-1.83 fold higher than in controls when exposed to sub lethal concentration of 2,4-DNT by 0.1 mg /L, 0.5 mg /L and 1 mg /L (Xu and Jiang 2010).

The accumulation and toxicity of 2,4-DNT and 2,6-DNT has been shown in selected earthworms *Enchytraetus crypticus* exposed to DNT amended weathered and aged sandy loam soil (Kuperman et al., 2005) with 2,4-DNT rendered more toxic than 2,6-DNT. TNT has been shown to accumulate in *Tubifex tubifex* through the contaminated soil (Lee et al., 2007). The study to report transport of DNT treated plants on primary consumers has not been shown yet.

Like other organic pesticides and insecticides, DNTs absorbed in the plants could potentially enter primary consumers when they feed upon the contaminated plant. A classic example for the transfer of pesticides from plant up into the food chain is the much studied organochlorine pesticide dichlorodiphenyltrichloroethane (DDT) (Dietz et al, 2000; Davies et al., 2007; Ondarza et al., 2012). Usually the hazardous xenobiotics along with nutrients enter the vascular tissues and are metabolised and stored by the plants (Neuhold and Ruggiero, 1976; Ondarza et al., 2012). These xenobiotics, if persistent, can get transported to the primary consumers and could be stored in their body fat. The continuous chain of predation of contaminated food can potentially result in the accumulation of these pollutants up in the trophic level. According to a study from the Ross Sea (East Antarctica) (Cho et al., 2008) the biomagnifications of chlorinated biphenyls was observed in the eggs of the resident birds adelie (*Pygoscelis adeliae*) and emperor (*Aptenodytes forsteri*) penguins. Comparative to that the migrating snow petrel (*Pagodroma nivea*) and south polar skua (*Catharacta*

maccormicki) showed less accumulation of the contaminants. This shows that the resident birds, since continuously consume the contaminated prey, have managed to accumulate lethal concentrations of the pollutants that may affect their reproduction and result in species extinction.

Central to the distribution of xenobiotics throughout ecosystems are the arthropods by virtue of their abundance (Pimental et al., 1980). Insects such as aphids belong to the order Hemiptera, exclusively feed on the phloem sap and have been shown to accumulate soil contaminants in their soft body parts (Colliney and Pimental, 1986; Babish et al., 1981; Raikow et al., 2011). These aphids or insects often become food of secondary consumers and transport these contaminants to them.

Several factors affect the distribution of xenobiotics from the aphid feeders to the organisms at higher trophic levels. For example, if the aphid is a specialist (feeding on selective closely related plant species; oligophagous) then it is highly likely to distribute the contaminant, in case the food range gets contaminated. However, in case of generalist (consuming a wide variety of plant species; polyphagous) the contaminant propagation can be avoided as they forage on broader food range (Ahmadi et al., 2011). One of the other factors to contribute in the contaminant distribution is the resistance in aphids. In case of aphids having high resistance (mostly reported for *Myzus persicae*) to the contaminants, the chances of propagation of hazardous substances increase if they manage to ingest and store the xenobiotics in their bodies (Pineda et al., 2012).

It has been observed that the survivorship and fecundity of the aphids get highly compromised when they are allowed to feed the contaminated plants. According to Cilliney and Pimental, 1986, the survival and fecundity of *M. persicae* was highly reduced when they were allowed to grow on *Brassica oleracea* plants treated with contaminated sewage. The

survival of *Lipaphis erysimi* was highly reduced when allowed to feed on plants contaminated with pyrethroids (Puinean et al., 2010)

For surviving aphids the accumulation of contaminants is highly likely and they play an important role in the transport of contaminants through trophic levels in an ecosystem. There have been extensive studies examining the uptake of pesticides and polychlorinated biphenyls (PCBs) in aphids feeding on contaminated host plants. Crawford et al., 1995 and Morington et al., 2001 have shown high levels of accumulation of cadmium in feeding aphids compared to the amount of heavy metal in the contaminated host black bean (*Phaseolus vulgaris*) and wheat (*Triticum* spp.) plants respectively. A study (Cojocarus et al., 1976) has shown the accumulation of chlorinated hydrocarbons in aphids feeding on the host plants grown on the contaminated municipal sludge.

The sequestration of contaminants from the contaminated food and its transport through tritrophic level of ecosystem has been established in several studies (Higginson et al., 2012; Jeger et al., 2012; Keathley et al., 2012). This relationship is important as it allows the researcher to determine the fate of xenobiotics in the ecosystem. According to a study (Jansen, 2010) the survival and fecundity of lady bird (*Adalia bipunctata*) was highly compromised when it was allowed to feed on the food treated with insecticides (Flonicamide, thiacloprid and pirimicarb) (Francis et al., 2000). This showed that the resistant xenobiotics can easily get transported to the higher trophic level after predation of contaminated food.

1.8 Hypothesis:

Keeping in view the environmental contamination and uptake of DNTs following hypotheses were suggested for whole thesis. All isomers of DNT have different toxicities.

- The DNT isomers are reduced to their respective metabolites in plants.
- Transformed DNTs undergo conjugation in plants.
- Specialists aphids and generalist aphids reproduction and growth is affected if they are allowed to feed on DNT treated plants.
- Aphids feeding on treated plants can accumulate DNT based metabolites in their bodies.

1.9 Aims:

The aims of the studies conducted through out the thesis were then designed to investigate the above hypotheses.

- Establish toxicity of four available isomers of DNT in axenic and non axenic culture for *A. thaliana*.
- Analyse the reduced transformed products of these isomers in *A. thaliana* by GC-MS analysis.
- Analyse the conjugates of considered isomers of DNTs in *A. thaliana* by LC-MS.
- Analyse the affects of DNT treated *A. thaliana* on specialist (*Brevicoryne brassicae*) and generalist (*Myzus persicae*) aphids to analyze the bioavailability of DNTs in ecosystem.
- Analyze the DNT based metabolites in both specialist (*Brevicoryne brassicae*) and generalist (*Myzus persicae*) aphids through GC-MS and LC-MS/MS analysis.

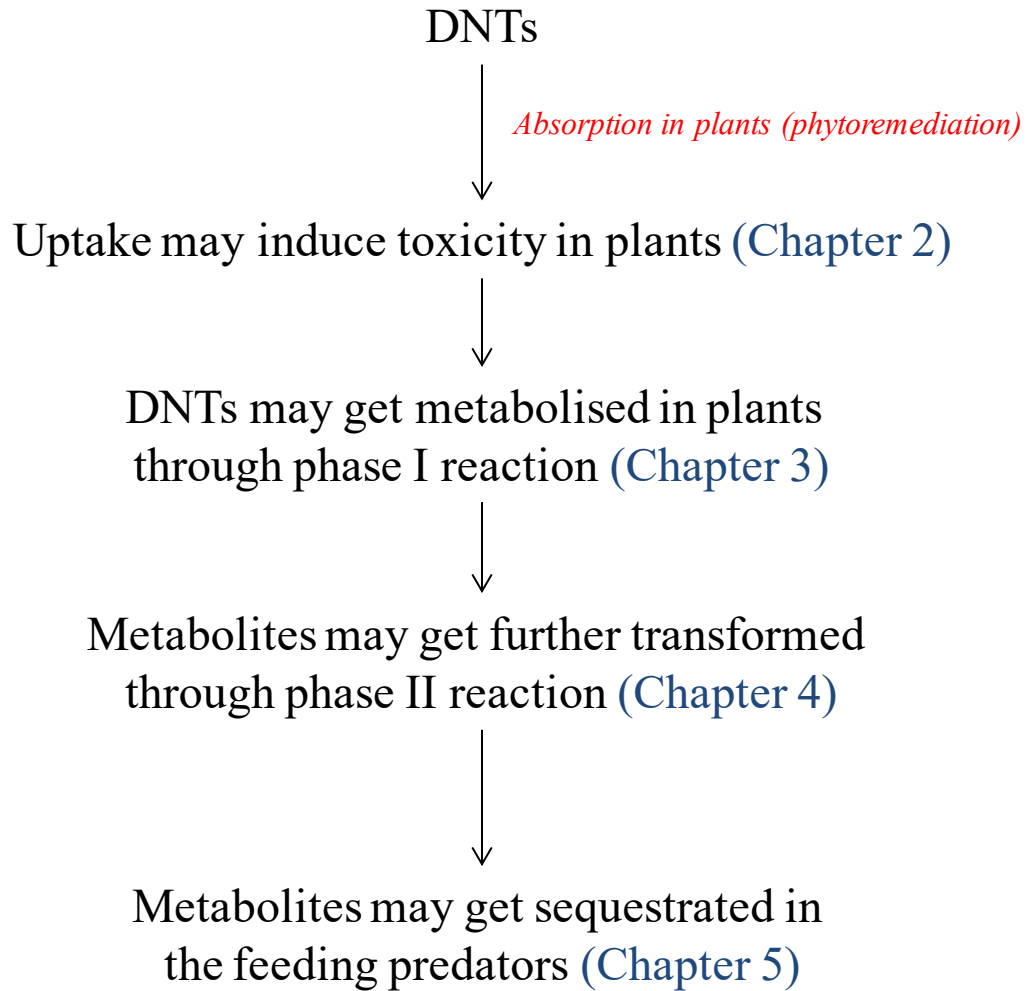


Figure 1.15: Overall structure of thesis. Each study (chapter) is provided with detailed hypothesis and aims of the study.

Chapter 2

Toxicity determination of four dinitrotoluene isomers on *Arabidopsis thaliana* grown in Phytagel and hydroponic media

2.1 Introduction:

Dinitrotoluenes (DNT) are introduced into the environment as a by product of 2,4,6-trinitrotoluene and synthesis of polyurethane, dyes and propellants (Chen et al., 2012). The contamination of environmental resources occurs as a result of dumping the partially treated wastewater and spillages of these industrial sites into water and land reservoirs. Leaching of DNTs from the sites with army ammunition activities can also contribute to soil and water pollution in surrounding areas (Yoon *et al.*, 2007).

DNTs are toxic to living cells and are potential carcinogens. According to the International Agency for Research on Cancer (IARC) it has been found that DNTs could be a potential carcinogen as 2,6-DNT has been shown to be hepatocarcinogenic in Fischer-344 rats (Rickert et al., 1984; George et al, 1996). High levels of toxicity have also been shown to cause low sperm counts and reduced fertility (International Agency for Research on Cancer, 1996). The toxicity of DNTs encouraged scientists to develop remediation strategies for contaminated resources. Phytoremediation was the most cost effective, easy to assemble and efficient technique and serves as a preferred approach over the more expensive and complex chemical methods which may be efficient but are themselves not the best representatives of eco-friendly compounds (Yoon et al., 2007).

The phytoremediation of trinitrotoluene (TNT) and other nitroaromatic explosives have been researched extensively (Van Aken et al., 2002) but research based on DNTs is scarce even

though DNT has been detected in the soil and water resources of at least 122 toxic wastes sites out of the 1467 enlisted sites of National Priorities List in the US alone (Agency for toxic substances and disease registry, 1999). This lack of research might be because the properties of DNTs are considered similar to TNT due to the similar molecular structure. However, all nitroaromatics have different fates of remediation that varies with the type and position of the side chains attached to the benzene ring (Yoon *et al.*, 2007). The phytoremediation approach varies even among the DNT isomers as changes in the electronic configuration varies the stability of the compound. Most toxicological studies with DNTs have been carried out on 2,6-DNT and 2,4-DNT with little or no research into the remaining isomers.

Restriction of the plants ability to grow on the contaminated soils has led researchers to focus on the toxicity of the compounds before approaching remediation so that the remediation approach can be achieved successfully without killing the plant altogether (Wang *et al.*, 2012). There are several methods for the characterization of the toxic levels, however, analysing root length and root shoot biomass in media containing the toxic compounds is the preferred approach.

The plant uptake of pollutants is primarily through the roots and secondarily through the shoot. The pollutants are first absorbed on the surface of the roots and are then absorbed into the adjoining cell organelles via the cell membrane (Dokken and Davis, 2010). Therefore, certain enzymes present at the surface of the roots or in the root cells can degrade the xenobiotics in the root growth media to reduce their toxicity, however, a large amount is also transported unprocessed to the shoot (Schwitzguebel *et al.*, 2011; Padmavathiamma and Li, 2007). It has been shown that the suberine layers of cells, which act as efficient barriers against the solutes *via* the apoplast, are not well developed in the root area, resulting in the uncontrolled apoplastic uptake of contaminants from the xylem (Mench *et al.*, 2009). These

partially degraded and unprocessed toxic compounds cause potential damage to the roots and root cells rendering them unable to carry out their function. Thus large amounts of unprocessed hazardous compounds are transported to the shoot for further degradation. When in the shoot these compounds (depending upon the nature and level of toxicity) can potentially damage the shoot cells but on metabolism to more polar compounds, for example by cytochrome P450, they can be stored within the vacuole or lignin components (Brazier-Hicks et al., 2009). The continuous influx of pollutants usually results in a build up of pollutant metabolites at higher concentrations than in the shoots (Fassler et al., 2010).

In view of the scarcity of toxicity data for 2,3-DNT and 3,4-DNT compared to the well researched 2,6-DNT and 2,4-DNT in *A. thaliana* it was decided to carry out a comparative study of all four DNT isomers.

Keeping in focus the above description, the following hypothesis were suggested for the present study,:

- *A. thaliana* has different tolerance levels for different isomers of DNTs.
- The toxicities of different isomers to *A. thaliana* vary with the nature of the growth media.
- The DNTs present in the root solutions have different toxicities for shoots compared to the roots.
- As exposure time to DNTs is increased, root growth is negatively affected.

Based on the hypotheses as described above, the aims of this study are therefore to

- Evaluate the toxicities of all isomers of DNT in *A. thaliana* grown in Phytagel and hydroponic media.

- Analyse root and shoot growth in *A. thaliana* grown in DNT amended Phytigel and hydroponic media.
- Carry out a time course study for *A. thaliana* grown in DNT amended hydroponic media to evaluate the affect of DNT.

2.2 Material and Methods:

2.2.1. Chemicals:

2,4-DNT (purity $\geq 98\%$), 2,3-DNT (purity $\geq 99\%$), 2,6-DNT (purity $\geq 99\%$) and 3,4-DNT (purity $\geq 99\%$) were purchased from Sigma Aldrich (UK). Acetonitrile (purity $\geq 97.8\%$, HPLC grade) and methanol (purity $\geq 97\%$) were reagent grade and obtained from VWR, UK. Murashige and Skoog salts (MS), Phytigel (P8169), Agrose (A9539), 2-(*N*-morpholino) ethanesulfonic acid (MES) and sucrose (99.8%) were bought from Sigma Aldrich (UK).

The ‘Araponic’ system for the hydroponic plant growth was purchased from Araponics SA, Belgium. The growth media- Flora micro (for root growth) and Flora gro (for biomass) were added to the system. Flora micro contained total nitrogen (5%), ammonical nitrogen 1%, nitrate nitrogen 4%, soluble potassium (K_2O) 1.3%, boron 0.01%, calcium (CaO), copper (chelated EDTA) 0.01%, iron (chelated 6% EDDHH) 0.12%, manganese 0.04%, molybdenum 0.004% and zinc (chelated EDTA) 0.015%. Flora gro contained total nitrogen 1%, ammonical nitrogen 3%, nitrate nitrogen 2%, available phosphate (P_2O_5) 1%, soluble potassium (K_2O) 7% and soluble magnesium (MgO) 0-8%. These nutrients were further diluted (0.75 ml of each Flora micro and Flora gro stock nutrients for each 1.5 L deionised water) for each hydroponic setup.

2.2.2. Bioassays

The toxicities of 2,6-DNT, 2,3-DNT, 2,4-DNT and 3,4-DNT were evaluated for *A. thaliana* grown in DNTs amended Phytagel and hydroponic media through root elongation (for 7 days exposure), root to shoot ratio (for 12 days exposure) and time course root elongation (28 days exposure in hydroponic media).

2.2.2.1 Cultivation of plants

i) Phytagel media

The *A. thaliana* ecotype Columbia 0 (Col-0) seeds were provided by Mrs Nina Grabov (Imperial College, London). The seeds were surface sterilized according to Xiang and Oliver, 1998, where 1 ml of 70% ethanol was added to an Eppendorf tube (2 ml) containing *A. thaliana* seeds and kept for 2 min with occasional vortexing. The supernatant was decanted and 25% sodium hypochlorite was added for 15 min with occasional vortexing and completely drained. The seeds were washed five times with sterile distilled water, and incubated at 4°C for 48 h. These seeds were grown for four days in vertically placed petri plates containing half strength MS, 1% sucrose, 0.025% MES (Sigma UK) and 0.75% Phytagel (P8169, Sigma UK) adjusted to pH 5.7 with 1M KOH in Petri plates under the constant light conditions of 200 $\mu\text{molm}^{-2}\text{s}^{-1}$ (Hanstech Quantitherm lightmeter) maintained by starcoat T5 high output (Hungry fluorescent bulbs) and temperature of 20-23°C for 16:8 h of a day:night period.

ii) Hydroponic media

The hydroponics experiment was performed in a 'Araponics' (Araponics, SA, Belgium system specialised for *A. thaliana* growth). The plants were grown in 'Araponic' containers of length 22.6 cm, width 17.6 cm height 10 cm and total volume of 1.8 L. The nutrient solutions (Flora micro and Flora gro), as provided by the manufacturer (described in section

2.2.1), were tested for their DNT reactivity (i.e. to make sure the diluted nutrient solution doesn't get turbid by the addition of DNT stock solutions) by adding 2ml of 25mg/l 2,6-DNT and 2ml of 0.5 mg/l 2,4-DNT, 2,3-DNT and 3,4-DNT separately in diluted growth media kept at room temperature.

Seeds of *A. thaliana* were placed in seed holders (diameter 2cm, height 2.5cm and filling volume 0.5ml) amended with 0.75% agar (Agrose, molecular biology tested, Sigma, UK) and incubated at 4°C for 48 h. These were then placed under constant light conditions of 200 $\mu\text{molm}^{-2}\text{s}^{-1}$ (Hanstech Quantitherm light meter) and a temperature of 20-23°C for 16:8 h of day:night period in the nutrient solution (Flora micro and Flora gro) amended according to the manufacturers protocol (0.5 ml of each stock nutrient for each 1L solution) for four days.

2.2.2.2 Toxicity evaluation by determining root length

i) Phytigel media

The stock solutions (Table 2.1) were prepared in ethanol (reagent grade, VWR, UK) and further diluted in Phytigel media so that the overall concentration of ethanol in the media was 0.25%.

Table 2.1: Series of dilutions of four isomers of DNTs considered for toxicity evaluation through root elongation of *A. thaliana* in the Phytigel media.

2,6-DNT	2,3-DNT	3,4-DNT	2,4-DNT
(mg /L)	(mg /L)	(mg /L)	(mg /L)
5	0.05	0.05	0.05
10	0.1	0.1	0.1
15	0.25	0.25	0.25
20	0.5	0.5	0.5

25	1	1	1
30	1.5	1.5	1.5
35	2	2	2
40	2.5	2.5	2.5
45	3	3	3
50	3.5	3.5	3.5
55	4	4	4
60	4.5	4.5	4.5
-	5	5	5
-	5.5	5.5	5.5
-	6	6	6

Five replicates were prepared for each nominal concentration of DNTs as described in the Table 2.1. This was achieved by pouring 0.05 ml of stock solutions in Petri plates with a 20 ml mixture of half strength MS, 1% sucrose, 0.025% MES (Sigma UK) and 0.75% Phytigel (P8169, Sigma UK) adjusted to pH 5.7 with 1M KOH. Three four day old *A. thaliana* seedlings, as cultivated earlier in Phytigel media, were placed evenly on one end of the Petri plates and their root tips marked on the outside of the Petri plate. The plates were placed vertically under the constant light conditions of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Hanstech Quantitherm light meter) and temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h of day:night period and allowed to grow under these conditions for 7 days. The ends of the root tips were marked before terminating the

experiment and the root length measured. The data (mean of root length from 15 seedlings) was normalised relative to the control values and data was plotted in GraphPad Prism 5 to determine the EC₅₀ value.

ii) Hydroponic media

The stock solutions (Table 2.1) were prepared in ethanol (reagent grade, VWR, UK) and further diluted in hydroponic media so that the overall concentration of ethanol in the media was 0.25%. Three replicates were prepared by pouring 3.75 ml of a DNT stock solution individually into hydroponic growth medium (0.75 ml of each Flora micro and Flora gro stock nutrients, as described in section 2.2.1, for each 1.5 L deionised water) contained in the Araponic containers. Seedlings that were germinated earlier in the hydroponic media, with were selected for similar root lengths and transferred along with their seed holders into the 'Araponic' containers. The seedlings were allowed to grow for 7 days under the constant light conditions of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Hanstech Quantitherm light meter) and a temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h of day:night period in this system. The initial root length was measured before setting up the experiment and the final increase in root length was measured before terminating the experiment.

The data was normalised relative to the control values and graphs were plotted in GraphPad Prism 5 to determine the EC₅₀ value.

2.2.2.3 Toxicity evaluation through analysis of root and shoot growth

i) Phytigel media

Six replicates were prepared for each concentration of DNT by pouring 0.15 ml of stock solutions (DNTs prepared in ethanol, concentration as described in Table 2.1) in polycarbonate Magenta (GA-7) tissue culture boxes (3×3×4") having 60 ml mixture of half

strength MS, 1% sucrose, 0.025% MES and 0.75% Phytigel (P8169, Sigma UK) adjusted to pH 5.7 with 1M KOH. Two four day old *A. thaliana* seedlings with even root lengths, as cultivated earlier in Phytigel media, were placed evenly in each of these boxes. These boxes were then placed under the constant light conditions of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Hanstech Quantitherm light meter) and temperature of $20 \pm 3^\circ\text{C}$ for 16:8 h of day:night period and allowed to grow under these conditions for 12 days.

After 14 days the plants were harvested from the boxes along with the agar adhered to the roots carefully so that root loss is minimised. The root and shoot were then separated at the base of shoots with a sharp razor. The roots were rinsed in deionised distilled water (Milli Q reagent grade) and gently agitated to remove the adhered agar to minimise any damage. The roots and shoots were blotted dry on filter paper (Whatman, No.2) and their fresh weights measured. Both roots and shoots were freeze dried for 48 h before measuring their dry weights.

ii) Hydroponic media

The procedure for cultivating plants in this experiment was as described earlier in the section 2.2.2.1. Three replicates were prepared by adding 3.75 ml of DNT stock solution individually into the 'Araponic' medium (0.75 ml of each of Flora micro and Flora gro stock nutrient, as described in section 2.2.1, for each 1.5 L deionised distilled water) present in the containers. The seedlings, as germinated earlier in the hydroponic media, with similar root lengths were selected and transferred along with their seed holders into Araponic containers. The seedlings were allowed to grow for 14 days under constant light conditions of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Hanstech Quantitherm light meter) and a temperature of $20 \pm 3^\circ\text{C}$ for 16:8 h of day:night period in this system.

After 14 days the plants were harvested and root and shoot were separated with a sharp razor. The rest of the procedure was followed as described in the section 2.2.2.3 (i).

2.2.2.4 Toxicity evaluation – a time course study in root growth inhibition in hydroponic media

The Arapronics system was set up as previously described (section 2.2.2.1) and a time course study (7, 14, 21 and 28 days) was carried out using different concentrations of DNTs.

After 7 days the root length was measured and data recorded. Then after 14 days the root length was recorded again and difference in root length increase was calculated. A similar procedure was repeated for 21 and 28 days with final root length measured on 28th day.

2.2.3 Statistical Analysis

Data analysis including calculation of average values, calculation of percentage rates and standard deviations were completed using Microsoft Excel 2007. The elongation rates (%) of root length were calculated through Excel 2007 following the equation as below:

$$\text{Root length (\%)} = \frac{[\text{Root length of treated plant}]}{[\text{Root length of untreated plant}]} \times 100 \quad (\text{Eq. i})$$

Non-linear regression (with best curve fit on the basis of $r > 0.9$) analysis was carried out to calculate the EC₅₀ value using GraphPad Prism 5 at 95% Confidence Interval (95% CI). The equation for the calculation of EC₅₀ (GraphPad Prism 5) is as follows:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{EC}_{50} - X) * \text{HillSlope})}) \quad (\text{Eq. ii})$$

EC50 is a halfway response between maximum and minimum values at Y-axis

HillSlope is the steepness of curve. A value of 1 for HillSlope is standard whereas greater than 1 is steeper and lesser than 1 is shallower slope.

Top and **Bottom** are values of the plateaus in the units of the Y axis.

EC10 and EC90 were also calculated through GraphPad Prism 5 at 95% CI. The reason was to give comparison of toxicities at the end of concentrations. The cut-off values (% reduction in root length) were calculated through the formula as follows:

$$(\%) \text{ reduction in root length} = 100 - \text{value from obtained from Eq. } i$$

The Lowest Observable Adverse Effect Concentration (LOAEC) were calculated through the analysis of variance and subsequent Duncan multiple range test at 5% level of significance through ASSISTAT version 7.6 (2011). The % root growths from treated plants were compared with the control and the least value having significant difference with the control data was referred to as LOAEC. The studentized range (SI) for Duncan multiple range test can be calculated as from the equation as follows:

$$R_p = r_p \sqrt{\frac{s^2}{n}}$$

Where R_p = least range significance for subsets of p sample means, r_p = is the least significant studentized range, it varies with the number of means in subsets and degrees of freedom, s = is the error mean from Analysis of variance and n = sample size for individual treatment.

2.3 Results

Preliminary study (visual observation of roots and shoots) showed that *A. thaliana* root and shoot growth is inhibited after the addition of the specified concentrations described in the Table 2.2. The root and shoot growth was stunted on and higher than these concentrations, therefore the concentrations selected for further analysis were equal and below these limits.

Table 2.2: Maximum concentrations (as obtained through visual observation) of four isomers of DNT, which inhibit *A. thaliana* seedling growth in Phytigel and hydroponic growth media. The data was derived from 7 days (post cultivation) seedlings grown at $20\pm 3^{\circ}\text{C}$ for 16:8 h day:night period in growth media treated with different concentrations of DNT isomers. A significant difference ($p < 0.05$) was obtained only when highest inhibition concentration for 2,6-DNT was compared for Phytigel and hydroponic media.

DNT isomers	Highest Concentration inhibiting the root growth (mg /L) for Phytigel media	Highest Concentration inhibiting the root growth (mg /L) for hydroponic media
2,6-DNT	45	60
2,3-DNT	4	4.5
3,4-DNT	4.5	5.5
2,4-DNT	5	6

Addition of DNTs in the plant growth media was visually observed to inhibit the root and shoot growth when compared to control. According to the data as obtained after the analysis, it was found that different isomers of DNT have different toxicities in *A. thaliana* that varies with the nature of the growth medium.

2.3.1 Toxicity evaluation by determining the reduction in root length

2.3.1.1 Toxicities determined in Phytigel media

All toxicity is based on a % reduction in root length (%) compared to a control (untreated plants).

i) Toxicity evaluation for 2,6-DNT

At a 2,6-DNT concentration of 50 mg /L of Phytigel media, the **root growth (Figure 2.1) of *A. thaliana* was totally inhibited**. The least considered 2,6-DNT concentration (5 mg /L of Phytigel media) inhibited the root growth by **15.15±5.43%**. This inhibition in root growth continued to increase with increasing 2,6-DNT concentrations in the media, with **99.53±0.03%** inhibition observed at a 2,6-DNT concentration of 50 mg /L of Phytigel media. The increased inhibition in root growth is reflective of the fact that plants are unable to tolerate relatively high concentrations of 2,6-DNT which are toxic to root cells. The root growth data obtained from the experiment was analysed using GraphPad Prism 5 to determine the EC₅₀ for 2,6-DNT treated *A. thaliana* grown in Phytigel media. The non-linear regression dose vs. response curve ($r^2=0.987$) for variable data showed a decrease in root length with the increase in dose. The analysis showed that EC₅₀ of 2,6-DNT to *A. thaliana* is **13.2 mg /L of Phytigel media**. A significant difference ($p < 0.05$) was obtained for the data after 2,6-DNT treatments of 20 mg /L of Phytigel media after the analysis of variance was applied. This indicates LOAEC for 2,6-DNT in Phytigel media were higher than **3.22 mg /L of Phytigel media**.

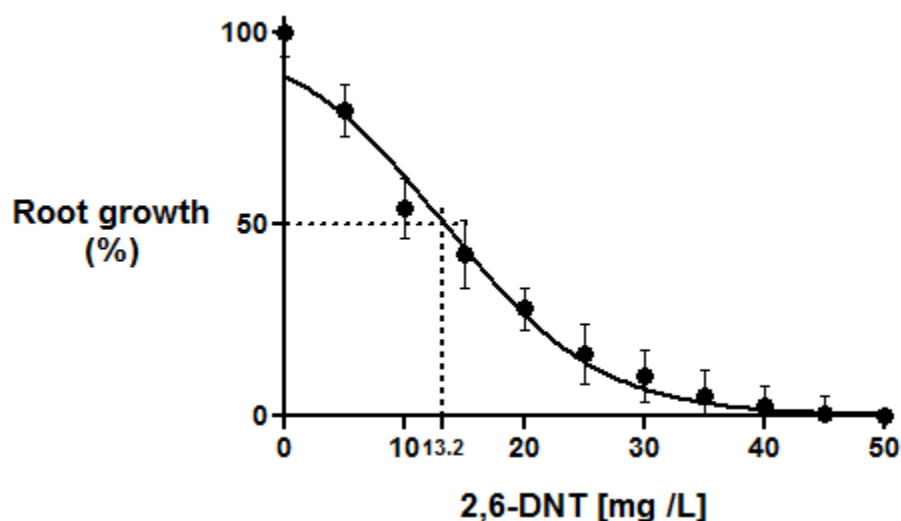


Figure 2.1: EC₅₀ determination of 2,6-DNT on *A. thaliana* by measuring root length (%) of the 7 day old (post cultivation) plants treated with different concentrations of 2,6-DNT (in Phytigel media) compared to control (unamended Phytigel media; absolute root length of 6.4 cm) as analysed through a dose-response curve ($r^2=0.987$) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$); GraphPad Prism 5). The plants were grown at a temperature of $20\pm3^{\circ}\text{C}$ for 16:8 h day:night period. Data points are the mean of roots length from 15 seedlings. The EC₅₀ for 2,6-DNT at which root growth inhibited by 50% of the control was 13.2 mg /L of Phytigel media.

ii) Toxicity evaluation for 2,3-DNT

Compared to control the root length (%) of *A. thaliana* grown in the media treated with 2,3-DNT concentration of 4 mg /L of Phytigel media prevented root growth (Figure 2.2). The lowest considered concentration of 2,3-DNT (0.05 mg /L of Phytigel media) inhibited root growth by $31.58\pm5.41\%$ compared to the root growth in control media. The highest inhibition was observed to be $99.70\pm1.33\%$ compared to the control at a 2,3-DNT concentration of 4 mg /L of Phytigel media, which shows that as the concentration of 2,3-DNT in the media increases the inhibition of root growth starts increasing. The EC₅₀ for 2,3-DNT treated *A. thaliana* grown in Phytigel media through non-linear regression dose vs. response curve ($r^2=0.967$) for variable data (GraphPad Prism 5) was found to be **0.13 mg /L of Phytigel media**. Since a significant difference ($p < 0.05$) was obtained for the data after

2,3-DNT treatments of 1 mg /L of Phytagel media compared to control after the analysis of variance was applied, therefore the LOAEC values for 2,3-DNT were higher than **0.02 mg /L of Phytagel media**.

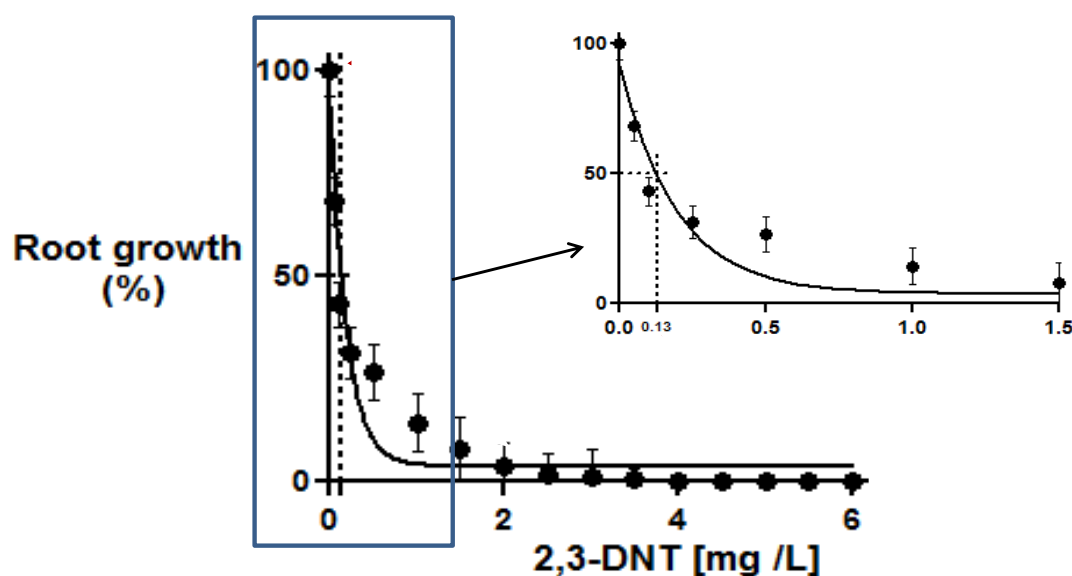


Figure 2.2: EC₅₀ determination of 2,3-DNT on *A. thaliana* by measuring root length (%) of the 7 day old (post cultivation) plants treated with different concentrations of 2,3-DNT (in Phytagel media) compared to control (unamended Phytagel media; absolute root length of 6.6 cm) as analysed through a dose-response curve ($r^2=0.967$) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$); GraphPad Prism 5). The plants were grown at a temperature of $20\pm3^{\circ}\text{C}$ for 16:8 h day:night period. Data points are the mean of roots length from 15 seedlings. The EC₅₀ for 2,3-DNT at which root growth inhibited by 50% of the control was 0.13 mg /L of Phytagel media.

iii) Toxicity evaluation for 3,4-DNT

The root length (%) data of *A. thaliana* grown in the media treated with 3,4-DNT showed that a 3,4-DNT concentration of 4.5 mg /L of Phytagel media inhibited root growth (Figure 2.3).

The lowest considered concentration of 3,4-DNT (0.05 mg /L of Phytagel media) reduced root growth by $14.69\pm6.29\%$ to that of the control. This reduction in root length increased with increasing 3,4-DNT concentrations in the media and reaching $99.69\pm1.66\%$ for the highest considered 3,4-DNT concentration (4.5 mg /L of Phytagel media) in the media. The

root growth data was analysed in the same way as in the previous section. The analysis showed that EC_{50} of 3,4-DNT to *A. thaliana* is **0.23 mg /L of Phytigel media**. The data obtained against 3,4-DNT concentration of 1.5 mg /L of Phytigel media was observed to have significant difference ($p < 0.05$) compared to control after the application of analysis of variance, therefore the LOAEC for 3,4-DNT were higher than - **0.02 mg /L of Phytigel media**.

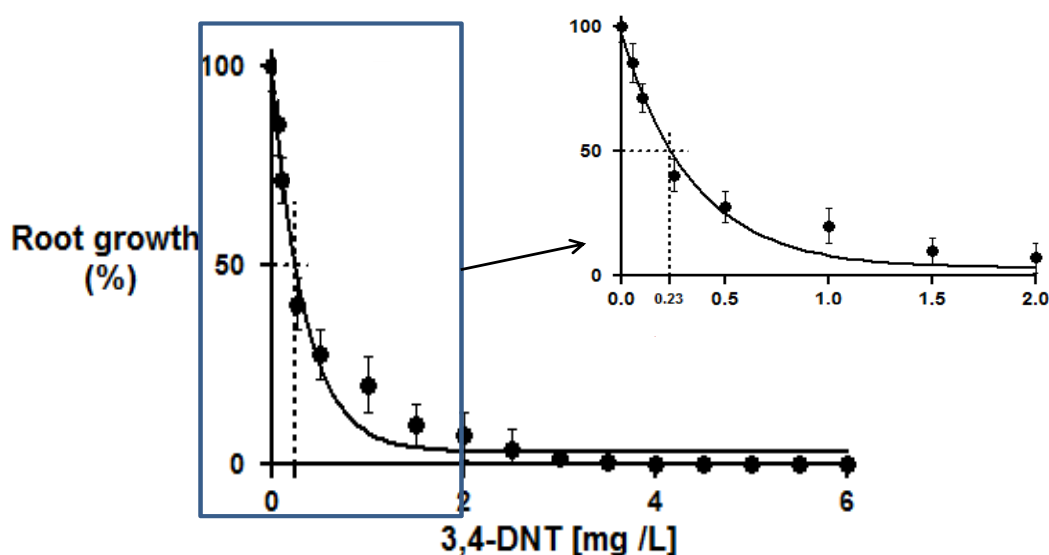


Figure 2.3: EC_{50} determination of 3,4-DNT on *A. thaliana* by measuring root length (%) of the 7 day old (post cultivation) plants treated with different concentration of 3,4-DNT (in Phytigel media) compared to control (unamended Phytigel media; absolute root length of 7.02 cm) as analysed through a dose-response curve ($r^2=0.983$) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$; GraphPad Prism 5). The plants were grown at a temperature of $20\pm3^{\circ}C$ for 16:8 h day:night period. Data points are the mean of roots length from 15 seedlings. The EC_{50} for 3,4-DNT at which root growth inhibited by 50% of the control was 0.23 mg /L of Phytigel media.

iv) Toxicity evaluation for 2,4-DNT

Figure 2.4 shows that the root length (%) data of *A. thaliana* grown in the media at a 2,4-DNT concentration of 5.5 mg /L of Phytigel media completely inhibited root growth. The

lowest considered concentration of 2,4-DNT reduced root growth of *A. thaliana* by $16.01 \pm 6.11\%$ compared to the root growth in control media, which increased to $99.43 \pm 1.56\%$ against the 2,4-DNT concentration of 5.5 mg /L of Phytigel media. This value shows that 2,4-DNT is very toxic at high concentrations and damage root cells. The data obtained for root growth *A. thaliana* treated with 2,4-DNT was analysed in the same way as previous DNTs giving an EC_{50} value of **0.33 mg /L of Phytigel media** for 2,4-DNT. A significant difference ($p < 0.05$) obtained after 2,4-DNT concentration of 2 mg /L in the Phytigel media showed that LOAEC values were higher than **0.03 mg /L of Phytigel media**.

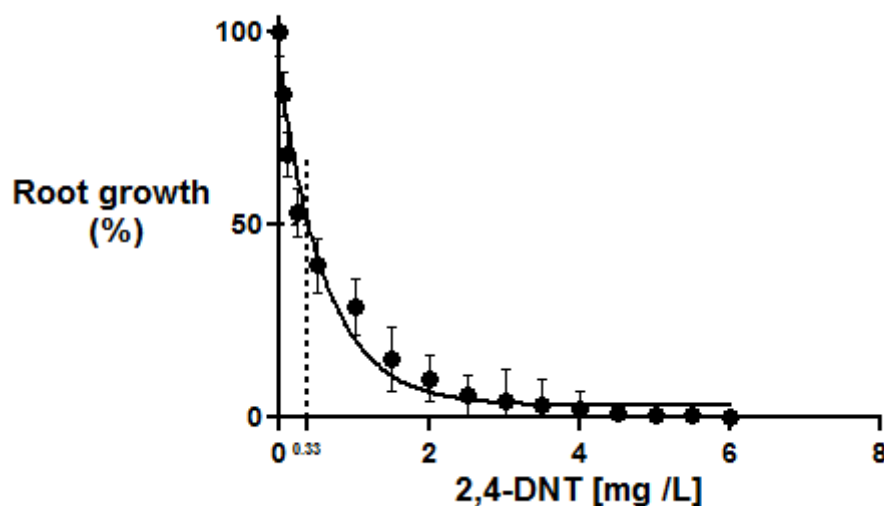


Figure 2.4: EC_{50} determination of 2,4-DNT on *A. thaliana* by measuring root length (%) of the 7 day old (post cultivation) plants treated with different concentrations of 2,4-DNT (in Phytigel media; absolute root length of 6.87 cm) compared to control (unamended Phytigel media) as analysed through a dose-response curve ($r^2=0.977$) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$; GraphPad Prism 5). The plants were grown at a temperature of $20 \pm 3^\circ C$ for 16:8 h day:night period. Data points are the mean of roots length from 15 seedlings. The EC_{50} for 2,4-DNT at which root growth inhibited by 50% of the control was 0.33 mg /L of Phytigel media.

2.3.2.2 Toxicities evaluation in hydroponic media

i) Toxicity evaluation for 2,6-DNT

A visual examination of the plants for root toxicity showed that at a 2,6-DNT concentration of 60 mg /L of hydroponic media growth was completely inhibited. The root length (%) measured (Figure 2.5) showed that at 2,6-DNT concentration of 50 mg /L of hydroponic media root growth was reduced by $97.24 \pm 0.34\%$ compared to the control. At the lowest concentration of 2,6-DNT (5 mg /L of hydroponic media) root length was reduced by $20.12 \pm 5.64\%$ to that of the control. The EC_{50} , as calculated by the method described above, for 2,6-DNT treated *A. thaliana* grown in hydroponic media was **24.8 mg /L of hydroponic media**. The LOAEC values were found to be higher than **2.56 mg /L of hydroponic media** ($p < 0.05$ compared to control) after the analysis of variance was applied on the all the data obtained.

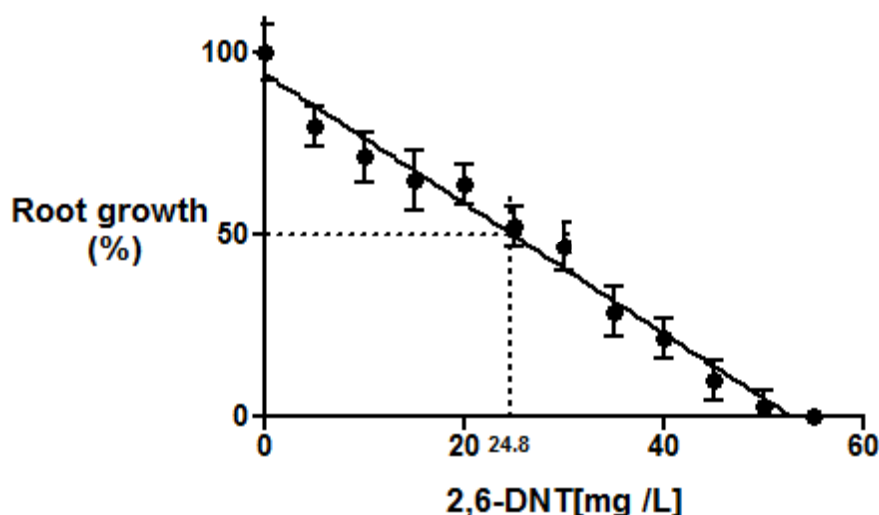


Figure 2.5: EC_{50} determination of 2,6-DNT on *A. thaliana* by measuring root length (%) of the 7 day old (post cultivation) plants treated with different concentrations of 2,6-DNT (in hydroponic media) compared to control (unamended Phytigel media; absolute root length of 12.34 cm) as analysed through a dose-response curve ($r^2=0.981$) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$); GraphPad Prism 5). The plants were grown at a temperature of $20 \pm 3^\circ C$

for 16:8 h day:night period. Data points are the mean of roots length from 15 seedlings. The EC_{50} for 2,6-DNT at which root growth inhibited by 50% of the control was 24.8 mg /L of hydroponic media.

ii) Toxicity evaluation for 2,3-DNT

No root growth was observed in *A. thaliana* against the maximum (6 mg /L of hydroponic media) tested 2,3-DNT concentration, which indicates that 2,3-DNT is very toxic that even such a low concentration can inhibit the root growth completely (Figure 2.6). The root inhibition (%) compared to control against the least considered concentration of 2,3-DNT of 0.05 mg /L of hydroponic media was $68.78 \pm 7.66\%$ that increased with the increase in 2,3-DNT concentration in the media and reached an amount of $98.24 \pm 1.32\%$ compared to control after 2,3-DNT concentration of 5.5 mg /L of hydroponic media. The EC_{50} , calculated through non-linear regression analysis of dose- response curve (GraphPad Prism 5), for 2,3-DNT was found to be to be **1.12 mg /L of hydroponic media**. The LOAEC value found through the analysis of variance on the all the data obtained for 2,3-DNT treated *A. thaliana* compared to control were higher than **0.01 mg /L of hydroponic media** ($p < 0.05$).

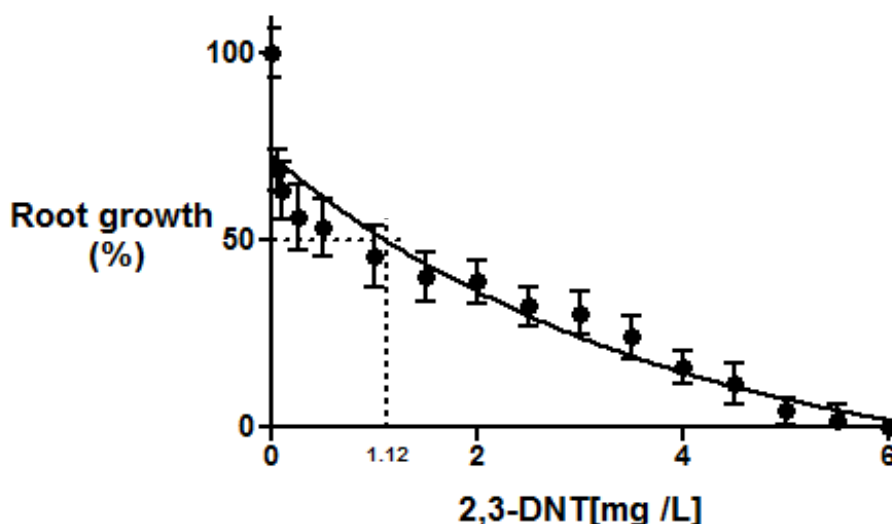


Figure 2.6: EC_{50} determination of 2,3-DNT on *A. thaliana* by measuring root length (%) of the 7 days old (post cultivation) plants treated with different concentrations of

2,3-DNT (in hydroponic media) compared to control (unamended Phytigel media; absolute root length of 12.37 cm) as analysed through a dose-response curve ($r^2=0.92$) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$; GraphPad Prism 5). The plants were grown at a temperature of $20\pm3^{\circ}\text{C}$ for 16:8 h day:night period. Data points are the mean of roots length from 15 seedlings. The EC_{50} for 2,3-DNT at which root growth inhibited by 50% of the control was 1.12 mg /L of hydroponic media.

iii) Toxicity evaluation for 2,4-DNT

At a 2,4-DNT concentration of 6 mg /L of hydroponic media, no root growth was observed.

The least considered concentration of 2,4-DNT (**0.05 mg /L of hydroponic media**) reduced the root growth by **$16.01\pm7.83\%$** compared to the root growth in control media, which increased to **$97.31\pm3.22\%$** by 2,4-DNT concentration of 5.5 mg /L of hydroponic media.

Analysis of the data gave an EC_{50} value of **1.63 mg /L of hydroponic media**. The LOAEC values were found to be higher than **0.05 mg /L of hydroponic media** of 2,4-DNT ($p < 0.05$) after the analysis of variance on the all the data obtained for 2,4-DNT treated *A. thaliana* compared to control.

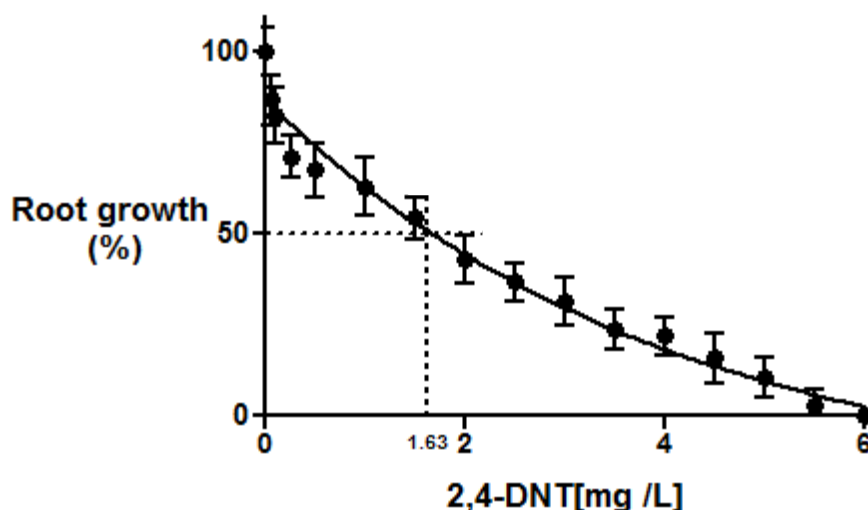


Figure 2.7: EC_{50} determination of 2,4-DNT on *A. thaliana* by measuring root length (%) of the 7 day old (post cultivation) plants treated with different concentrations of 2,4-DNT (in hydroponic media) compared to control (unamended Phytigel media; absolute root length of 12.54 cm) as analysed through a dose-response curve ($r^2=0.976$) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$).

$X) * HillSlope))$; GraphPad Prism 5). The plants were grown at a temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period. Data points are the mean of roots length from 15 seedlings. The EC_{50} for 2,4-DNT at which root growth inhibited by 50% of the control was 1.63 mg /L of hydroponic media.

iv) Toxicity evaluation for 3,4-DNT

Figure 2.8 shows that the root length (%) data of *A. thaliana* grown in the media treated with 3,4-DNT concentration of 6 mg /L of hydroponic media inhibited root growth. The least considered concentration of 3,4-DNT (0.05 mg /L of hydroponic media) reduced the root growth by $18.47 \pm 8.22\%$ compared to the root growth in control media. Increasing concentrations of 3,4-DNT concentration in the media decreased the root length and at a concentration of 5.5 mg /L of hydroponic media a maximum reduction of $98.21 \pm 0.21\%$ was obtained. The analysis of data through the dose-response curve fit using non-linear regression gave an EC_{50} value for 3,4-DNT of 1.39 mg /L of hydroponic media. The LOAEC value found through the analysis of variance on the all the data obtained for 3,4-DNT treated *A. thaliana* compared to control were higher than 0.01 mg /L of hydroponic media ($p < 0.05$).

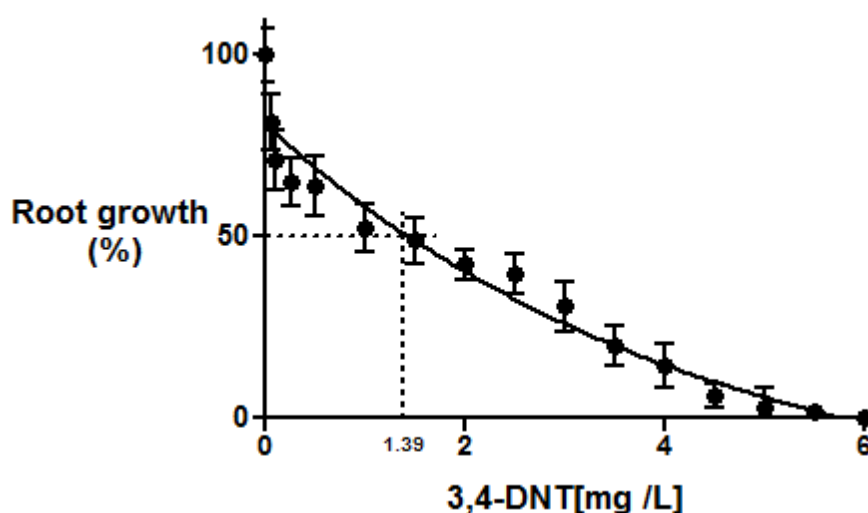


Figure 2.8: EC_{50} determination of 3,4-DNT on *A. thaliana* by measuring root length (%) of the 7 day old (post cultivation) plants treated with different concentrations of 3,4-DNT (in hydroponic media) compared to control (unamended Phytigel media absolute root length of 12.55 cm) as analysed through a dose-response curve ($r^2=0.953$)

fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$); GraphPad Prism 5). The plants were grown at a temperature of $20\pm 3^{\circ}\text{C}$ for 16:8 h day:night period. Data points are the mean of roots length from 15 seedlings. The EC_{50} for 3,4-DNT at which root growth inhibited by 50% of the control was 1.39 mg /L of hydroponic media.

Overall toxicities:

The toxicity of DNT isomers in Phytigel and hydroponic media showed that 2,3-DNT is most toxic however 2,6-DNT is least toxic (Table 2.3). According to EC_{10} , EC_{50} and EC_{90} the toxicities of the isomers vary in the order of 2,6-DNT < 2,4-DNT < 3,4-DNT < 2,3-DNT .

Table 2.3: Estimation of EC_{10} , EC_{50} and EC_{90} and CI 95% of four DNT isomers for *A. thaliana* cultivated in Phytigel and non-axenic hydroponic media. Calculations were performed using a dose-response curve ($r^2 > 0.9$) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$); GraphPad Prism 5).

EC values (mg /L) for <i>A. thaliana</i> in Phytigel media						
DNT isomers	EC_{10}	CI 95%	EC_{50}	CI 95%	EC_{90}	CI 95%
2,6-DNT	3	0.45-7.62	13.3	2.33-18.54	30	16.45-45.61
2,3-DNT	0.02	0.004-1.21	0.13	0.14-1.24	0.83	0.14- 1.45
3,4-DNT	0.02	0.012-1.24	0.23	0.06-1.45	0.96	0.45-2.21
2,4-DNT	0.03	0.014- 0.14	0.33	0.07-1.43	1.55	0.34-3.41
EC values (mg /L) for <i>A. thaliana</i> in hydroponic media						
DNT	EC_{10}	CI 95%	EC_{50}	CI 95%	EC_{90}	CI 95%

isomers

2,6-DNT	2.4	1.24- 4.52	24.8	12.13- 30.24	48	17.45- 54.73
2,3-DNT	0.01	0.01-2.44	1.12	0.87- 2.31	4.64	2.4-6.54
3,4-DNT	0.01	0.005-1.17	1.39	0.82-3.62	4.69	2.2-5.63
2,4-DNT	0.05	0.03-4.24	1.63	0.1-4.32	5.01	3.1-6.44

*EC= Effective dose

**CI= Confidence Interval

2.3.2 Toxicity evaluation by measurement of root and shoot biomass

The root and shoot biomass was evaluated as an alternate method of assessment along with the root length. The roots have direct contact with the DNT containing roots solution. Therefore, it was speculated that *A. thaliana* shoots can tolerate higher concentrations of DNTs in hydroponic and Phytigel media compared to roots.

2.3.2.1 Phytigel media

i) Root shoot growth *A. thaliana* treated with 2,6-DNT

The analytical data of Figure 2.9 shows that *A. thaliana* shoot can tolerate very high concentrations of 2,6-DNT in the Phytigel media. The addition of 2,6-DNT in the media caused an reduction in both root mass ($14.52 \pm 3.42\%$) and shoot ($11.61 \pm 4.14\%$) mass compared to the root and shoot mass in the control media against the least considered concentration of 2,6-DNT (5 mg /L of Phytigel media), which increased with the increase in 2,6-DNT concentration in the media and reached to an inhibition value of $98.20 \pm 3.44\%$ for

shoot mass compared to control corresponding to 2,6-DNT concentration of 55 mg /L of Phytigel media. The highest inhibition in root mass was $92.72 \pm 4.33\%$ obtained from plants at 2,6-DNT concentration of 45 mg /L of Phytigel media. No root growth was observed beyond this concentration of 2,6-DNT in the media.

The analysis of variance and subsequent Duncan test showed the LOAEC values for 2,6-DNT concentration were higher than 30 mg /L and 35 mg /L of Phytigel media for root and shoot data respectively compared to control (significant difference ($p < 0.05$) was observed when analysis of variance was applied).

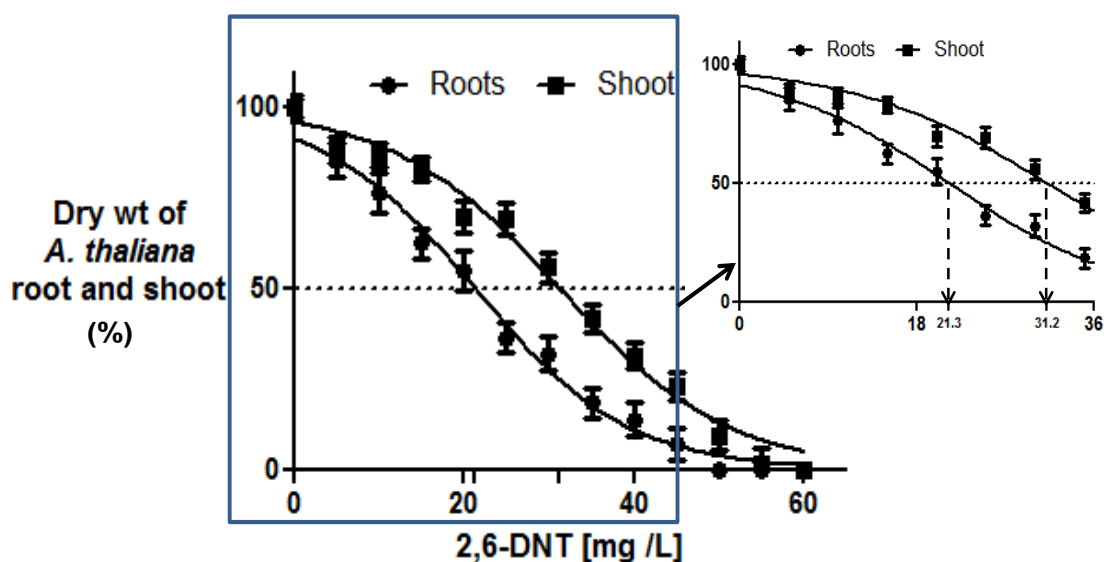


Figure 2.9: EC₅₀ determination of 2,6-DNT on *A. thaliana* by measuring dry wt (mg) of root and shoot of the 14 day old (post cultivation) plants treated with different concentrations of 2,6-DNT (in Phytigel media) compared to control (unamended Phytigel media) as analysed through a dose-response curve ($r^2=0.98$ for root data and $r^2=0.978$ for shoot data) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$; GraphPad Prism 5). The plants were grown at a temperature of $20 \pm 3^\circ\text{C}$ for 16:8 h day:night period. Data points are the mean of root and shoot dry wt. from 12 seedlings. The EC₅₀ for 2,6-DNT at which root mass inhibited by 50% of the control was 21.3 mg /L and shoot mass inhibited by 50% of the control was 31.2 mg /L of Phytigel media.

ii) Root shoot growth *A. thaliana* treated with 2,4-DNT

The inhibition in root and shoot mass against the least considered 2,4-DNT concentration of 0.05 mg /L of Phytigel media was $14.32 \pm 4.11\%$ and shoot mass was $12.22 \pm 3.91\%$ compared to the root and shoot mass in the control media. This value then increased with the increase in 2,4-DNT concentration in the media and reached to the reduction by $98.2 \pm 3.44\%$ for shoot mass corresponding to 2,4-DNT concentration of 6 mg /L of Phytigel media and by $94.12 \pm 3.51\%$ at the 5.5 mg /L of Phytigel media. No root mass was obtained at 6 mg /L of Phytigel media.

The EC_{50} value obtained through non-linear regression analysis of dose-response curve through GraphPad Prism 5 was **1.74 mg /L of Phytigel media** for roots and **2.22 mg /L of Phytigel media** for shoots. The LOAEC as calculated through the analysis of variance and subsequent Duncan's test was found to higher then **0.03 mg /L of Phytigel media** ($p < 0.05$) for both root and shoot data.

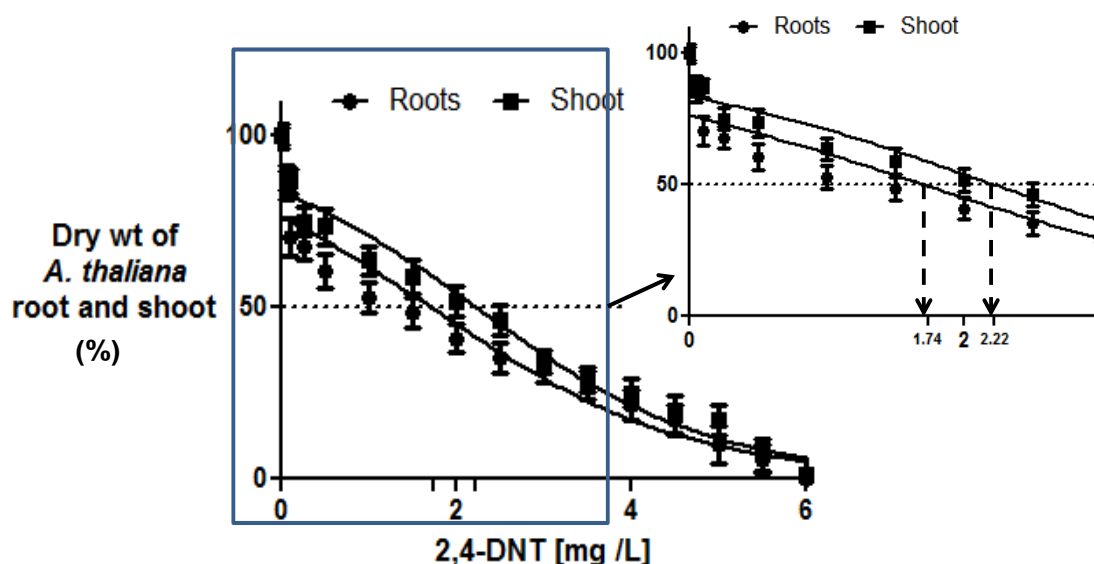


Figure 2.10: EC_{50} determination of 2,4-DNT on *A. thaliana* by measuring dry wt (mg) of root and shoot of the 14 day old (post cultivation) plants treated with different concentrations of 2,4-DNT (in Phytigel media) compared to control (unamended Phytigel media) as analysed through a dose-response curve ($r^2=0.977$ for root data and

$r^2=0.981$ for shoot data) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$; GraphPad Prism 5). The plants were grown at a temperature of $20\pm3^{\circ}\text{C}$ for 16:8 h day:night period. Data points are the mean of root and shoot dry wt. from 12 seedlings. The EC_{50} for 2,4-DNT at which root mass inhibited by 50% of the control was 1.74 mg /L and shoot mass inhibited by 50% of the control was 2.22 mg /L of Phytigel media.

iii) Root shoot growth *A. thaliana* treated with 3,4-DNT

The analytical data in Figure 2.11 shows that addition of 3,4-DNT caused an inhibition in root and shoot mass with the inhibition of $18.24\pm3.81\%$ in root mass compared to control and inhibition of $21.31\pm3.66\%$ in shoot mass against the least considered 3,4-DNT concentration of 0.05 mg /L of Phytigel media compared to the root and shoot mass in the control media. These values then increased with the increase in 3,4-DNT concentration in the media and reached to an inhibition value of $99.17\pm1.42\%$ for shoot mass compared to control corresponding to 3,4-DNT concentration of 6 mg /L of Phytigel media. No root mass was obtained at this concentration.

The EC_{50} value obtained through non-linear regression analysis of dose-response curve through GraphPad Prism 5 was 2.45 mg /L for shoots and 1.92 mg /L of Phytigel media for roots. The LOAEC values for root and shoot data as found through the analysis of variance were higher than 0.02 mg /L of Phytigel media.

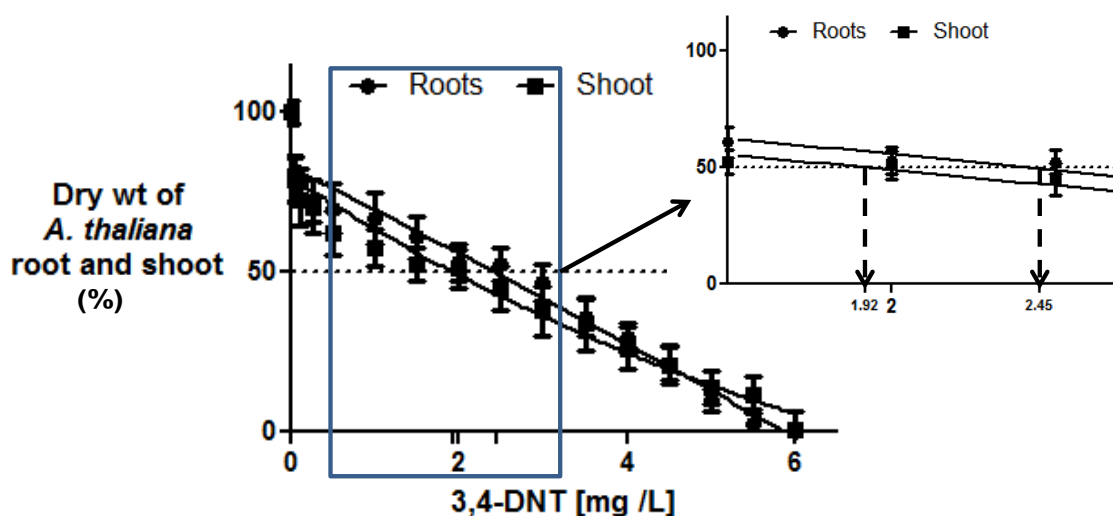


Figure 2.11: EC₅₀ determination of 3,4-DNT on *A. thaliana* by measuring dry wt (mg) of root and shoot of the 14 day old (post cultivation) plants treated with different concentrations of 3,4-DNT (in Phytigel media) compared to control (unamended Phytigel media) as analysed through a dose-response curve ($r^2=0.967$ for root data and $r^2=0.974$ for shoot data) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC_{50}-X)*HillSlope)})$; GraphPad Prism 5). The plants were grown at a temperature of $20\pm3^{\circ}\text{C}$ for 16:8 h day:night period. Data points are the mean of root and shoot dry wt. from 12 seedlings. The EC₅₀ for 3,4-DNT at which root mass inhibited by 50% of the control was 2.45 mg /L and shoot mass inhibited by 50% of the control was 1.92 mg /L of Phytigel media.

iv) Root shoot growth in *A. thaliana* treated with 2,3-DNT

The trend of root and shoot growth from Figure 2.12 in 2,3-DNT treated *A. thaliana* plants shows that *A. thaliana* shoot can tolerate very high concentrations of 2,3-DNT (4.5 mg /L of Phytigel media) in the growth media compared to which no root growth was observed corresponding to same concentration. No root and shoot growth was observed at highest tested concentration of 2,3-DNT (5, 5.5 and 6 mg /L of Phytigel media) in the growth media. The analytical data in Figure 2.12 shows that addition of 2,3-DNT caused an inhibition in root and shoot mass with the inhibition of **$22.56\pm3.89\%$** in root mass compared to control and inhibition of **$13.24\pm4.26\%$** in shoot mass obtained against 2,3-DNT concentration of 0.05 mg /L of Phytigel media compared to the root and shoot mass in the control media. These values then increased with the increase in 2,3-DNT concentration in the media and reached to an inhibition value of **$94.45\pm1.61\%$** for shoot mass and **$96.69\pm1.23\%$** for root mass compared to control corresponding to 2,3-DNT concentration of 4.5 mg /L and 4 mg /L of Phytigel media.

The EC₅₀ value obtained through non-linear regression analysis of dose-response curve through GraphPad Prism 5 was **0.86 mg /L** for roots and **1.48 mg /L of Phytigel media** for shoots. The LOAEC was found to be higher than 2,3-DNT concentration of **0.02 mg /L of Phytigel media** for both root and shoot data as obtained through analysis of variance.

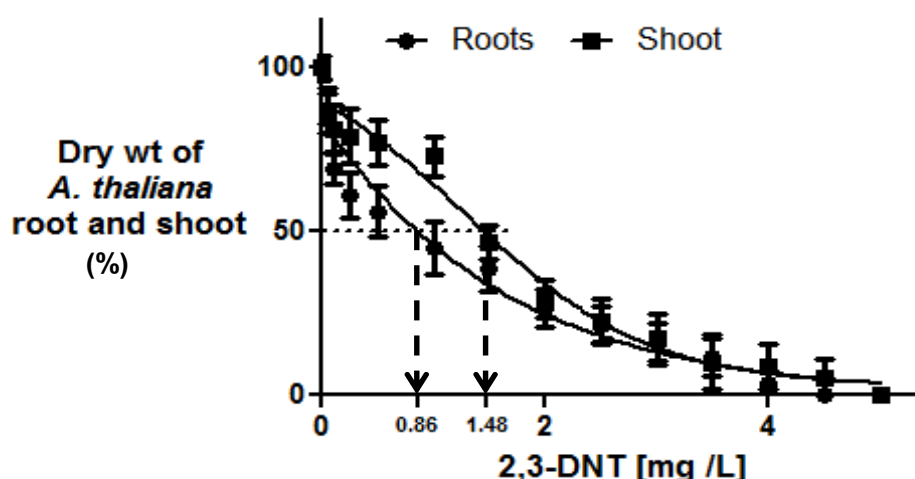


Figure 2.12: EC₅₀ determination of 2,3-DNT on *A. thaliana* by measuring dry wt (mg) of root and shoot of the 14 day old (post cultivation) plants treated with different concentrations of 2,3-DNT (in Phytigel media) compared to control (unamended Phytigel media) as analysed through a dose-response curve ($r^2=0.968$ for root data and $r^2=0.972$ for shoot data) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$); GraphPad Prism 5). The plants were grown at a temperature of $20\pm3^{\circ}\text{C}$ for 16:8 h day:night period. Data points are the mean of root and shoot dry wt. from 12 seedlings. The EC₅₀ for 2,3-DNT at which root mass inhibited by 50% of the control was 0.86 mg /L and shoot mass inhibited by 50% of the control was 1.48 mg /L of Phytigel media.

2.3.2.2 Hydroponic media

The toxicity evaluation of root and shoot growth in *A. thaliana* treated with different concentrations of the DNT isomers in non-axenic hydroponic media indicated that the all DNT isomers have varied toxicities to *A. thaliana* with 2,6-DNT was found to be least toxic and 2,3-DNT most toxic in hydroponic medium.

i) Root shoot growth in *A. thaliana* treated with 2,6-DNT

The analytical data of Figure 2.13 shows that *A. thaliana* shoot can tolerate very high concentrations of 2,6-DNT in the hydroponic media. The addition of 2,6-DNT in the media caused an inhibition in both root mass ($2.27\pm3.89\%$) and shoot ($5.88\pm3.91\%$) mass

compared to the root and shoot mass in the control media against the least considered concentration of 2,6-DNT (5 mg /L of hydroponic media), which increased with the increase in 2,6-DNT concentration in the media.

The mass of root throughout the tested concentrations of 2,6-DNT in the hydroponic media was comparatively less than mass of shoots therefore the EC_{50} value obtained through non-linear regression analysis of dose-response curve through GraphPad Prism 5 was **30.9 mg /L** for shoots and **23.3 mg /L of hydroponic media** for roots. The LOAEC as calculated through the analysis of variance and subsequent Duncan test was higher than **2.44 mg /L of hydroponic media** for root data and **2.91 mg /L of hydroponic media** for shoot data.

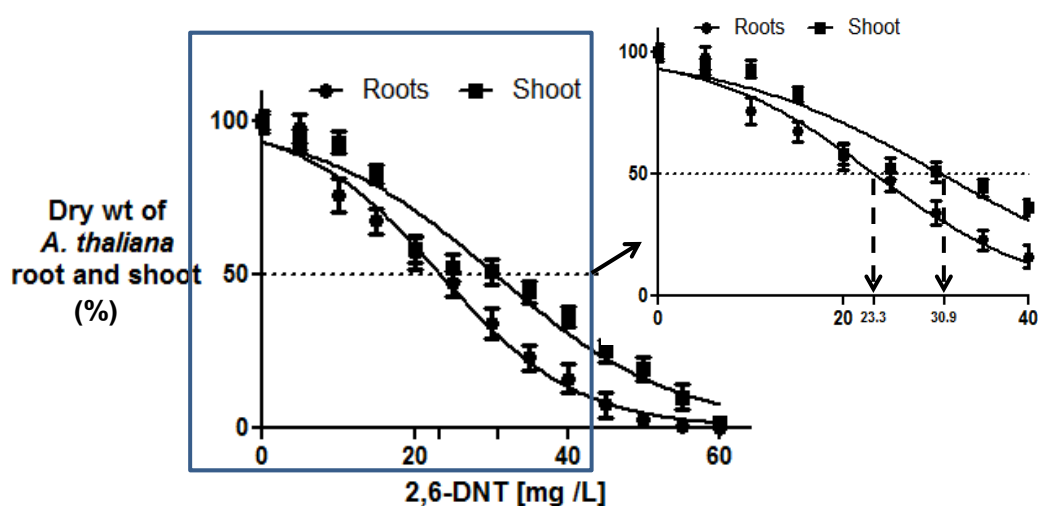


Figure 2.13: EC_{50} determination of 2,6-DNT on *A. thaliana* by measuring dry wt (mg) of root and shoot of the 14 day old (post cultivation) plants treated with different concentrations of 2,6-DNT (in hydroponic media) compared to control (unamended hydroponic media) as analysed through a dose-response curve ($r^2=0.982$ for root data and $r^2=0.981$ for shoot data) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC_{50}-X)*HillSlope)})$; GraphPad Prism 5). The plants were grown at a temperature of $20\pm3^{\circ}C$ for 16:8 h day:night period. Data points are the mean of root and shoot dry wt. from 12 seedlings. The EC_{50} for 2,6-DNT at which root mass inhibited by 50% of the control was 23.3 mg /L and shoot mass inhibited by 50% of the control was 30.9 mg /L of hydroponic media.

ii) Root shoot growth in *A. thaliana* treated with 2,4-DNT

The inhibition in root and shoot mass against the least considered 2,4-DNT concentration of 0.05 mg /L of hydroponic media was $12.35 \pm 4.01\%$ and shoot mass was $14.25 \pm 3.45\%$ compared to the root and shoot mass in the control media (Figure 2.14). This value then increased with the increase in 2,4-DNT concentration in the media and reached to an inhibition value of $98.82 \pm 1.54\%$ for shoot mass compared to control corresponding to 2,4-DNT concentration of 6 mg /L of hydroponic media. The highest inhibition in root mass was $98.92 \pm 3.88\%$ obtained at the 2,4-DNT concentration of 5.5 mg /L of hydroponic media. No root growth was observed after this concentration of 2,4-DNT in the media.

The EC_{50} value obtained through non-linear regression analysis of dose-response curve through GraphPad Prism 5 was 1.74 mg /L for shoots and 1.5 mg /L of hydroponic media for roots. The LOAEC as calculated through the analysis of variance and subsequent Duncan test was higher than **0.05 mg /L of hydroponic media** for both root and shoot data.

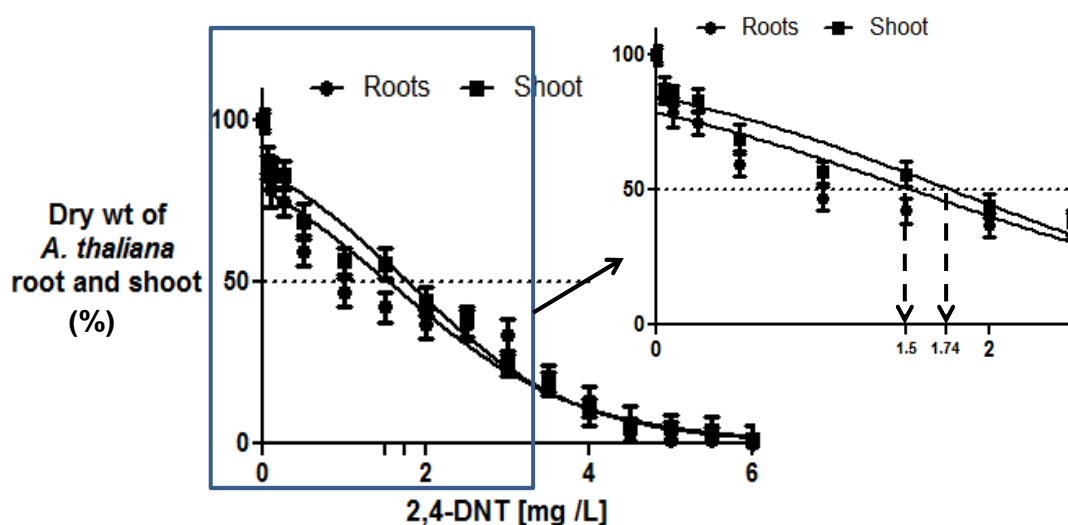


Figure 2.14: EC₅₀ determination of 2,4-DNT on *A. thaliana* by measuring dry wt (mg) of root and shoot of the 14 day old (post cultivation) plants treated with different concentrations of 2,4-DNT (in hydroponic media) compared to control (unamended hydroponic media) as analysed through a dose-response curve ($r^2=0.971$ for root data and $r^2=0.969$ for shoot data) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$; GraphPad Prism 5). The plants were grown at a temperature of 20 ± 3^0C for 16:8 h day:night period. Data points are the mean of root and shoot dry wt. from 12 seedlings. The EC₅₀ for 2,4-DNT at which root mass inhibited by 50% of the control was 1.5 mg /L and shoot mass inhibited by 50% of the control was 1.74 mg /L of hydroponic media.

iii) Root shoot growth in *A. thaliana* treated with 3,4-DNT

The analytical data in Figure 2.15 shows the trend in reduction of root and shoot mass with the increase of 3,4-DNT in the media compared to the root and shoot growth in the control media. The EC₅₀ value obtained through non-linear regression analysis of dose-response curve through GraphPad Prism 5 was **1.45 mg /L** for roots and **1.54 mg /L of hydroponic media** for shoots. The LOAEC values for root and shoot data were higher than **0.01mg /L of hydroponic media** ($p < 0.05$).

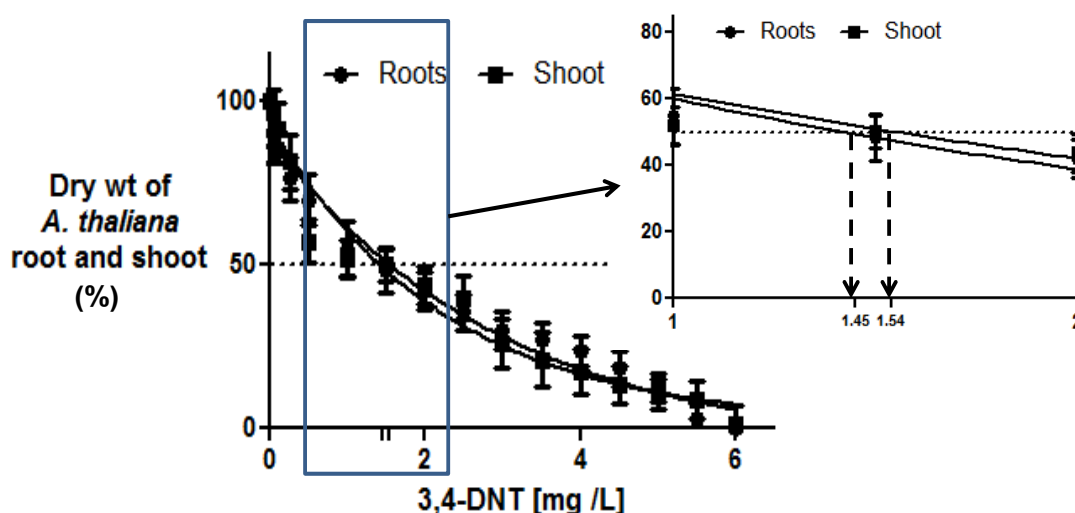


Figure 2.15: EC₅₀ determination of 3,4-DNT on *A. thaliana* by measuring dry wt (mg) of root and shoot of the 14 day old (post cultivation) plants treated with different concentrations of 3,4-DNT (in hydroponic media) compared to control (unamended hydroponic media) as analysed through a dose-response curve ($r^2=0.968$ for root data and $r^2=0.952$ for shoot data) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$; GraphPad Prism 5). The plants were grown at a

temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period. Data points are the mean of root and shoot dry wt. from 12 seedlings. The EC_{50} for 3,4-DNT at which root mass inhibited by 50% of the control was 1.45 mg /L and shoot mass inhibited by 50% of the control was 1.54 mg /L of hydroponic media.

Iv) Root shoot growth in *A. thaliana* treated with 2,3-DNT

The analytical data in Figure 2.16 shows that addition of 2,3-DNT caused an inhibition in root and shoot mass with the inhibition of $23.9 \pm 3.44\%$ in root mass compared to control and inhibition of $6.79 \pm 4.72\%$ in shoot mass obtained against 2,3-DNT concentration of 0.05 mg /L of hydroponic media compared to the root and shoot mass in the control media. These values then increased with the increase in 2,3-DNT concentration in the media and reached to reduction in biomass by $98.2 \pm 1.34\%$ for shoot mass and $93.83 \pm 1.65\%$ for root mass compared to control corresponding to 2,3-DNT concentration of 4.5 mg /L and 4 mg /L of hydroponic media. The EC_{50} value obtained through non-linear regression analysis of dose-response curve through GraphPad Prism 5 was 1.26 mg /L for roots and 1.51 mg /L of hydroponic media for shoots. The LOAEC values were found to be higher than 0.01 mg /L of hydroponic media ($p < 0.05$ compared to control) after analysis of variance and subsequent Duncan test.

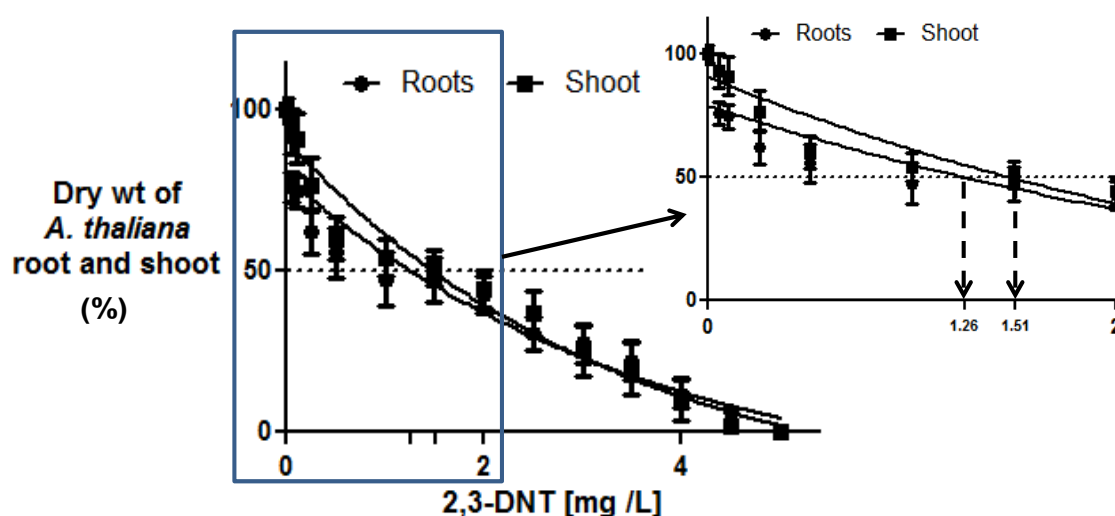


Figure 2.16: EC₅₀ determination of 2,3-DNT on *A. thaliana* by measuring dry wt (mg) of root and shoot of the 14 day old (post cultivation) plants treated with different concentrations of 2,3-DNT (in hydroponic media) compared to control (unamended hydroponic media) as analysed through a dose-response curve ($r^2=0.954$ for root data and $r^2=0.942$ for shoot data) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$); GraphPad Prism 5). The plants were grown at a temperature of $20\pm3^\circ\text{C}$ for 16:8 h day:night period. Data points are the mean of root and shoot dry wt. from 12 seedlings. The EC₅₀ for 2,3-DNT at which root mass inhibited by 50% of the control was 1.26 mg /L and shoot mass inhibited by 50% of the control was 1.51 mg /L of hydroponic media.

2.3.2.3 Overall toxicities:

Through the biomass analysis it was found that the toxicities of DNT isomers in Phytigel media for both root and shoot varied in the pattern 2,6-DNT<3,4-DNT<2,4-DNT<2,3-DNT with 2,6-DNT was the least toxic and 2,3-DNT most toxic (Table 2.4). However, the toxicities of DNT isomers in hydroponic media for both root and shoot varied in the pattern 2,6-DNT<2,4-DNT<3,4-DNT<2,3-DNT with 2,6-DNT was the least toxic and 2,3-DNT most toxic (Table 2.4). A significant difference ($p < 0.05$) when toxicities of 2,6-DNT for root and shoot data against different concentrations in Phytigel and hydroponic media were compared. However no significant difference were obtained among the values obtained through different growth media in case of other isomers. A significant value ($p < 0.05$) was also obtained when EC₅₀ value obtained for 2,6-DNT was compared with the EC₅₀ values for other isomers in Phytigel and hydroponic media when calculated individually.

Table 2.4: Estimation of EC₅₀ and CI 95% of four DNT isomers for *A. thaliana* root and shoot cultivated in Phytigel and non-axenic hydroponic media. Calculations were performed using a dose-response curve ($r^2 > 0.9$) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$); GraphPad Prism 5).

EC ₅₀ values (mg /L) for <i>A. thaliana</i> in Phytigel media				
DNT	Roots	CI 95%	shoots	CI 95%
isomers				

2,6-DNT	21.3	2.3-76.5	31.2	25-78.2
2,3-DNT	0.84	0.1-76	1.84	1-57.1
3,4-DNT	1.92	0.5-76	2.45	1.44-65.7
2,4-DNT	1.74	0.67-74	2.22	1.2-76

EC₅₀ values (mg /L) for *A. thaliana* in hydroponic media

DNT isomers	Roots	CI 95%	shoots	CI 95%
2,6-DNT	23.3	15-75.4	30.9	18.7-78.22
2,3-DNT	1.26	0.78-68.7	1.51	1.2-67.3
3,4-DNT	1.45	0.77-82.1	1.54	1.32-71.23
2,4-DNT	1.5	0.67-78.91	1.74	1.2-67.2

*EC= Effective dose

**CI= Confidence Interval

2.3.3 Toxicity evaluation through time course study in root growth inhibition for hydroponic media

The toxicity evaluation through time course root growth inhibition was used to estimate the toxicity of DNTs to *A. thaliana* over a period of exposure to different concentrations of the treatments. This study showed that as the time of exposure to the treatments increased the inhibition of root growth of *A. thaliana* increased.

i) Toxicity evaluation through time course study in *A. thaliana* treated with 2,6-DNT

The time course toxicity of *A. thaliana* grown in non-axenic hydroponic media cultivated with different concentrations of 2,6-DNT showed that root elongation was comparatively high on 14th day of treatment that reduced with the increase in exposure to the treatment and very low root growth was observed on 28th day of exposure (Figure 2.17). The results indicated that compared to control the root elongation in *A. thaliana* was observed to decrease ($16.24 \pm 5.66\%$) on 7th day exposure to the lowest considered 2,6-DNT concentration (5 mg /L of hydroponic media). The trend in Figure 2.17 indicates that root growth ($88.79 \pm 5.21\%$) was observed for *A. thaliana* only on 7th day of exposure to the maximum considered concentration for 2,6-DNT (55 mg /L of hydroponic media) and no root growth was observed as the exposure extended to 14th day.

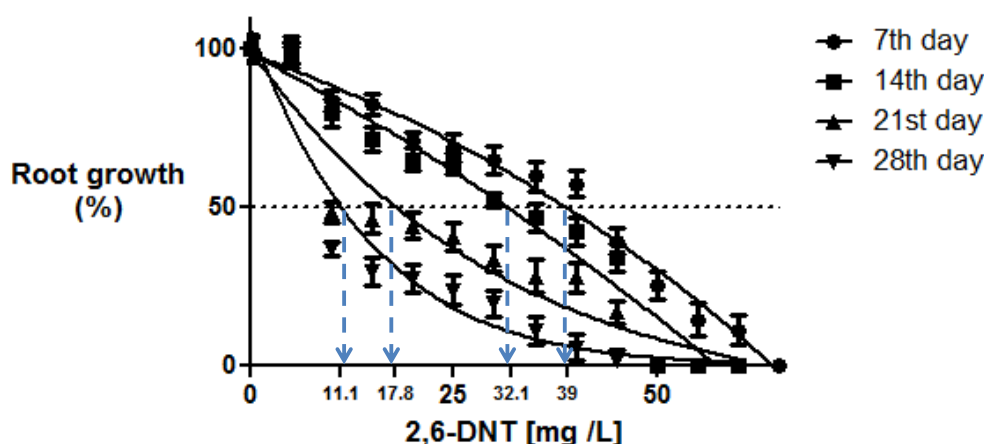


Figure 2.17: Time course toxicity analysis and EC₅₀ determination of 2,6-DNT in *A. thaliana* through root growth (%) compared to control grown in hydroponic media. The data points are mean of technical duplicates (root and shoot lengths of 36 seedlings) of the 28 day old (post cultivation) plants treated with different concentrations of 2,6-DNT grown at 20 ± 3 °C temperature and 16:8 h day night period. EC₅₀ was determined through the dose-response curve ($r^2=0.94$ for 7th day, $r^2=0.92$ for 14th day, $r^2=0.931$ for 21st day and $r^2=0.91$ for 28th day) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$; GraphPad Prism 5). The EC₅₀ for 2,6-DNT at which root mass inhibited by 50% of the control was 39 mg /L for 7th day, 32.1 mg /L for 14th day, 17.8 mg /L for 21st day and 11.1 mg /L of hydroponic media for 28th day.

ii) Toxicity evaluation through time course study in *A. thaliana* treated with 2,4-DNT

The trend in Figure 2.18 indicates that the root elongation of *A. thaliana* grown in non-axenic hydroponic media cultivated with different concentrations of 2,4-DNT was comparatively high on 14th day of treatment that reduced with the increase in exposure to the treatment and very low root growth was observed on 28th day of exposure. The results indicated that compared to control the root elongation was inhibited ($98.21 \pm 1.21\%$) in *A. thaliana* was observed at 14th day of exposure of *A. thaliana* to the maximum considered concentration of 2,4-DNT (5.5 mg /L of hydroponic media). This elongation then decreased ($99.32 \pm 0.21\%$) on 21st day of exposure and further decreased to no root length was observed on 28th day of exposure. From the trend in Figure 2.18 it can also be observed that compared to the root elongation in plants cultivated in the treated media continued to inhibit with the increase in concentration and time of exposure to the media.

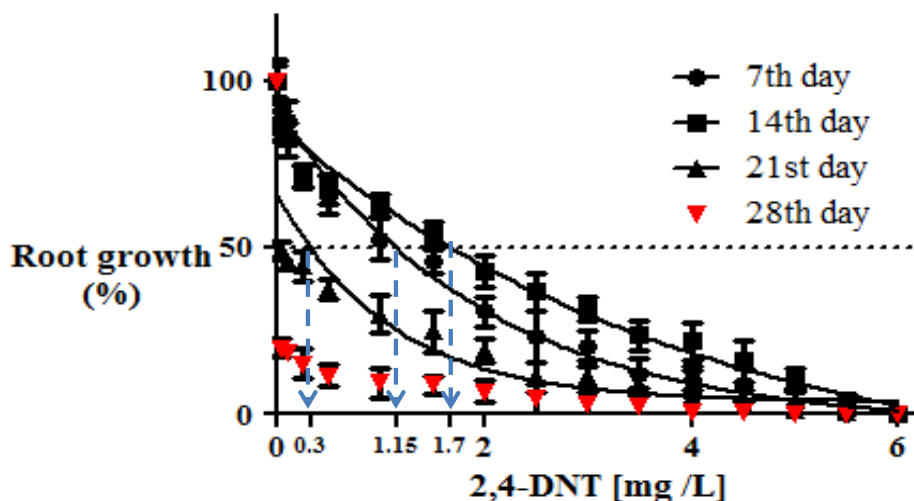


Figure 2.18: Time course toxicity analysis and EC₅₀ determination of 2,4-DNT in *A. thaliana* through root growth (%) compared to control grown in hydroponic media. The data points are mean of technical duplicates (root and shoot lengths of 36 seedlings) of the 28 day old (post cultivation) plants treated with different concentrations of 2,4-DNT grown at 20 ± 3 °C temperature and 16:8 h day night period. EC₅₀ was determined through the dose-response curve ($r^2=0.951$ for 7th day, $r^2=0.933$ for 14th day, $r^2=0.91$ for 21st day and $r^2=0.9$ for 28th day) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$; GraphPad Prism 5). The EC₅₀ for 2,4-DNT at

which root mass inhibited by 50% of the control was 1.7 mg /L for 7th day, 1.15 mg /L for 14th day and 0.3 mg /L for 21st day. Most of the plant died on 28th day therefore EC₅₀ was not determined.

iii) Toxicity evaluation through time course study in *A. thaliana* treated with 3,4-DNT

Like the other DNT isomers the time course toxicity of *A. thaliana* grown in non-axenic hydroponic media cultivated with different concentrations of 3,4-DNT showed that root elongation was comparatively high on 14th day of treatment that reduced with the increase in exposure to the treatment and very low root growth was observed on 28th day of exposure (Figure 2.19). The results indicated that compared to control the root elongation ($98.23 \pm 0.23\%$) in *A. thaliana* was observed on 14th day of exposure to maximum considered 3,4-DNT concentration (5.5 mg /L of hydroponic media). No further root elongation was then observed on 21st and 28th day of exposure at similar concentration.

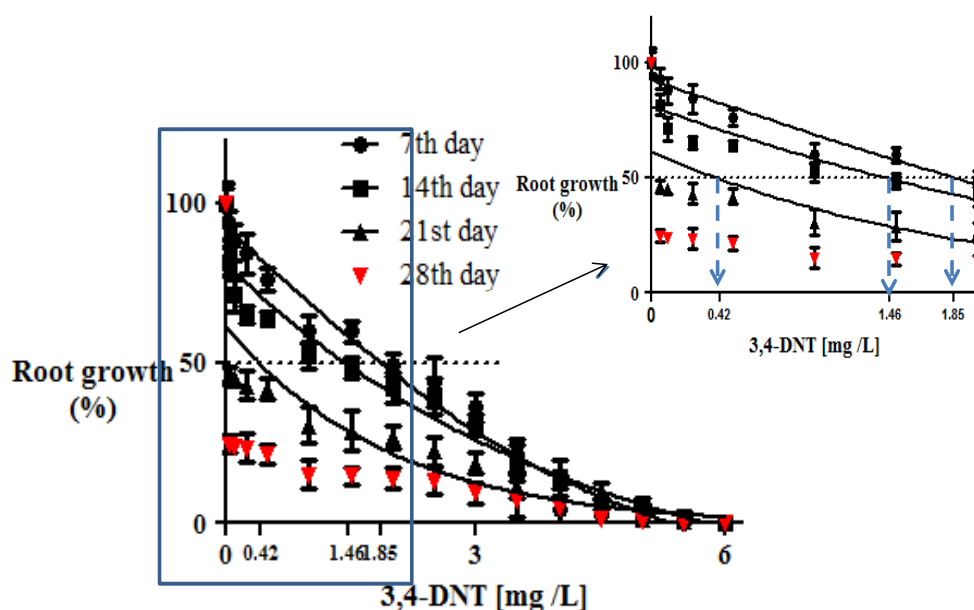


Figure 2.19: Time course toxicity analysis and EC₅₀ determination of 3,4-DNT in *A. thaliana* through root growth (%) compared to control grown in hydroponic media. The data points are mean of technical duplicates (root and shoot lengths of 36 seedlings) of the 28 day old (post cultivation) plants treated with different concentrations of 3,4-DNT grown at 20 ± 3 °C temperature and 16:8 h day night period. EC₅₀ was determined through the dose-response curve ($r^2=0.96$ for 7th day, $r^2=0.92$ for 14th day and $r^2=0.92$ for 21st day) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-$

$X) * HillSlope))$; GraphPad Prism 5). The EC_{50} for 3,4-DNT at which root mass inhibited by 50% of the control was 1.85 mg /L for 7th day, 1.46 mg /L for 14th day and 0.42 mg /L for 21st day. Most of the plant died on 28th day therefore EC_{50} was not determined.

iv) Toxicity evaluation through time course study in *A. thaliana* treated with 2,3-DNT

The time course toxicity of *A. thaliana* grown in non-axenic hydroponic media cultivated with different concentrations of 2,3-DNT showed that root elongation was comparatively high on 14th day of treatment that reduced with the increase in exposure to the treatment and very low root growth was observed on 28th day of exposure (Figure 2.20). The results indicated that compared to control the root elongation ($97.3 \pm 0.23\%$) in *A. thaliana* was observed on 14th day of exposure to maximum considered 2,3-DNT concentration (5.5 mg /L of hydroponic media). No root elongation was observed on 21st and 28th day of exposure at similar concentration.

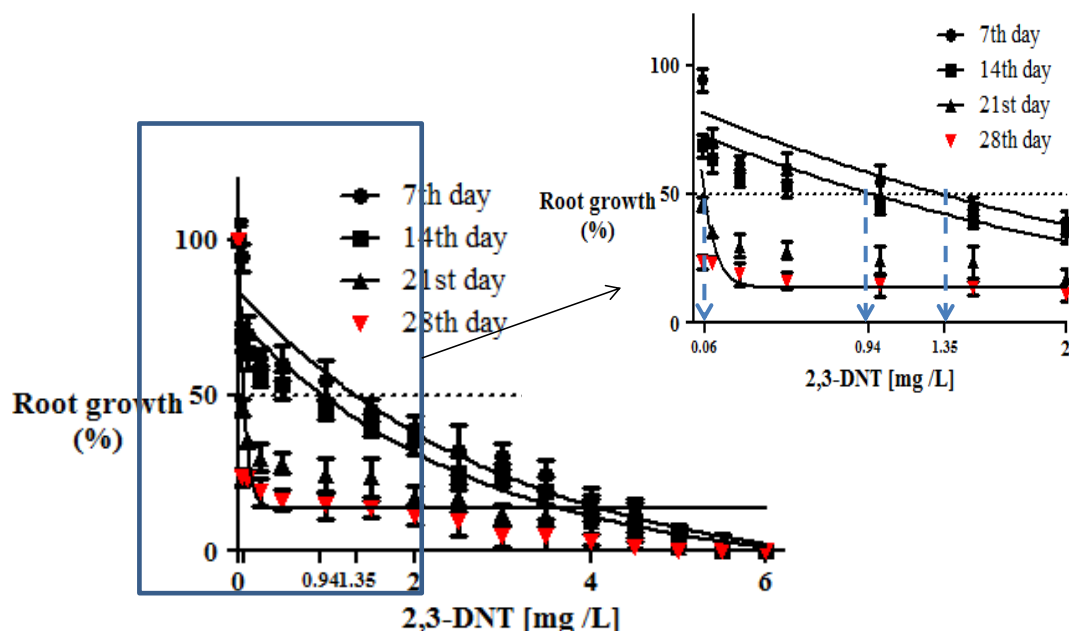


Figure 2.20: Time course toxicity analysis and EC_{50} determination of 2,3-DNT in *A. thaliana* through root growth (%) compared to control grown in hydroponic media. The data points are mean of technical duplicates (root and shoot lengths of 36 seedlings) of the 28 day old (post cultivation) plants treated with different concentrations of 2,3-DNT grown at 20 ± 3 °C temperature and 16:8 h day night period. EC_{50} was determined

through the dose-response curve ($r^2=0.95$ for 7th day, $r^2=0.96$ for 14th day and $r^2=0.91$ for 21st day) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$); GraphPad Prism 5). The EC₅₀ for 2,3-DNT at which root mass inhibited by 50% of the control was 1.35 mg /L for 7th day, 0.94 mg /L for 14th day and 0.06 mg /L for 21st day. Most of the plant died on 28th day therefore EC₅₀ was not determined.

2.3.3.1 Overall toxicities:

The overall toxicities show that the EC₅₀ values as determined through the time course analysis of the root growth (%) tend to decrease with the increase in time of exposure (Table 2.5). The highest EC₅₀ were determined on 7th day of exposure and lowest on 21st day of exposure. Most of the plant died on 28th day therefore, there were not enough time points to determine the EC₅₀ for them. On 7th day, 14th and 21st day of exposure the toxicities varied from 2,6-DNT < 3,4-DNT < 2,4-DNT < 2,3-DNT, with 2,6-DNT least toxic and 2,3-DNT most toxic.

Table 2.5: Estimation of EC₅₀ and CI 95% of four DNT isomers for *A. thaliana* root and shoot cultivated in Phytigel and non-axenic hydroponic media. Calculations were performed using a dose-response curve ($r^2 > 0.9$) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$); GraphPad Prism 5).

DNT isomers	EC ₅₀ values (mg /L) for <i>A. thaliana</i> in hydroponic media							
	7 th	CI	14 th day	CI	21 st day	CI	28 th day	CI
	day	95%		95%		95%		95%
2,6-DNT	39.03	30.01-	32.11	27.21-	17.82	12.31-	11.12	9.8-
		42.31		42.13		18.76		11.42
2,3-DNT	1.35	1.22-	0.94	0.76-	0.06	0.05-	-	-
		3.21		1.45		0.12		
3,4-DNT	1.85	1.42-	1.46	1.21-	0.42	0.13-	-	-

		2.35		2.44		2.13		
2,4-DNT	1.71	1.21-	1.15	0.2-	0.31	0.11-	-	-
		2.33		2.31		67.5		

2.4 Discussion:

Previous studies have already demonstrated that DNTs have an inhibitory affect on root and shoot growth in plants (Dokken and Davis, 2011; Yoon et al., 2006 and 2007; Tognetti et al., 2007). The current study considered four isomers, 2,4-DNT and 2,6-DNT, 2,3-DNT and 3,4-DNT to establish toxicity in root and shoots of *A. thaliana* grown in axenic Phytagel media and non-axenic hydroponic media. The commercially available composition of these isomers is 1.54% of 2,3-DNT, 0.69% 2,5-DNT, 0.04% 3,5-DNT and 2.43% 3,4-DNT) (Burrows et al., 1989; Bayer, 2003; Lent et al., 2012).

The axenic Phytagel media was considered to solely see plants own interaction with DNT isomers as opposed to soil and other non axenic cultures (where other external interactions such as microbes are highly likely). Since DNTs are considered slightly hydrophilic (K_{ow} for 2,4-DNT is 1.98 and 2,6-DNT is 1.72 to 2.1) ($\log K_{ow} < 1$ = hydrophilic; $\log K_{ow} > 3$ = hydrophobic) (Su and Liang, 2011; Ortega-Calvo et al., 1999; Mussenbrock and Kleibohmer, et al., 1995; ATSDR report 2,4-DNT and 2,6-DNT), therefore a hydroponic media (non-axenic) was used to examine variations in the toxicity of DNT isomers. The EC_{50} , the value where the enzyme machinery of plants works efficiently and is capable of absorbing maximum pollutants (Sulaiman and Ooi, 2012; Zhuang et al., 2009; Rocheleau et al., 2010), is a measure of toxicity of the isomers of DNT tested against *A. thaliana*. Three different

approaches, root growth, biomass weight and a time course of root growth were used to determine and compare the toxicities of the four isomers of DNT.

The plants survived up to certain concentrations of DNTs in the media after which they showed prominent chlorosis and stunted root growth (Figure 2.21). Among the four isomers, 2,6-DNT was the least toxic tolerable up to 50 mg /L of Phytigel media and rest of the isomers on average survived up till 5 mg /L of Phytigel media with slight variations. Similar survival rates have also been reported in earlier studies (Yoon et al., 2006 and 2007) with highest tolerable concentration of 2,6-DNT (approximately 46 mg /L of Phytigel media) and 2,4-DNT (approximately 6 mg /L of Phytigel media) for *A. thaliana*. In the case of other plants such as sunflower (*Helianthus annuus*) and maize (*Zea mays*) were found to tolerate 5 mg /L of 2,4-DNT in hydroponic media (Dokken and Davis, 2011), in tomato (*Lycopersicon esculentum* Miller) and wheat (*Triticum aestivum*) 6 mg /L of 2,4-DNT in non-axenic nutrient solution was the maximum tolerable concentration (Su and Liang, 2011). The prominent features of plant stress due to the toxicity of DNT are the symptoms of chlorosis and stunted root growth. The reason for chlorosis is the lack of non-haem iron necessary to make sufficient chlorophyll, which is also required in the production of nitroreductase (involved in the transformation of nitroaromatics) (Hannink et al., 2007; Yang et al., 2004).

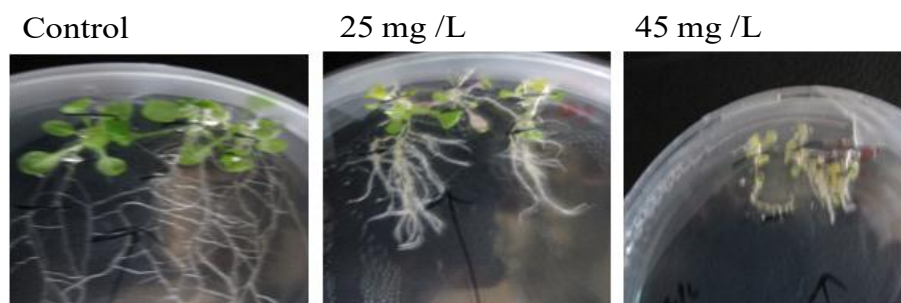


Figure 2.21: Stunted root growth and chlorosis in 2,6-DNT treated *A. thaliana* compared to control on fourth day of observation in Phytigel media.

Increased concentrations of DNT in the media increases the competition for the available non-haem iron, which results in reduced chlorophyll production and resulting in plant death. The first de-nitration of nitroaromatics has been shown to be catalyzed by dioxygenases, a Rieske non-haem iron oxygenase, in bacterial system (Symons and Bruce, 2006). The stunted root growth is due to inhibition in mitotic division. As the concentration of DNT increases, the cell division is hindered. The amines formed during DNT metabolism could potentially act as microtubule inhibitors hindering tissue growth and the blocked mitotic division causes the increased density of the cells due to accumulation of abnormal microtubules (Landa et al., 2010; Hannink et al., 2001).

According to root growth analysis in Phytigel media 2,3-DNT was observed to be most toxic and 2,6-DNT least toxic. Similar variations in toxicities of isomers have been reported (Valentovic et al., 1995; Ewertowska and Mlynarczyk, 2002; Monti et al., 2005) in different media. The EC_{50} values obtained in this study are in good agreement with previous studies (Yoon et al., 2006 and 2007). This variation in toxicities of isomers is due to the position of nitro-groups attached to the toluene ring. The reduced products of DNTs (ANTs) and active metabolites interact with biological molecules which determine their toxicities (Lent et al., 2011; Picka and Friedl et al., 2004). In bacteria the variation in toxicities of 2,4-DNT and 2,6-DNT has also been reported (Nishino et al., 1999 and 2000) due to the toxicities associated with intermediate products of degradation. The preference of nitroreductases and resonance energy associated with nitro groups at specific positions on the benzene ring also affects the toxicity variation between isomers (Lent et al., 2011).

Along with EC_{50} , EC_{10} and EC_{90} values were calculated to determine the Lowest Observable Adverse Effect Concentration (LOAEC), a 50% toxicity value and highest toxic value before the plant is completely inhibited. LOAEC determines very slight influence of a contaminant without completely inhibiting its growth and compromising the metabolic machinery (Peric

et al., 2011; Wilson and Wilson, 2010). EC₁₀ values indicate low concentrations of 2,3-DNT in both hydroponic and Phytigel media is tolerable for *A. thaliana* (Table 2.3). This concentration can be effective for a successful phytoremediation as it preserves the crucial pathways of DNT transformation. EC₉₀ is important as this is the maximum concentration without killing the plant altogether.

The EC₅₀ values for different DNT isomers as determined through root and shoot biomass was found to be slightly different from the root growth assay. Although the roots are stunted at high concentrations of DNTs their density still increases. The ANT_s interact with microtubules during mitotic cell division resulting in accumulation of abnormal cell division products (Sarlauskas et al., 2004). This increases the density of the cells; therefore the EC₅₀ values found from the biomass analysis are slightly different from root length analysis. Both the root length and biomass analysis are equally important in toxicity determination. The choice of experiment varies with the stability of the compound in the media and type of toxicity analysis require. For DNTs, root growth analysis in sterile media is enough to provide the toxicity data for different isomers.

The results indicate that plants grown in hydroponic media have a slightly higher tolerance compared to the plants grown in Phytigel media. This suggests that variations in DNT toxicities depend upon the type of media as well (possibly the solubility affect). The EC₅₀ for lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*) and oats (*Avena sativa*) was found less in the nutrient solution compared to the soils (Adema and Henzen, 1989). There is a significant and inversely proportional relationship between EC₅₀ of the plants and the soil organic matter content (Rocheleau et al., 2010; Waisner et al., 2008). This shows that the binding of 2,4-DNTs to the organic contents reduces their availability to plants. The EC₅₀ for 2,4-DNT was found to be 30 mg /kg for alfalfa and 7 mg /kg for Barnyard grass grown on same soil amended with similar concentration of 2,4-DNT (Rocheleau et al., 2010). No data

for phytotoxicities of 2,3-DNT and 3,4-DNT is available therefore, they cannot be compared with any previous study.

The root and shoot biomass comparison showed that *A. thaliana* shoots can tolerate higher concentrations compared to roots. The results for EC₅₀ of 2,6-DNT (31.2 mg /L of Phytigel media) for shoot biomass in the present study agree with other studies (Yoon et al., 2007) (29 mg /L for *A. thaliana* in axenic culture). Similar results have been found (Picka and Friedl et al., 2004) with different plant species (EC₅₀ of 2,6-DNT ranges from 26-61 mg /kg for shoot dry mass). Root and shoot have different levels of absorptions of DNTs and therefore they have different levels of toxicities. The plants roots being in direct contact with the root solution are likely to be affected quickly compared to shoots where contaminants are transported through roots (after being slightly processed). It has been shown that (Gong et al., 1999) toxicity in roots is comparatively more than in shoots for TNT. The study showed 20 times elevation in TNT in roots compared to shoot associated with high toxicity to roots for TNT. According to the distribution of nitroaromatics (Yanyu et al., 2010) in *Phragmites australis*, the concentration was elevated in shoots first but then decreased presumably due to translocation of compounds reducing their toxicities.

As the time of exposure to DNT increased, the EC₅₀ value was observed to decrease. The toxicity was therefore increased with the time of exposure. This suggests that with the increase in time of exposure there is an increase in tissue damage. Like other nitroaromatics, the continuous influx of DNTs through vascular tissues (Su and Liang, 2011) saturates the enzymes and hinders their activity, which leads to increased tissue damage due to untransformed DNTs (Nehrenheim et al., 2011; Landa et al., 2010; Durringer et al., 2010). The highest inhibition in root growth was observed on 28th day of exposure. Not enough tissues could be recovered on 28th day of exposure from all the considered isomers; therefore it was hard to determine EC₅₀ with too few data. According to the present study in

hydroponic media, 2,4-DNT is more toxic than 3,4-DNT however in Phytagel media 2,4-DNT was less toxic than 3,4-DNT. This variation in behaviours of isomers with media could also be explained by their variation in availability according to their structure.

In conclusion, all DNT isomers have different toxicities for *A. thaliana* with 2,6-DNT is the least toxic and 2,4-DNT is the most toxic. These values of the toxicity slightly vary with the plant growth media. The shoot biomass toxicity analysis shows that it is likely that roots absorbing the DNTs and transporting them to shoot which is causing inhibition in biomass growth.

Chapter 3

Uptake and metabolism of four dinitrotoluene isomers in *Arabidopsis thaliana* in Phytigel and hydroponic medium (a time course study) to trace the efficiency of plant towards dinitrotoluene metabolism

3.1 Introduction

As previously discussed (Chapter 2; section 2.1) dinitrotoluenes (DNTs) are both resistant to degradation and toxic and are therefore prime targets for remediation. Phytoremediation is a cost effective and environmental friendly technique and has great potential to clean up environmental resources contaminated with DNTs and its metabolites (Yoon et al., 2006 and 2007). Being toxic to plant cells, the phytoremediation of DNTs can be limited. For the effective use of plants in phytoremediation it is important to understand the toxicity limitations and vital biochemical processes involved in the detoxification of these chemicals in the plant following absorption (Zhu et al., 2012).

Plants being sessile have evolved a wide range of different mechanisms to cope with the affects of toxic compounds. Thus plants utilise highly complex and compartmentalized networks of different cellular organelles each of which house a unique set of enzymes for detoxification. Once DNTs gain entry into plant cells, they follow a putative three phase route, categorized as oxidation or reduction, conjugation and compartmentalization as for other xenobiotics (Sandermann, 1992).

The presence of DNTs in plant cells triggers the expression of several enzymes such as nitroreductases, which use flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) as a prosthetic group and nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH) to reduce the nitro group (Hannink et al., 2001). This enzyme activity affects the bioactivity and mobility of compounds in plant cells and makes them available for further metabolism (Oliveira et al., 2010). Nitro groups attached to the toluene backbone have highly electronegative nitrogen and oxygen atoms. The electronegativity of oxygen compared to nitrogen increases the polarity of NO bond creating a partial positive charge on the nitrogen atom. This partial positive charge on the nitrogen atom allows it to undergo reduction catalysed by nitroreductases (Figure 3.1) (Esteve-Nunez et al., 2001; Chandor et al., 2008; Blasco et al., 2001).

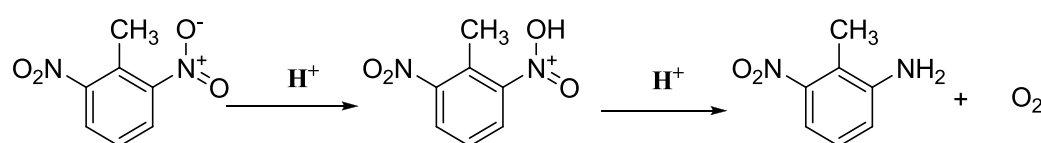


Figure 3.1: A generalised reduction mechanism of 2,6-DNT.

Nitroaromatics such as DNTs can undergo reduction in prokaryotic and eukaryotic cells through single and double electron transfers, reactions that are catalysed by nitroreductases (Esteve-Nunez et al., 2001; Bryant et al., 1981; Peterson et al., 1979). Single electron transfers, usually catalysed by Type II (oxygen sensitive) nitroreductases, yields a nitro-anion radical which is reduced to give either a nitroso group or reacts with oxygen to produce super oxide anion (Zenno et al., 1996 and 1998). Double electron transfer reactions, usually catalysed by Type I (oxygen insensitive) nitroreductases are common in higher plants and animals tissues and yield

reduced DNTs products (Figure 3.1). This process is oxygen independent and yields no radicals. Here, along with nitroreductases, aldehyde oxidase (Wolper et al., 1973), cytochrome P450s and dihydrophilic amide dehydrogenase have also been shown (Tatsumi et al., 1979) to catalyse the reduction of nitro-group of nitro-aromatic compounds in the presence of a reducing agent and produce aromatic nitro-amines (Spanggord et al., 1991).

There are several investigations, which have reported amino-nitrotoluenes (ANTs) to be the main degradation products when the plants are exposed to DNT (Yoon et al., 2006 and 2007). Yoon et al (2006 and 2007) showed the transformation of 2,6-DNT and 2,4-DNT to 2-amino-6-nitrotoluene (2A6NT) and 2-amino-4-nitrotoluene (2A4NT), respectively when *A. thaliana* was exposed to DNT containing growth medium. Similar studies with TNT reported the formation of ANTs in *A. thaliana* (Gandia-Herrero et al., 2008) and *Myriophyllum spicatum* (Gao et al., 2000; Susarla et al., 2002). This shows that ANTs are the most abundant metabolites in the process of the detoxification of nitroaromatics such as DNT.

Studies (Haack et al., 2001; Darwish et al., 2010) have shown that complete reduction of DNTs to amino-nitrotoluenes appears in response to reduce the toxicity of DNTs. The nitroso and hydroxylamino compounds formed after the DNT metabolism have been found to be comparatively more toxic than the nitro-amines. The conjugation of hydroxylamino compounds with biological molecules can lead to mutagenesis, the risk of which is reduced by the reduction of nitroaromatics to nitro-amines (Salamanca-Pinzon et al., 2010; Dolores et al., 2008).

In biotic systems there are several potential electron donors that contribute to the reduction of DNT and other nitroaromatic molecules (Figure 3.2) (Esteve-Nunez et al., 2001).

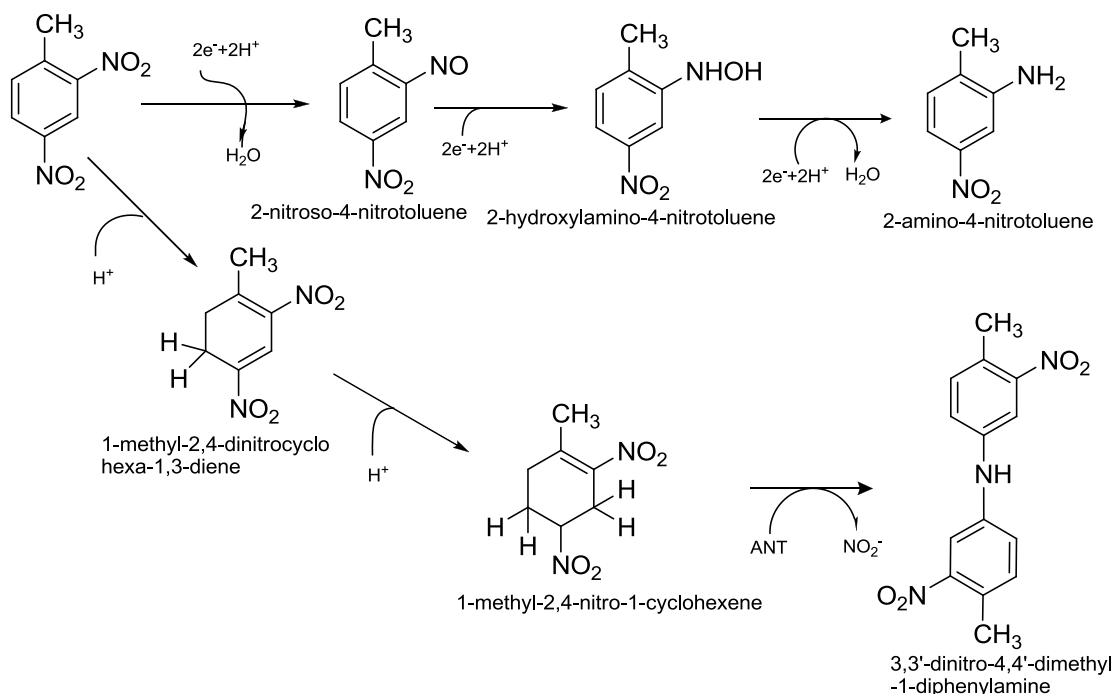


Figure 3.2: The reduction of 2,4-DNT to 2A4NT and 3,3'-dinitro-4,4'-dimethyl-1-diphenylamine. The reduction of nitro group can yield nitroso, hydroxylamino and amino compounds. The reduction of toluene ring leads to cyclohexadiene and cyclohexene and finally to phenylamine (derived from Beynon et al., 2009).

Nitroreductases along with transferases are regioselective in their reduction of nitro-groups present in the aromatic ring. This property leads to enhanced toxicity of certain isomers of DNTs compared to others. However, plant based toxicity and detoxification data is limited for all isomers of DNT. Several studies have reported the properties and toxicities of 2,4-DNT and 2,6-DNT and their metabolites in living systems (Yoon et al., 2007; 2006; Johnson et al., 2002; Snellinx et al., 2003). Less information, however, is available in relation to the toxicities of 3,4-DNT and 2,3-DNT and their metabolites in plants compared to other isomers.

The root stress response of *Arabidopsis thaliana* (Chapter 2, section 2.3) has indicated that all isomers of DNT are phytotoxic and only low concentrations are tolerable. The

survival and growth of plants in media amended with low DNT concentrations indicate that they have the metabolic machinery to mediate the detoxification of the absorbed DNT enabling survival (Rylott et al., 2006).

The present study is designed to examine four isomers of DNTs and analyse their metabolites in *A. thaliana*. Further this study looks at the rate of uptake and metabolite formation in *A. thaliana* when the plants are exposed to different concentrations of these compounds. This study utilises two systems to observe metabolite formation. The first is Phytigel (sterile media), which provides solely plant based DNT interactions (Yoon et al., 2007), however, the growth of plant is limited due to less available growth space and nutrients. The other system involves plants grown in hydroponic media, which being non-sterile can have plant-microbe DNT interactions. However, root and shoot growth can be for more prolonged than in Phytigel media and nutrients easily replenished. The analysis was carried out by Gas Chromatography Mass Spectrometry (GC-MS), which provides a powerful analytical technique.

There are several analytical techniques available for the detection of DNT metabolites in plant extracts but they vary in sensitivity depending on the analytical procedure. The most commonly used are HPLC (high performance liquid chromatography) (Dodard et al., 1998; Emmrich, 2001; Yoon et al., 2006 and 2007; Gandia-Herrero et al., 2008) and GC (gas chromatography) coupled with MS (mass spectrometry). Analysis by HPLC requires extensive sample purification followed by separation of metabolites using reverse phase chromatography. The detection of metabolites is normally done by mass spectrometry or by UV absorbance and to achieve higher sensitivity fluorescence detector (Ormazabal et al., 2005). In contrast to this, GC-MS

provides a simple direct method of analysis for the volatile metabolites of DNT and does not require extensive sample purification steps. Selective ion monitoring can further increase the sensitivity by selecting specific ions associated with each metabolite and thus filters out unnecessary peaks in the chromatogram (Gerber et al., 2012).

3.1.2 Hypotheses

The present study was based on following hypotheses:

1. All four isomers of DNTs are metabolised to ANTs in *A. thaliana*.
2. The uptake and metabolism of each DNT isomer is different in the plant and is dose dependant.

3.1.3 Aims:

Based on the above hypothesis **the aims** of this study are therefore:

- I. To develop methods to analyse the four different isomers of DNT and their metabolites by GC-MS.
- II. To analyse the formation of metabolites in plants grown at different concentrations for all isomers of DNT.
- III. To estimate the variation in a single plant's capacity to take up and metabolise different isomers of DNT, exposed to different concentrations.
- IV. To examine the difference in the plant's ability to take up and metabolize different isomers of DNT at different concentrations using a hydroponic system during a time course of exposure.

The second and third aim will be achieved by setting up an experiment in a sterile Phytigel system, where no microbial metabolism of DNTs could occur. The fourth

aim will be achieved using a hydroponic system, which can support plant growth for prolonged periods so that a time course of exposure can be investigated.

3.2 Materials and Methods

3.2.1 Chemicals

The chemicals used were exactly as described in the Chapter 2 (section 2.2, subsection 2.2.1).

2,4-DNT (purity $\geq 98\%$), 2,3-DNT (purity $\geq 99\%$), 2,6-DNT (purity $\geq 99\%$) and 3,4-DNT (purity $\geq 99\%$) were purchased from Sigma Aldrich (UK). Acetonitrile (purity $\geq 97.8\%$, HPLC grade) and methanol (purity $\geq 97\%$) were reagent grade and obtained from VWR, UK. Murashige and Skoog salts (MS), Phytigel (P8169), Agrose (A9539), 2-(*N*-morpholino) ethanesulfonic acid (MES) and sucrose (99.8%) were purchased from Sigma Aldrich (UK).

The ‘Araponic’ system for the hydroponic plant growth was purchased from Araponics SA, Belgium. The growth medium- Flora micro (for root growth) and Flora Gro (for biomass) were supplemented with the system and were of commercial grade. Flora micro contained total nitrogen 5%, ammonical nitrogen 1%, nitrate nitrogen 4%, soluble potassium (K_2O) 1.3%, boron 0.01%, calcium (CaO), copper (chelated EDTA) 0.01%, iron (chelated 6% EDDHH) 0.12%, manganese 0.04%, molybdenum 0.004% and zinc (chelated EDTA) 0.015%. Flora Gro contained total nitrogen 1%, ammonical nitrogen 3%, nitrate nitrogen 2%, available phosphate (P_2O_5) 1%, soluble potassium (K_2O) 7% and soluble magnesium (MgO) 0 - 8%.

3.2.2 Cultivation of the plants

3.2.2.1 Phytigel media

3.2.2.1.1 DNT stock solutions for the plant growth media

The concentrations of DNT isomers, for the uptake and metabolism studies in *A. thaliana* grown in Phytigel, were selected to include the IC₅₀ value. The stock solutions were prepared in 100% ethanol and were further diluted accordingly, such that each growth media (except the control) contained 0.25% ethanol. The pipettes were calibrated once every week to ensure correct measurements.

Table 3.1: Concentrations of four isomers of DNTs considered for GC-MS analysis of metabolites for plants grown in Phytigel media. The highlighted concentrations in bold indicate the IC₅₀ value (Chapter 2; section 2.1)

2,6-DNT	2,3-DNT	3,4-DNT	2,4-DNT
(mg/l)	(mg/l)	(mg/l)	(mg/l)
3	0.05	0.02	0.03
13	0.1	0.2	0.1
23	0.25	0.4	0.3
33	0.5	0.8	0.9
43	1	1.6	1.8
53	2.5	3.2	2.7
-	5	4.6	3.6
-	-	6.4	4.6

3.2.2.1.2 Cultivation of the plants

The *A. thaliana* ecotype Columbia 0 (Col-0) seeds were kindly provided by Mrs Nina Grabov (Imperial College, London). The seeds were surface sterilized according to Xiang and Oliver, 1998, where 1ml of 70% ethanol was added to an Eppendorf tube containing *A. thaliana* seeds and kept for 2 min with occasional vortexing. The supernatant was decanted and 25% sodium hypochlorite was added for 15 min with occasional vortexing and then completely drained. The seeds were washed five times with sterile distilled water, and incubated at 4°C for 48 h to break the dormancy. These seeds were sown on the medium containing half strength MS, 1% sucrose, 0.025% MES and 0.75% Phytigel (P8169, Sigma UK) adjusted to pH 5.7 with 1M KOH. The seeds were placed onto the sterile agar solution contained in petri dishes and allowed to grow for four days under constant light conditions of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Hanstech Quantitherm lightmeter; attained by Starcoat T5 high output Hungry fluorescent bulbs) and temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h of a day:night period.

After four days, the seedlings of similar root length were carefully transferred to polycarbonate Magenta tissue culture boxes (3×3×4") with 60 ml of half strength MS, 1% sucrose, 0.025% MES and 0.75% Phytigel adjusted to pH 5.7 with 1M KOH and amended with a specific concentration of the DNT isomer. Each magenta box received two seedlings of similar size and age. The procedure was repeated for all concentrations of DNT isomers (Table 3.1) with nine replicates. The plants were allowed to grow for 18 days in the growth room under light conditions of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Hanstech Quantitherm light meter) and temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h of day:night period. These plants were processed further and analysed to calculate the concentration of DNT metabolites per gram dry weight of DNT treated *A. thaliana* tissues.

To observe the amount of DNTs absorbed and metabolised in a single *A. thaliana* plant exposed to different concentrations of different DNT isomers, a single four day old seedling was transferred to a polycarbonate Magenta tissue culture boxes (3×3×4") with 60ml of half strength MS, 1% sucrose, 0.025% MES and 0.75% Phytigel adjusted to pH 5.7 with 1M KOH and amended with a specific concentration of the DNT isomer. The procedure was repeated for all concentrations (Table 3.1) of DNT isomers with four replicates. The plants were allowed to grow for 18 days in the growth room under the light conditions of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Hanstech Quantitherm light meter) and temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h of day:night period. These plants were then further processed and analysed to determine the concentration of DNT metabolites per single dry *A. thaliana* plant.

3.2.2.2 Hydroponic Media

3.2.2.2.1 DNT stock solutions for the plant growth media

The concentrations of DNT isomers for hydroponic media were selected on the basis of the highest toxic concentration as derived from Chapter 2. The concentrations of 2,3-DNT, 3,4-DNT and 2,4-DNT were kept the same to give a comparison (their toxicities are closely related Chapter 2, section 2.3). The concentrations of DNT isomers are as described in the Table 3.2.

Table 3.2: Concentrations of four isomers of DNTs considered for GC-MS analysis of metabolites for plants grown in hydroponic media during a time course of exposure.

2,6-DNT	2,3-DNT	3,4-DNT	2,4-DNT
(mg /L)	(mg /L)	(mg /L)	(mg /L)
5	0.05	0.05	0.05

15	0.1	0.1	0.1
25	0.25	0.25	0.25
35	0.5	0.5	0.5
45	1	1	1
55	2.5	2.5	2.5
-	5	5	5

3.2.2.2.2 Cultivation of the plants

The hydroponic experiments were performed in a system specialised for *A. thaliana* growth called ‘Araponics’ (Araponics, SA, Belgium). The plants were grown in containers of length 22.6 cm, width 17.6 cm and height 10 cm. The nutrient solutions, as provided by the manufacturer, were tested for their DNT reactivity by adding 2 ml of 25 mg /L 2,6-DNT and 2 ml of 0.5 mg /L 2,4-DNT, 2,3-DNT and 3,4-DNT separately in diluted growth mediums kept at room temperature.

Seeds were placed in the seed holders (diameter 2cm, height 2.5cm and filling volume 0.5 ml) amended with 0.75% agar (Agrose, molecular biology tested, Sigma, UK) under the constant light conditions of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Hanstech Quantitherm light meter) and temperature of 20-23°C for 16:8 h of day:night period in the nutrient solution amended according to the manufacturer advice (0.5 ml of each stock nutrient for each 1L solution). After four days seedlings with similar root lengths were selected for the experiment. These seedlings were placed in ‘Araponic’ tanks with nutrient solutions amended with a specific concentration of DNT. This procedure was

repeated for all isomers of DNTs for the considered concentrations (Table 3.2). The *A. thaliana* plants were allowed to grow in this system under constant light conditions of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Hanstech Quantitherm light meter) and temperature of $20\text{-}23^{\circ}\text{C}$ for 16:8 h of day:night period. The plants were removed and processed for analysis after every seven days to establish a time course study. The nutrient solutions were also changed every seven days to avoid any fungal growth interfering with the experiment.

3.2.3 Method development, extraction and analysis of metabolites

3.2.3.1 Method development

3.2.3.1.1 Analytical technique (GC-MS)

A full scan acquisition was performed to find retention times, quant ions and qualitative for DNT isomers and ANTs to develop methods for SIM acquisition. The details are given in Table 3.3.

Table 3.3: GC-MS conditions to determine dinitrotoluene isomers and their metabolites.

Gas Chromatography	
GC instrument	Hewlett Packard Series 6890 GC system
Carrier gas	He
Injector	
Injector mode	Back Injector
Injection volume	1.0 μl
Syringe size	10.0 μl
	Syringe washed (acetone) a total of 8 Xs between

injections

Inlets

Mode	Pulsed splitless
------	------------------

Carrier gas	Helium
-------------	--------

Heater	250 ⁰ C
--------	--------------------

Pressure	7.54 psi
----------	----------

Total flow	52.9 ml/min
------------	-------------

Injection

Pulse pressure	20 psi until 1.00 min
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Purge flow to split vent	49.5ml/min @0 min
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Gas server	20.0 ml/min @ 2:50 min
------------	------------------------

Column

Name	HP-5MS, 0.25mm×30m, film:0.25μm
------	---------------------------------

Type	Agilent 19091S-433
------	--------------------

Mode	Constant flow
------	---------------

Inlet	Back
-------	------

Detector	MS
----------	----

Helium flow

Pressure	7.54 psi
Flow	1 ml/min
Average velocity	36 cm/sec

Oven temperature

Initial temperature	50 ⁰ C, hold for 3 min
Ramp temperature	250 ⁰ C-300 ⁰ C in 30min
Final temperature	310 ⁰ C, hold for 5 min
Run time	33 min
Maximum post oven configuration temperature	310 ⁰ C
Equilibration time	50 s

Detector

Name	Hewlett Packard 5973 Mass Selective Detector
------	--

Mass Spectrometer

Acquisition mode	Full Scan
Solvent delay	6 min
Time window	33 min

3.2.3.1.1.1 Selection of Ions for SIM acquisition

SIM was used for the analysis of DNTs and their metabolites in *A. thaliana* treated with the four DNT isomers. This technique was expected to increase sensitivity of detection for target compounds through the selective detection of the most indicative ions. To obtain this, a full scan acquisition was performed on two test mixtures. The first contained a mixture of 1 mg /L of each of 2,4-DNT, 2,6-DNT, 2,3-DNT and 3,4-DNT (solution prepared in acetonitrile) adjusted to a volume of 1ml and added to GC vials and the second contained a mixture of 2 mg /L of each of 2A4NT, 4A2NT, 3A4NT, 4A3NT, 2A6NT and 2A3NT (solution prepared in acetonitrile) adjusted to the volume of 1ml and added to GC vials. The retention times, dwell times, quant ions and qualitative ion concentrations were determined. The ions of interest were selected in a group of four on the basis of mass per charge ratio (m/z).

3.2.3.1.2 Optimization of extraction procedure (necessity of internal standard while extraction)

A preliminary experiment was conducted to determine the extraction efficiency for the samples with and without an internal standard. For this purpose two 60 mg of samples of freeze dried 0.5 mg /L 3,4-DNT treated *A. thaliana* leaf tissues were taken and 1 ml of acetonitrile was added to each of them. One of the samples received internal standard of 1 mg /L of 2,6-DNT. A stainless steel ball (5/32") was added to each Eppendorf tube (2 ml) and processed using a QIAGEN Retsch Tissue Lyser with a frequency of 30.0 Hz/sec for 2 min. The samples were sonicated for 12 h and spun in Eppendorf centrifuge 5415D (13.2×1000 rpm, 7 min). The extracts were decanted and concentrated under nitrogen to a volume of 200 µl and analysed by GC-MS along with the standard solutions (2,6-DNT in acetonitrile) and analysed. The samples were processed in triplicate. The percentage recoveries were calculated from the areas of

the peaks of internal standard (2,6-DNT) obtained after analysing the samples by GC-MS to show the difference using the formula as described below:

$$\% \text{ recovery of compound} = \frac{[\text{Area of 2,6-DNT peak in sample}]}{[\text{Area of 2,6-DNT peak in standard}]} \times 100 \quad (\text{Eq. i})$$

To analyse the amount of 3A4NT obtained from the samples, the standard (3,4-DNT dissolved in acetonitrile) run with varying concentrations was conducted along with sample run on GC-MS. Linear regression analysis was carried out for these standards. The amount of metabolites and their corrected concentration relative to internal standard were calculated as follows:

$$\text{The total volume of the extract} = 200 \mu\text{l} \quad (\text{Eq. ii})$$

From the standard curve the concentration of the metabolite is in mg /L = Y

$$\text{Absolute amount of metabolite in } 200 \mu\text{l} = Y \times (200/10^6) = X$$

$$\text{Amount of metabolite } (\mu\text{g or ng}) / \text{g dry wt tissue} = (1000 / \text{wt of tissues analysed in mg}) \times X = Z$$

$$\text{Corrected concentrations of metabolites relative to standards} = (\text{Z/ratio of standards recovered in Eq. i})$$

The corrected concentrations were then compared to figure out any difference.

3.2.3.1.3 Determination of the stability of DNT and its metabolites

A time course study was conducted for plant tissues amended with a specific amount of 2,6-DNT and 2A6NT using phenylethyl alcohol (99%, Sigma Aldrich, UK) as an internal standard. Samples were grouped into two sets; one set was stored at room temperature and the other at -20°C. Phenylethyl alcohol (10 µl of 100 mg /L stock solution in acetonitrile), 2,6-DNT (10 µl of 100 mg /L stock solution in acetonitrile) and 2A6NT (10 µl of 200 mg /L stock solution in acetonitrile) were added to 60 mg of freeze dried *A. thaliana* tissues such that each Eppendorf tube had a concentration

of 1 mg /L of phenylethyl alcohol, 1 mg /L of 2,6-DNT and 2 mg /L of 2A6NT and the volume adjusted to 1 ml with acetonitrile.

Samples (triplicate) were processed using a QIAGEN Retsch Tissue Lyser with a frequency of 30.0 Hz/sec for 2 min after adding a stainless steel ball (5/32") in each Eppendorf tube. The tubes with homogenised slurry were vortexed for 50 s and then centrifuged in an Eppendorf centrifuge 5415D (13.2×1000 rpm for 7 min). The solvent phase was decanted and collected using a glass syringes (Agilent glass syringe, 1 ml capacity). The samples were re-extracted and the solvent phase was combined with the previous extraction. The resulting extract was concentrated by solvent evaporation under nitrogen using a Techne DRI-Block DB 3A to a volume of 200 µl. After analysis by GC-MS the peak areas of 2,6-DNT and 2A6NT were compared with the peak area of phenylethyl alcohol and with the standard solutions prepared in acetonitrile. The samples were extracted and analysed in triplicate. The efficiency was calculated as a percentage recovery by comparing the area of the peaks (DNT or ANT) obtained from the sample to the peaks (DNT or ANT) obtained through analysing stock solutions (same concentration as the sample) by GC.

$$\% \text{ recovery of compound} = \frac{[\text{Area of DNT/ANT from sample}]}{[\text{Area of DNT/ANT from stock}]} \times 100$$

Unprocessed samples having standard (1 mg /L phenylethyl alcohol) 2,6-DNT (1 mg /L) and 2A6NT (2 mg /L) were left at room temperature and at -20⁰C and were analysed and compared following the same procedure as described above every 24 h.

3.2.3.1.4 Determination of potential non specific binding of DNT and its metabolites

An experiment was conducted to verify any non specific binding of DNTs and ANTs in samples of different masses. This was achieved by taking homogenous freeze dried *A. thaliana* tissue samples weighing 20, 40, 60, 80 and 100 mgs in 2 ml Eppendorf tubes separately. 2,6-DNT (10 µl of 100 mg /L stock solution in acetonitrile) and 2A6NT (10 µl of 200 mg /L stock solution in acetonitrile) were added to the plant material and the volume made up to 1 ml with acetonitrile. The samples were vortexed and sonicated (Sonicator USC 100T, VWR, UK) for 12 h. The samples were centrifuged in Eppendorf centrifuge 5415D (13.2×1000 rpm) for 7 min and the supernatant decanted. The supernatant was concentrated under nitrogen using a Techne DRI-Block DB 3A to a volume of 200 µl (volume measured by Agilent Glass Syringe, 250 µl). The samples were stored in sample inserts (Agilent, 400 µl flat bottom glass inserts) within HP GC vials and analysed by GC-MS. The samples were processed in triplicate.

3.2.3.2 Extraction of metabolites from *A. thaliana* grown in Phytigel media

3.2.3.2.1 Extraction of metabolites from homogenised leaf tissue

After 18 days (full foliage stage that reaches magenta box limit) plants were removed from the agar and washed with deionised water, blotted dry on filter paper and their fresh weights recorded. The plants were frozen for 3 h at -20⁰C and freeze dried (HETO Dry Winner) for 48 h. Precautions were taken while handling plants to avoid cross contamination. After freeze drying, the plants from three boxes (each box had two plants) of the same treatment were combined together and ground in a coffee grinder (Wahl James Martin ZX595 coffee grinder) to obtain a homogeneous mixture i.e. one replicate is an homogenate of three individual plants. Sample (80 mg) was

taken from the homogenous mixture of freeze dried treated *A. thaliana* plants and placed into 2 ml Eppendorf tubes. A stainless steel ball (5/32") and acetonitrile (1ml) was added to the tubes and they were processed using a QIAGEN Retsch Tissue Lyser at a frequency of 30.0 Hz/sec for 2 min. The tubes were dipped in an ultrasonic bath (USC 100T, VWR, UK) and sonicated for 12 h. The extracts were vortexed for 1 min and centrifuged in an Eppendorf centrifuge 5415D (13.2×1000 rpm, 15 min). The solvent phase was decanted and collected using glass syringes (Agilent 250 µl glass syringe). The samples were re-extracted and the solvent phase was combined with the previous extraction. The resulting extract was then concentrated by solvent evaporation under nitrogen at Techne DRI-Block DB 3A to a volume of 200 µl (Agilent Glass Syringe, 250 µl). The sample was stored in GC vials and analysed by GC-MS. The process was repeated for a further two replicates and data was plotted as a mean of these three replicates.

3.2.3.2.2 Extraction of metabolites from a single plant

For the analysis of DNT metabolites in a single plant of *A. thaliana*, the treated plants were removed individually from the agar and washed with deionised water, blotted dry on filter paper and weighed. The plants were frozen individually for 3 h at -20°C and freeze dried (HETO Dry Winner) and homogenised by grinding in a coffee grinder (Wahl James Martin ZX595 coffee grinder). The homogenised dried leaf tissues of a single plant were considered as one replicate. The complete sample was placed in an Eppendorf tube together with a stainless steel ball (5/32"), acetonitrile (1ml) and processed using a QIAGEN Retsch Tissue Lyser at a frequency of 30.0 Hz/sec for 2 min. The rest of extraction procedure was as described in the paragraph above (section 3.2.3.2.1). The samples were stored in GC vials for further analysis. Graphical plots were a mean of four replicates.

3.2.3.3 Extraction of DNT and its metabolites from *A. thaliana* grown in hydroponic media (time-course study)

Eight plants were removed along with their seed holders after 7, 14, 21 and 28 days from the Araponic tanks. The plants were washed with deionised water and carefully blotted dry on filter paper. The roots and shoots were excised from the shoot base with a sharp razor and weighed individually. They were then flash frozen and kept at -20°C for 3 h and freeze dried (HETO Dry Winner) for 48 h. After freeze drying, the samples were ground individually in a coffee grinder (Wahl James Martin ZX595 coffee grinder) to obtain a homogeneous mixture of plant tissue. Sample (80 mg) was taken from the homogenous mixture of freeze dried treated *A. thaliana* plants and placed into a 2 ml Eppendorf tube. A stainless steel ball (5/32") and acetonitrile (1ml) was added to the tubes and they were processed using a QIAGEN Retsch Tissue Lyser at a frequency of 30.0 Hz/sec for 2 min. The tubes with homogenous slurry were dipped in an ultrasonic bath (USC 100T, Sonicator, VWR, UK) and sonicated for 12 h. The extracts were vortexed for 1 min and centrifuged Eppendorf centrifuge 5415D (13.2×1000 rpm, 15 min). The solvent phase was decanted and collected using a glass syringe (Agilent Glass Syringe). The samples were re-extracted and the solvent phase was combined with the previous extraction. The resulting extract was then concentrated by solvent evaporation under nitrogen using a Techne DRI-Block DB 3A to a volume of 200 µl (Agilent glass Syringe). The samples were stored in sample inserts (Agilent, 400 µl flat bottom glass inserts) within GC vials and analysed by GC-MS.

3.2.3.3.1 Analysis of metabolites by GC-MS

The conditions of the GC-MS were as described in Table 3.3. The GC data thus obtained was calculated to give the concentrations of metabolites obtained for each sample.

3.2.3.3.2 Calculations of unknown concentrations of DNT/ANT extracted from plant tissue

A linear regression analysis was carried for each set of standards (see Appendix for the standard curves) and the formula below used to calculate the concentration in mg /g dry wt of *A. thaliana* and mg/single plant of *A. thaliana* grown in treated Phytigel media.

The total volume of the extract = 200 μ l

From the standard curve the concentration of the metabolite is in mg /L = Y

Absolute amount of metabolite in 200 μ l = $Y \times (200/10^6) = X$

Amount of metabolite (μ g or ng) /g dry wt tissue = $(1000 / \text{wt of tissues analysed in mg}) \times X$

Concentrations were calculated to give μ g/g for 2,6-DNT plants and ng/g for 2,3-DNT, 2,4-DNT and 3,4-DNT treated plants.

3.3 Results

3.3.1 Method development

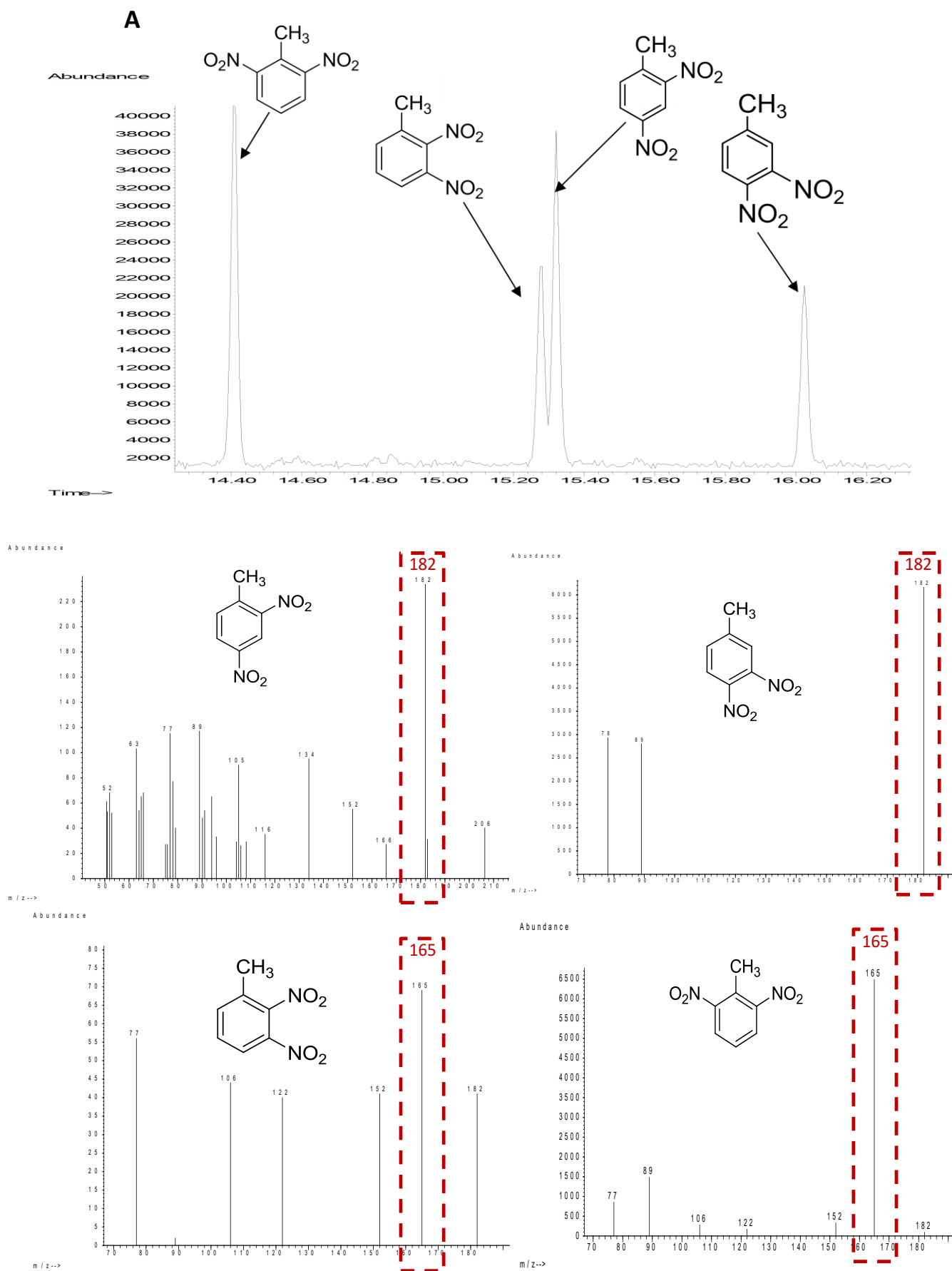
GC-MS methods were developed for the analysis of DNT and its metabolites extracted from the plants, grown on DNT treated media. The retention times of parent ions and qualitative ions were selected for all compounds undergoing analysis using a full scan mode. The conditions and dwell times were adjusted for SIM detection to

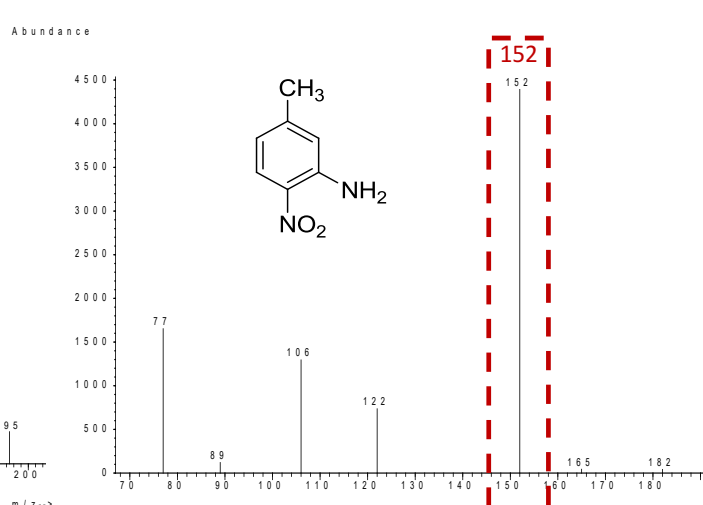
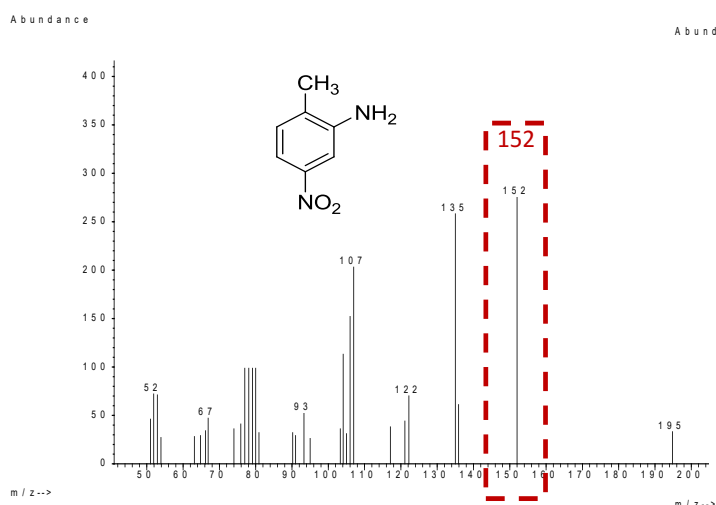
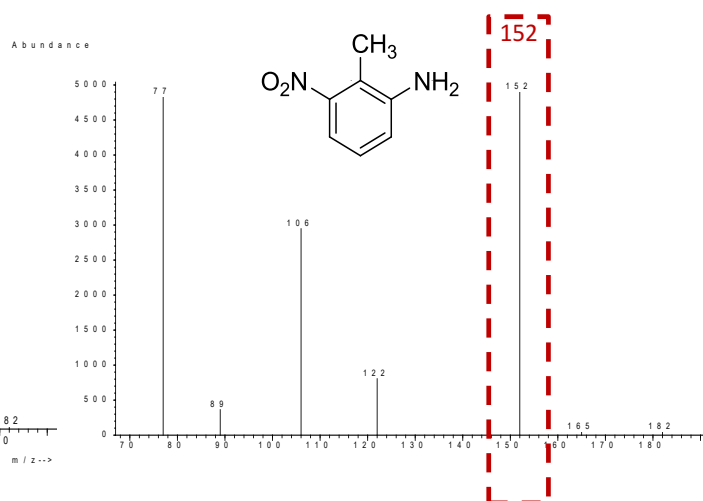
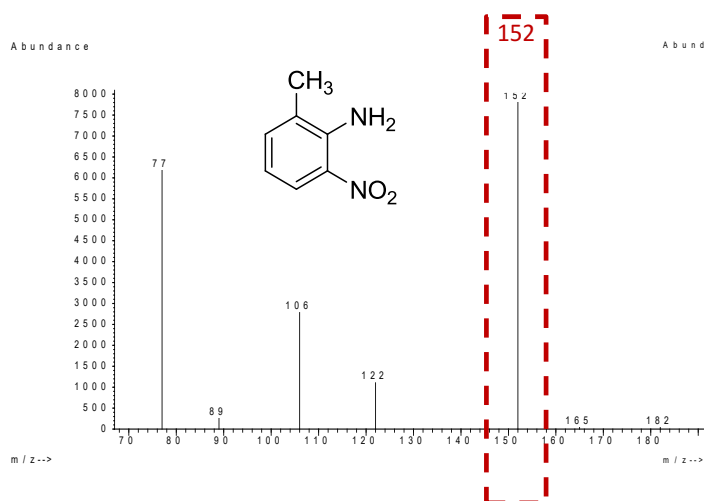
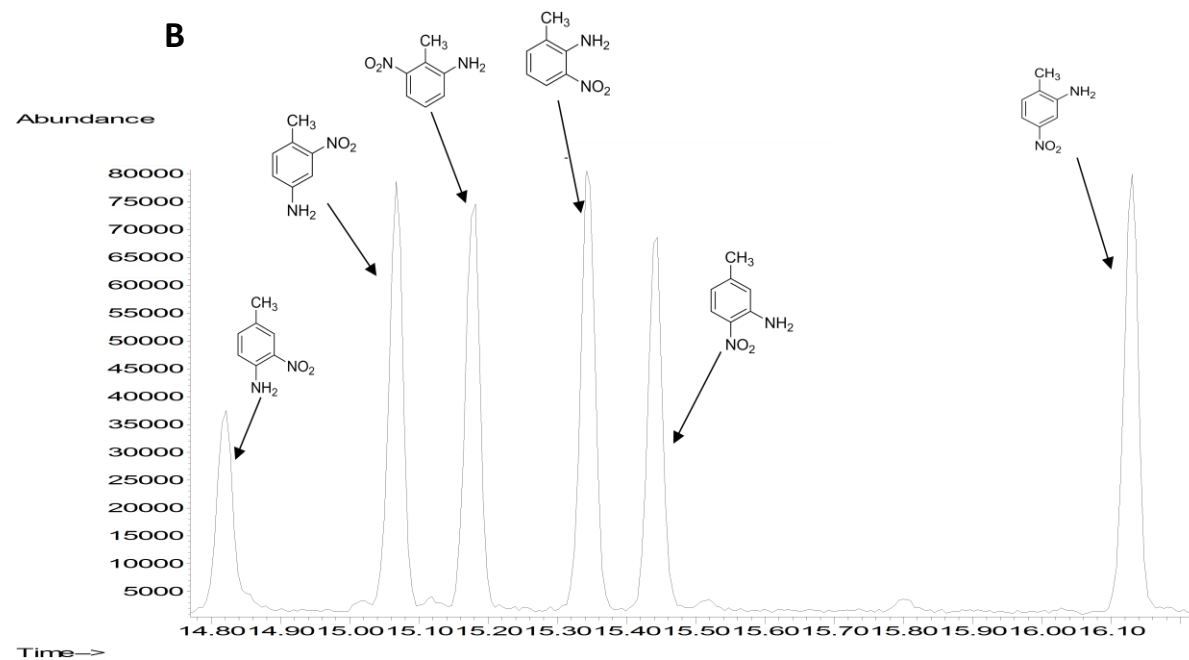
maximise the sensitivity of the instrument for the targeted compounds. The solvent efficiency and accuracy of the extraction procedure were determined to indicate the reliability of the method.

3.3.1.1. Selection of Ions for SIM acquisition

A full scan acquisition was performed on two samples, one was a mixture containing four isomers of DNTs (solution prepared in acetonitrile) and the other their respective metabolites (standard solutions prepared in acetonitrile). The GC conditions employed for the analysis are summarized in Table 3.3.

From the full scan run of the mixture of compounds the retention times for 2,4-DNT (15.30 min), 2,3-DNT (15.25 min), 2,6-DNT (-14.4 min) and 3,4-DNT (16. 25 min) were identified (Figure 3.3). The parent ions and quant ions of different DNT isomers and ANTs were differentiated. The percentage abundance of quant ions was determined relative to the parent ions for reference to the mass spectrum of each compound.





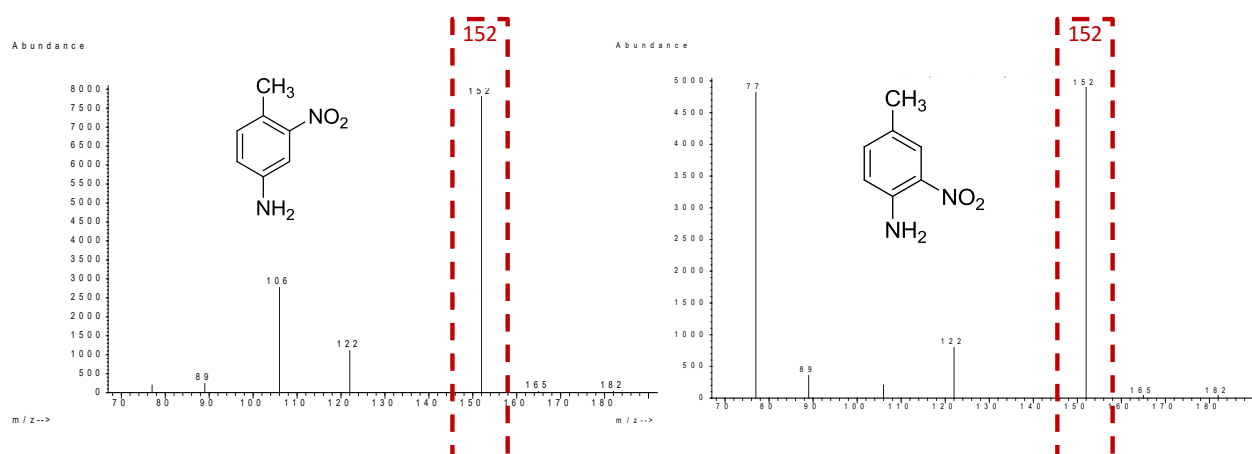


Figure 3.3: Full scan acquisition of four isomers of (A) DNT (2,4-DNT, 2,3-DNT, 3,4-DNT and 2,6-DNT) with their mass spectra. (B) The reduced metabolites of four isomers of DNT (2A4NT, 2A3NT, 3A4NT, 4A2NT, 4A3NT and 2A6NT) with their mass spectra. The GC chromatogram is normalized along the y-axis for the total ion current and the x-axis for time while the MS is normalised along y-axis for abundance and x-axis for m/z ratio.

After identification of parent ions and qualitative ions for the compounds of interest, the ions were selected for SIM acquisition (Table 3.4) of their mass spectrum and edited in the parameters. The purpose of this was to enhance the sensitivity of the analysis by reducing the unnecessary background peaks and noise (Figure 3.4).

Table 3.4: Quantification of ions for Selective Ion Monitoring, through Scan run of Standards. The qualitative ions are given as percentage of quant ions.

Name of the compound	Molecular weight (m/z)	Retention time	Quant Ion	Qualitative ions
2,4-dinitrotoluene	182	15.35	182	89 (54.5%)
				77 (51.6%)
				63 (47.17%)

2,6-dinitrotoluene	182	14.40	165	89 (26.19%)
				77 (15.6%)
				108 (14.3%)
2,3-dinitrotoluene	182	15.25	165	77 (74.5%)
				108 (56.3%)
				182 (52.23%)
3,4-dinitrotoluene	182	16.03	182	77 (32.85%)
				89 (31.75%)
2-amino- 4-nitrotoluene	152	16.12	152	136 (96.36%)
				107 (66.6%)
				122 (21.22%)
2-amino- 6-nitrotoluene	152	15.19	152	77 (98.97%)
				108 (25.1%)
				122 (21.7%)
4-amino-3-nitrotoluene	152	14.85	152	77 (99.95%)
				122 (12.3%)
				89 (7.6%)
3-amino-4-	152	15.45	152	77 (37.78%)

nitrotoluene				108 (33.2%)
				122 (16.26%)
4-amino-2-	152	15.09	152	108 (36.22%)
nitrotoluene				122 (16.3%)
2-amino-3-	152	15.35	152	77 (77.49%)
nitrotoluene				108 (35.02%)
				122 (12.56%)

After the selection of the relevant molecular and fragmentation ions, the mass to charge ratio of the selected ions were divided in four groups and SIM acquisition was modified to focus only those ions which are the most indicative of the DNT isomers and their metabolites. These groups on the basis of mass per charge ratio (m/z) of ions are group I - 65, 91, 107, 120; group II- 94, 104, 106, 122 and group III- 63, 77, 89, 106, 107, 122, 135, 152, 165, 182. The start dwell time was 6 min at which 4 ions (group I) were to be detected, then 12:50 min where again 4 ions (group II) were to be detected and then at 14:30 min 10 ions (group III) were detected. It was assumed that the column conditions (optimum temperature, maximum hold and ideal gas flow) are favourable for the maximum separation of ANTs and DNTs at 12:50 min therefore the maximum ions were adjusted to be detected at this time (Table 3.5).

Table 3.5: Detector specifications and group of ions for SIM acquisition

Detector	
Name	Hewlett Packard 5973 Mass Selective Detector

Mass Spectrometer

Acquisition mode Selective Ion Monitoring (SIM)

Solvent delay 6 min

Time window 33 min

Group of ions for
detection as divided
according to time 4

	<i>Time range</i> <i>(min)</i>	<i>No. of ions</i>	<i>m/z ratio of ions</i>
I	6:00-12:50	4	65, 91, 107, 120
II	12:50-14:30	4	94, 104, 106, 122
III	14:30-16:90	10	63, 77, 89, 106, 107, 122, 135, 152, 165, 182

The mass spectrum produced after this selection provided individual peaks of DNTs and ANTs compounds with no unnecessary peaks in the background.

3.3.1.2 Optimization of extraction procedure (necessity of internal standard while extraction)

After the selection of appropriate conditions for GC-MS to detect the ions of interest, the efficiency of extraction procedure was determined to account for any loss of analyte.

The extraction in acetonitrile was verified by comparing extractions with and without an internal standard. This was carried out by spiking a sample of *A.thaliana* (grown in Phytigel media with 20 mg 3,4-DNT) with 2,6-DNT.

The recovery of standard was 100% from the samples and negligible difference was observed when the area of 3A4NT with internal standard (2,6-DNT) was compared with the area of 3A4NT without internal standard (Table 3.6). Based on these results the use of an internal standard in any further analysis was considered unnecessary. It can be noted that since there are no clean up steps involved in processing the sample, where loss of sample could occur, addition of internal standard is not normally necessary for this type of analysis.

Table 3.6: The % recovery of 2,6-DNT(internal standard) and corrected concentrations of 3A4NT as recovered from the 3,4-DNT treated samples extracted with and without internal standard.

No. of obs.	% recovery of standard = (Area of peak from sample /area of peak of standard of the same concentration) ×100)	Corrected concentration of metabolites with internal standard (ng /g dry tissue wt of <i>A. thaliana</i>)	Uncorrected concentration of metabolites without internal standard (ng /g dry tissue wt of <i>A. thaliana</i>)
1	99.99 %	324.58	324.55
2	99.98%	324.61	324.56
3	100%	324.56	324.55

3.3.1.2.1. Determination of the storage stability of the DNT and its metabolites

To determine the stability of DNT isomers and their respective ANTs the percentage recovery was observed for 2,6-DNT (1 mg/L concentration) and 2A6NT (2 mg/L concentration) in *A. thaliana* tissue samples kept at -20°C and room temperature during a

time course of study. The phenylethyl alcohol (1 mg /L concentration) was used as an internal standard (since it does not co-run with DNTs or ANTs) to show the extraction efficiency.

The areas of the peaks for 2,6-DNT, 2A6NT and phenylethyl alcohol as obtained from the GC-MS analysis of samples (stored at room temperature and -20°C during different days of analysis) showed no difference ($p > 0.05$). The percentage recovery of the samples stored at room temperature and analysed during different days (Table 3.7), which is indicative of the stability of ANTs and DNTs at room temperature, showed that the compounds were very stable over the course of time.

Table 3.7: The percentage recoveries of 2,6-DNT, 2A6NT and phenylethyl alcohol in 60 mg plant extract, as obtained during different days of exposure from samples (n=3) kept at room temperature

Days of analysis	% recovery of 2,6-DNT	% recovery of 2A6NT	% recovery of phenylethyl alcohol
2	98.65 $\pm$ 0.02	98.02 $\pm$ 0.02	99.59 $\pm$ 0.01
4	99.05 $\pm$ 0.01	99.02 $\pm$ 0.02	98.49 $\pm$ 0.01
6	98.55 $\pm$ 0.01	98.99 $\pm$ 0.02	98.84 $\pm$ 0.01
8	98.35 $\pm$ 0.02	98.45 $\pm$ 0.02	99.89 $\pm$ 0.01
10	98.12 $\pm$ 0.02	98.61 $\pm$ 0.01	99.32 $\pm$ 0.01
12	99.12 $\pm$ 0.01	99.07 $\pm$ 0.01	99.74 $\pm$ 0.01
14	98.95 $\pm$ 0.01	99.33 $\pm$ 0.01	99.64 $\pm$ 0.01

For the samples stored at -20°C the recoveries of ANT, DNT and phenylethyl alcohol were observed to be same throughout the analysis during different days (Table 3.8), which concludes that ANT and DNT remained stable at both room temperature and -20°C for several days. Although only one isomer and metabolite was used for this study it would seem highly unlikely that any of the other isomers would behave in a different manner.

Table 3.8: The percentage recoveries of 2,6-DNT, 2A6NT and phenylethyl alcohol in a 60 mg plant extract, as obtained during different days of exposure from samples (n=3) kept at -20°C .

Days of analysis	% recovery of 2,6-DNT	% recovery of 2A6NT	% recovery of phenylethyl alcohol
2	98.23 $\pm$ 0.03	98.34 $\pm$ 0.01	98.32 $\pm$ 0.02
4	99.22 $\pm$ 0.02	98.82 $\pm$ 0.03	99.76 $\pm$ 0.03
6	98.87 $\pm$ 0.02	99.65 $\pm$ 0.03	99.02 $\pm$ 0.03
8	98.55 $\pm$ 0.02	98.43 $\pm$ 0.03	98.94 $\pm$ 0.03
10	99.02 $\pm$ 0.01	99.61 $\pm$ 0.02	99.34 $\pm$ 0.03
12	99.67 $\pm$ 0.02	98.88 $\pm$ 0.02	98.21 $\pm$ 0.03
14	98.25 $\pm$ 0.01	99.31 $\pm$ 0.03	99.14 $\pm$ 0.02

3.3.1.3. Determination of potential non specific binding of DNT and its metabolites

The non specific binding of the 2A6NT and 2,6-DNT with plant tissues was tested by adding 10 μl of 200 mg /L of 2A6NT (to give a final concentration of 2 mg /L) and 10 μl of 100 mg /L of 2,6-DNT (to give a final concentration of 1 mg /L) to 20 mg, 40 mg, 60 mg, 80 mg and 100 mg (dry wt) of *A. thaliana* and sonicated (Sonicator VWR, UK) for 12 h. All peak areas

were of a similar size throughout the experiment indicating that no compound losses were occurring due to non-specific binding of metabolites to plant material (Table 3.9).

Table 3.9: The comparison of the areas of the peaks of 2A6NT (2 mg /L) and 2,6-DNT (1 mg /L) as obtained through GC-MS analysis of samples (n=3) with different masses sonicated for 12 h.

Mass of dried <i>A. thaliana</i> tissue (mg)	Area of 2,6-DNT	Area of 2A6NT
20	2385 $\pm$ 2.02	1324 $\pm$ 1.11
40	2389 $\pm$ 1.42	1322 $\pm$ 1.34
60	2289 $\pm$ 1.65	1324 $\pm$ 1.65
80	2381 $\pm$ 1.71	1356 $\pm$ 2.11
100	2365 $\pm$ 2.33	1345 $\pm$ 2.04

3.3.2 DNT and its metabolites from *A. thaliana* grown in Phytigel media

The analysis of *A. thaliana* plants treated with DNTs showed that the main metabolites produced were ANTs. Primary confirmation of the peaks, as observed by MS, was made by matching the mass spectrum with the standards. Initial identification was indicated by the retention times and confirmation made by matching their mass fragmentation patterns. The target ions were added to the SIM method and further analysis of plant samples was conducted through SIM acquisition. The metabolites were measured in per gram dry tissue wt. of treated *A. thaliana* plants and single dry plant to get the variation in concentrations of metabolites obtained through two different ways.

3.3.2.1 Uptake and metabolism of 2,6-DNT in *A. thaliana*

3.3.2.1.1 Analysis using homogenised leaf tissues

GC-MS analysis showed that *A. thaliana* takes up and reduces 2,6-DNT to its metabolite 2A6NT. The concentration of metabolite measured varied with the concentration of 2,6-DNT (Figure 3.4) in Phytagel media. The maximum concentration of 2A6NT obtained was **5.23±0.18 µg /g dry wt** of *A. thaliana* at a 2,6-DNT concentration of 23 mg /L of Phytagel media (Figure 3.4). As expected control experiments (no DNT added to media) with and without 0.25% ethanol gave no traces of 2,6-DNT or its metabolites. The data from these experiments are shown in Table 3 of Appendix II. All subsequent control experiments are shown in the relevant Appendix.

At the lower concentrations (3 mg /L of Phytagel media) of 2,6-DNT in the media no untransformed 2,6-DNT was obtained only 2A6NT, which indicates that plants at this concentration can efficiently metabolise DNTs to ANTs. The concentration of 2A6NT increased to a maximum at a 2,6-DNT concentration of 23 mg /L of Phytagel media after which it decreased to **1.75±0.18 µg /g dry wt** at a 2,6-DNT concentration of 43 mg/L of Phytagel media. At a 2,6-DNT concentration of 53 mg /L of Phytagel media the plants were completely stunted without adequate tissue for analysis.

Untransformed 2,6-DNT appeared in the plants at a concentration of 13 mg /L of Phytagel media (IC₅₀ value for 2,6-DNT) and continues to increase until a 2,6-DNT concentration of 43 mg /L of Phytagel media reaching a concentration of **1.22±0.14 µg /g dry wt**.

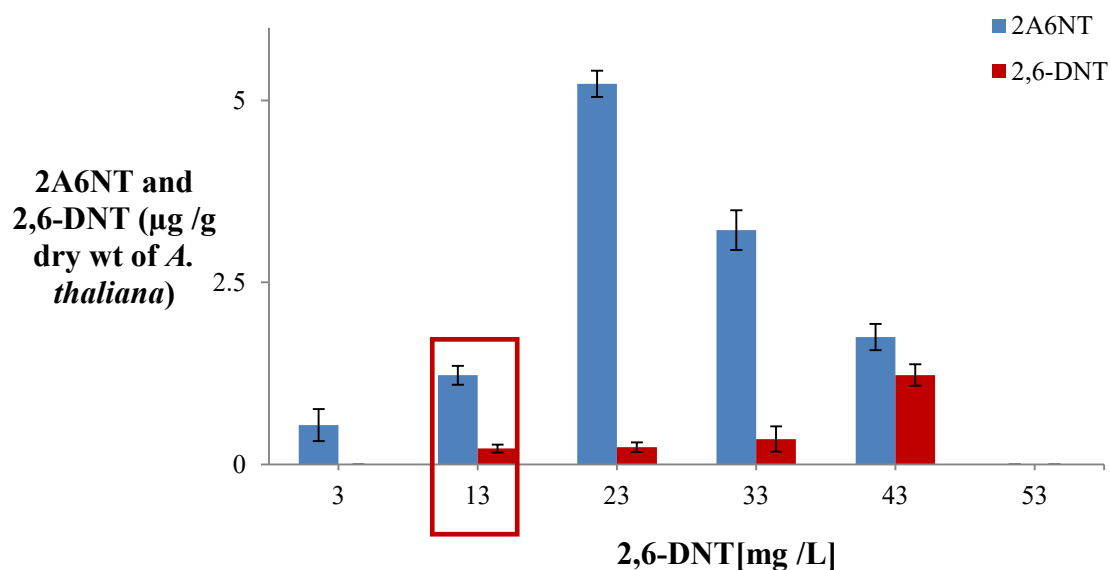


Figure 3.4: 2,6-DNT(■) and 2A6NT(■) concentrations recovered from 18 day (post cultivation) old *A. thaliana* plants grown in different concentrations of 2,6-DNT in sterile Phytigel media (growth temperature was $20\pm 3^{\circ}\text{C}$ for 16:8 h day:night period) and analysed by GC-MS. Data was plotted as a mean of three replicates (plants from three pots were combined, freeze dried and homogenized to give one replicate). The red box indicates the IC_{50} as calculated from root growth analysis (Chapter 2; section 2.3). The control and 0.25% mock control treated plants were analysed with exactly the same procedures as DNT treated plants and the values of all results are shown in Table 3 of Appendix II.

3.3.2.1.2 Analysis using whole single plants

It was decided to examine whole single plants rather than just analytical replicates of homogenised tissue. This would enable the variation between single plants to be examined.

The results showed that individual *A. thaliana* plants (mean of data from four plants analysed individually) grown in media amended with a 2,6-DNT concentration of 23 mg /L of Phytigel had the greatest capacity to metabolise 2,6-DNT producing 2A6NT at a concentration of 1.16 ± 0.03 µg /single dried plant (Figure 3.5).

At lower concentrations (3 and 13 mg /L of Phytigel media) of 2,6-DNT in the media no untransformed 2,6-DNT was obtained, which indicates that at this concentration plants can

efficiently metabolise DNTs to ANTs. The concentration of 2A6NT increased to a maximum ($0.87 \pm 0.065 \mu\text{g} / \text{single dried plant}$) at a 2,6-DNT concentration of 23 mg /L of Phytagel media after which it decreased to $0.24 \pm 0.054 \mu\text{g} / \text{single dried plant}$ at 2,6-DNT concentration of 43 mg /L of Phytagel media. At a 2,6-DNT concentration of 53 mg /L of Phytagel media the plants were completely stunted without adequate tissue for analysis.

Untransformed 2,6-DNT appears in plants at a concentration of 23 mg /L of Phytagel media and continues to increase until a 2,6-DNT concentration of 43 mg /L of Phytagel media reaching a concentration of $0.21 \pm 0.03 \mu\text{g} / \text{single dried plant}$. This indicates that the ability of the plant to detoxify 2,6-DNT is impaired as the concentration of 2,6-DNT in the media increases.

At the IC₅₀ value there appears to be only 2A6NT and no 2,6-DNT was obtained. At this concentration the plants can efficiently metabolise 2,6-DNT to 2A6NT, however, it is possible that some metabolites are below the detection limit..

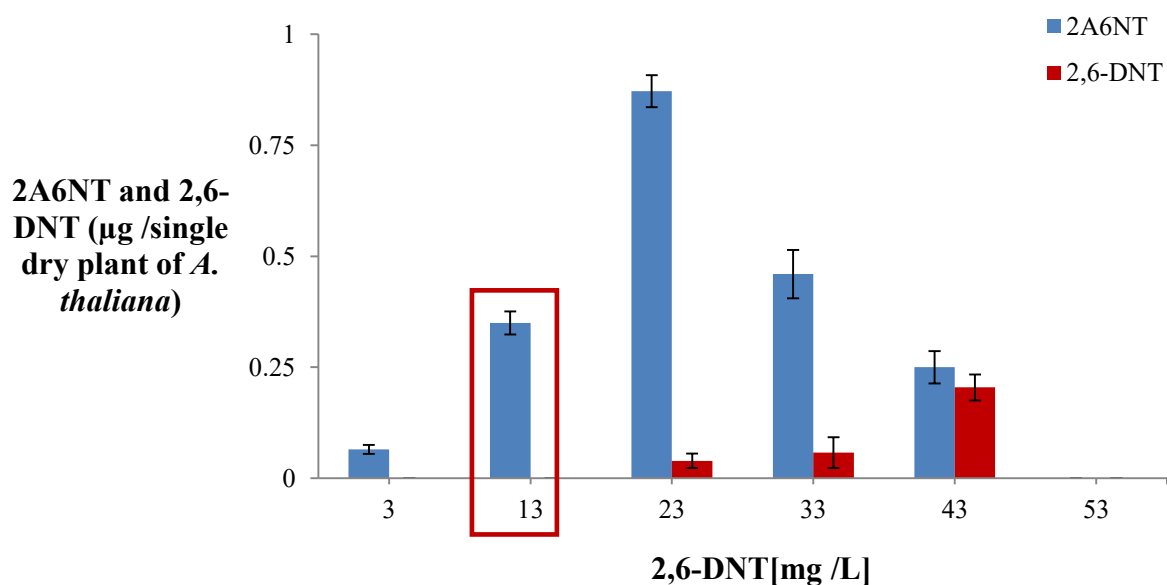


Figure 3.5: 2,6-DNT(■) and 2A6NT(■) concentrations recovered from a single 18 day (post cultivation) old *A. thaliana* plant grown in different concentrations of 2,6-DNT in

sterile Phytigel media and analysed by GC-MS (growth temperature was $20\pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as a mean of four replicates (only one plant from one pot was freeze dried and homogenized to give one replicate). The red box indicates the IC_{50} as calculated from root growth analysis (Chapter 2; section 2.3.1). The control and 0.25% mock control treated plants were analysed with exactly the same procedures as DNT treated plants and the values of all results are shown in Table 3 of Appendix II.

It would appear that the metabolite profile of the two sampling techniques i.e. per gram dried tissue weight (Figure 3.4) and per single dried plant of *A. thaliana* (Figure 3.5) are similar while there does not appear to be a large amount of variation between individual plants based on the standard error (SE) of mean.

3.3.2.2 Uptake and metabolism of 2,4-DNT in *A. thaliana*

3.3.2.2.1 Analysis using homogenised leaf tissue

When *A. thaliana* was exposed to 2,4-DNT containing media, two reduced metabolites (2A4NT and 4A2NT) were obtained through GC-MS analysis (Figure 3.6). The concentration of metabolites measured, varied with the concentration of 2,4-DNT (Figure 3.6) in Phytigel media. The maximum concentration of 2A4NT and 4A2NT obtained was 280.93 ± 26.68 ng /g dry wt and 508.3 ± 43.93 ng /g dry wt of *A. thaliana* respectively, at a 2,4-DNT concentration of 0.9 mg /L of Phytigel media. At lower concentrations (0.03 mg /L of Phytigel media) of 2,4-DNT in the media only 4A2NT was observed. This indicates the plant's efficiency to convert 2,4-DNT to 4A2NT compared to 2A4NT. At the IC_{50} value (0.3 mg /L of Phytigel) there appears to be only the metabolites 2A4NT and 4A2NT and no untransformed 2,4-DNT, which shows that at this concentration the plants can efficiently metabolise 2,4-DNT to 2A4NT and 4A2NT. At a 2,4-DNT concentration of 4.5 mg /L of Phytigel media the plants were completely stunted without adequate tissue for analysis.

The untransformed 2,4-DNT appears in plants at a concentration of 1.8 mg /L of Phytigel media and continues to increase until a 2,4-DNT concentration of 3.6 mg /L Phytigel media reaching a concentration of **311.82±25.45 ng /g dry weight**. This indicates that the ability of the plant to detoxify 2,4-DNT is most likely impaired as the concentration of the ANT isomers decreases with increasing 2,4-DNT concentration in the media.

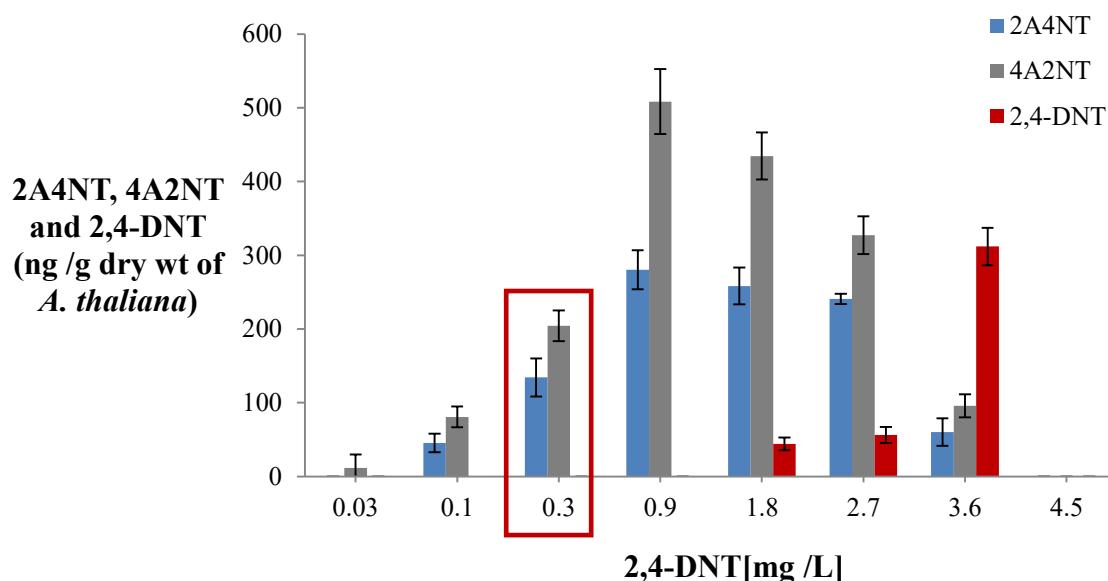


Figure 3.6: 2,4-DNT (■), 4A2NT (■) and 2A4NT (■) concentrations recovered from 18 day (post cultivation) old *A. thaliana* plants grown in different concentrations of 2,4-DNT in sterile Phytigel media and analysed by GC-MS (growth temperature was 20±3°C for 16:8 h day:night period). Data was plotted as a mean of three replicates (plants from three pots were combined, freeze dried and homogenized to give one replicate). The red box indicates the IC₅₀ as calculated from root growth analysis (Chapter 2; section 2.3). The control and 0.25% mock control treated plants were analysed with exactly the same procedures as DNT treated plants and the values of all results are shown in Table 7 of Appendix II.

3.3.2.2.2 Analysis using whole single plants

The data showed that the individual *A. thaliana* plants (mean of data from four plants analysed individually) grown in media amended with a 2,4-DNT concentration of 0.9 mg /L of Phytigel had the greatest capacity to metabolise 2,4-DNT producing 2A4NT at a

concentration of **106.66±5.01 ng /single dried plant** and 4A2NT at a concentration of **183.24±6.35 ng /single dried plant** (Figure 3.7).

At the lowest concentration of 2,4-DNT (0.03 mg /L of Phytigel media) only 4A2NT was observed and no 2A4NT and untransformed 2,4-DNT was observed showing a similar trend to the homogenised plant tissue analysis.

The concentrations of 2A4NT and 4A2NT increased to a maximum concentration (**106.66±5.01 µg /single dried plant** and **183.24±6.35 µg /single dried plant**, respectively) at a 2,4-DNT concentration of 0.9 mg /L of Phytigel media. After this it started to decrease reaching a value of **20.03±2.23 ng /single dried plant** and **45.2±2.91 ng /single dried plant** respectively. At the IC₅₀ value (0.3 mg /L of Phytigel) only the metabolites 2A4NT and 4A2NT were obtained (no untransformed 2,6-DNT was obtained), which shows that at this concentration the plants can efficiently metabolise 2,4-DNT to 2A4NT and 4A2NT. At a 2,4-DNT concentration of 4.5 mg /L of Phytigel media the plants were completely stunted without adequate tissue for analysis.

Untransformed 2,4-DNT starts to appear in plants at a concentration of 1.8 mg /L of Phytigel media and continues to increase up until a 2,4-DNT concentration of 4.5 mg /L of Phytigel media reaching a concentration of **24.13±4.87 ng /single dried plant**. This indicates that the ability of the plant to detoxify 2,4-DNT is impaired as the concentration of 2,4-DNT in the media increases.

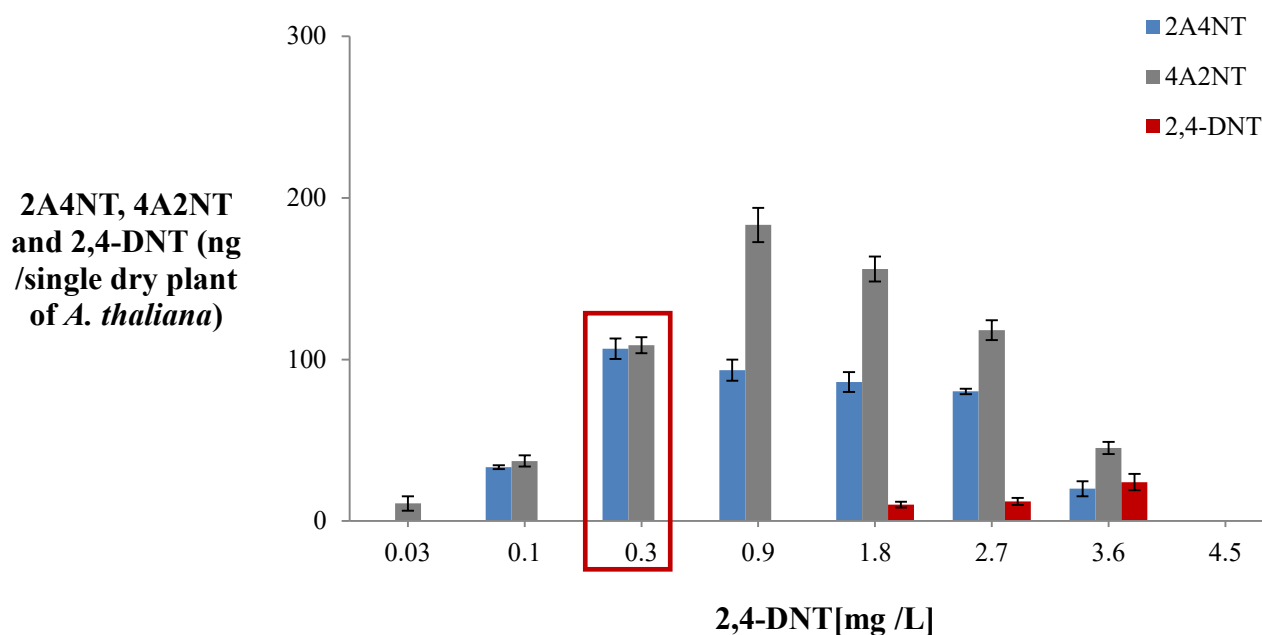


Figure 3.7: 2,4-DNT (■), 4A2NT (■) and 2A4NT (■) concentrations recovered from a single 18 day (post cultivation) old *A. thaliana* plant grown in different concentrations of 2,4-DNT in sterile Phytigel media and analysed by GC-MS (growth temperature was $20\pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as a mean of four replicates (only one plant from one pot was freeze dried and homogenized to give one replicate). The red box indicates the IC_{50} as calculated from root growth analysis (Chapter 2; section 2.3.1). The control and 0.25% mock control treated plants were analysed with exactly the same procedures as DNT treated plants and the values of all results are shown in Table 7 of Appendix II.

Comparing the trends (Figure 3.6 and 3.7) of metabolites and untransformed material as obtained through two different methods, it was observed that there is slight variation in the trends as comparatively lower concentrations of untransformed 2,4-DNT were obtained for single 2,4-DNT treated *A. thaliana* plants.

3.3.2.3 Uptake and metabolism of 2,3-DNT in *A. thaliana*

3.3.2.3.1 Analysis using homogenised leaf tissue

Figure 3.8 indicates that the highest concentration of 2A3NT observed was 675.22 ± 20.13 ng /g dry wt of *A. thaliana* for plants treated with a 2,3-DNT concentration of 1 mg /L of Phytigel media. At the lower concentrations (0.05 mg /L of Phytigel media) of 2,3-DNT in

the media only 2A3NT was observed and no untransformed 2,3-DNT was detected. At the IC_{50} value 2A3NT and untransformed 2,3-DNT were observed which shows that at this concentration the plants were stressed and could not completely detoxify 2,3-DNT to 2A3NT. The concentration of 2A3NT after reaching a maximum of 675.22 ± 20.13 ng /g dry wt at a 2,3-DNT concentration of 1 mg /L of Phytigel media, decreased to 103.2 ± 27.12 ng /g dry wt at a 2,3-DNT concentration of 2.5 mg /L of Phytigel media.

Untransformed 2,3-DNT appeared in the plants at a concentration of 0.1 mg /L of Phytigel media and continues to increase until a 2,3-DNT concentration of 2.5 mg /L of Phytigel media reaching a concentration of 162 ± 30.84 ng /g dry wt.

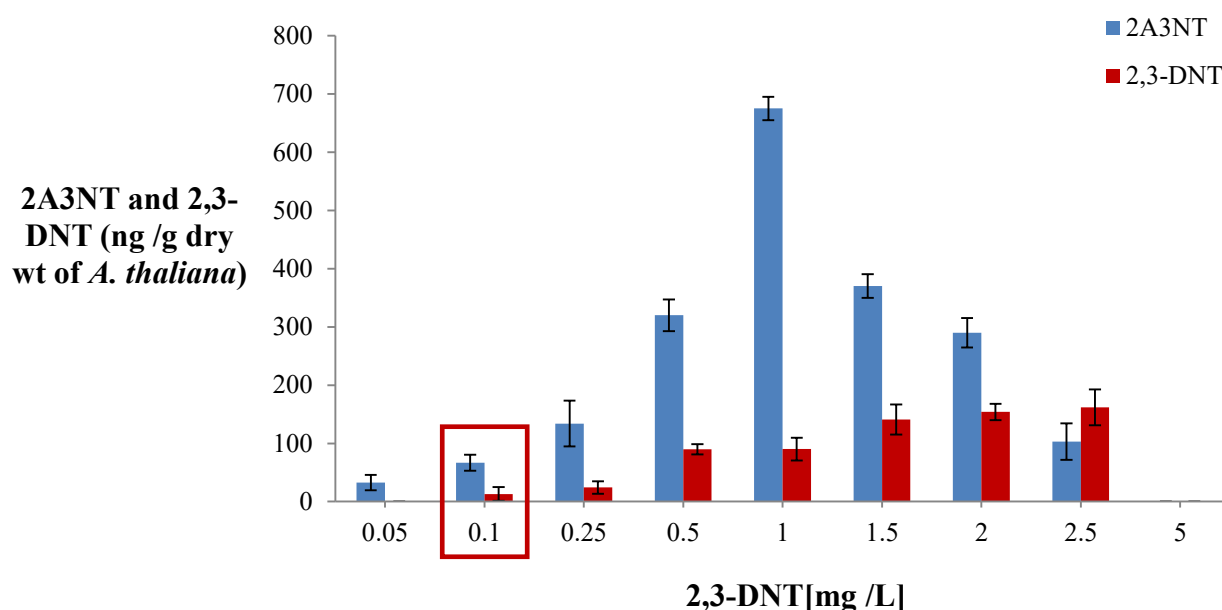


Figure 3.8: 2,3-DNT (■) and 2A3NT (■) concentrations recovered from 18 day (post cultivation) old *A. thaliana* plants grown in different concentrations of 2,3-DNT in sterile Phytigel media and analysed by GC-MS (growth temperature was $20 \pm 3^{\circ}C$ for 16:8 h day:night period). Data was plotted as a mean of three replicates (plants from three pots were combined, freeze dried and homogenized to give one replicate). The red box indicates the IC_{50} as calculated from root growth analysis (Chapter 2; section 2.3). The control and 0.25% mock control treated plants were analysed with exactly the same

procedures as DNT treated plants and the values of all results are shown in Table 10 of Appendix II.

3.3.2.3.2 Analysis using whole single plants

The results show that individual *A. thaliana* plants (mean of data from four plants analysed individually) grown in media amended with a 2,3-DNT concentration of 0.25 mg /L of Phytigel had the greatest capacity to metabolise 2,3-DNT producing 2A3NT at a concentration of **86.54±4.82 µg /single dried plant** (Figure 3.9) after which it decreased to **22.33±2.84 µg /single dried plant** at the 2,3-DNT concentration of 2.5 mg /L of Phytigel media. This concentration is higher than the IC₅₀ value (0.1 mg /L of Phytigel media). This shows that at concentrations higher than the IC₅₀ value plants can detoxify 2,3-DNT to its reduced product. At the lower concentrations (0.05, 0.1, 0.25 and 0.5 mg /L of Phytigel media) of 2,3-DNT in the media only 2A3NT was observed and no untransformed 2,3-DNT was obtained. This indicates that the plants can completely detoxify 2,3-DNT at low concentrations in the media. At 2,3-DNT concentration of 5 mg /L of Phytigel media the plants were completely stunted without adequate tissue for analysis.

Untransformed 2,3-DNT appears in plants at a concentration of 1 mg /L of Phytigel media and continues to increase up until a 2,3-DNT concentration of 2.5 mg /L of Phytigel media reaching a concentration of **45.66±3.22 ng /single dried plant**. This indicates that the ability of the plant to detoxify 2,3-DNT is impaired as the concentration of 2,3-DNT in the media increases.

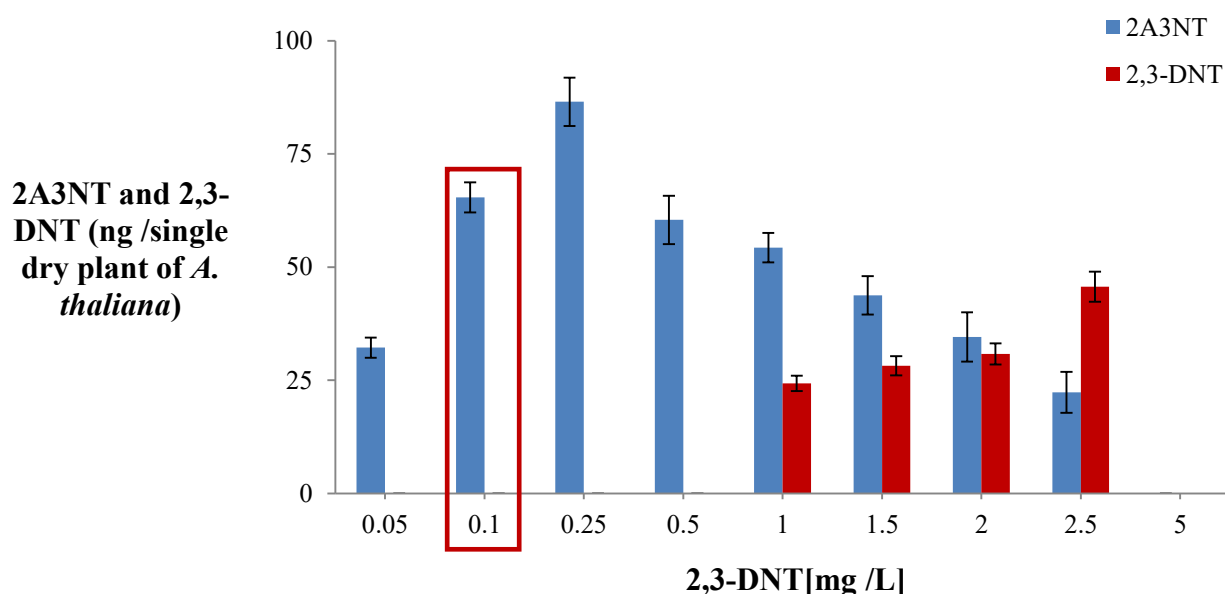


Figure 3.9: 2,3-DNT (■) and 2A3NT (■) concentrations recovered from a single 18 day (post cultivation) old *A. thaliana* plant grown in different concentrations of 2,3-DNT in sterile Phytigel media and analysed by GC-MS (growth temperature was $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as a mean of four replicates (only one plant from one pot was freeze dried and homogenized to give one replicate). The red box indicates the IC_{50} as calculated from root growth analysis (Chapter 2; section 2.3.1). The control and 0.25% mock control treated plants were analysed with exactly the same procedures as DNT treated plants and the values of all results are shown in Table 10 of Appendix II.

By comparing the profiles of the two analyses i.e. Figure 3.8 and 3.9, it can be seen that the pattern of DNT uptake and formation of the 2A3NT is different. In the Figure 3.8 the highest concentration of 2A3NT was observed at 2,3-DNT concentration of 1 mg /L of Phytigel media, however, according to data presented in Figure 3.9, the highest metabolites were observed at 0.25 mg /L of Phytigel media.

2.3.2.4. Uptake and metabolism of 3,4-DNT in *A. thaliana*

2.3.2.4.1 Analysis using homogenised leaf tissue

GC-MS analysis showed that *A. thaliana* takes up and reduces 3,4-DNT to its metabolite 3A4NT. The concentration of metabolite measured varied with the concentration of 3,4-DNT

(Figure 3.10) in Phytagel media. The maximum concentration of 3A4NT obtained was **564.32±17.98 ng /g dry wt** of *A. thaliana* at a 3,4-DNT concentration of 0.8 mg /L of Phytagel media which is higher than the IC₅₀ value (0.2 mg /L of Phytagel media) (Figure 3.8). At lower concentrations (0.02 mg /L of Phytagel media) of 3,4-DNT in the media no 3A4NT and untransformed 3,4-DNT was obtained indicating that the uptake mechanism or mobility of this compound is less efficient compared to the other DNT isomers.

The concentration of 3A4NT increased to a maximum (**564.32±17.98 ng /g dry wt**) at a 3,4-DNT concentration of 0.8 mg /L of Phytagel media after which it decreased to **41.23±11.32 ng /g dry wt** at a 3,4-DNT concentration of 4.6 mg /L of Phytagel media. At the IC₅₀ (0.2 mg /L of Phytagel media) no untransformed 3,4-DNT was obtained, which indicates that at this concentration the plants can detoxify 3,4-DNT completely. At a 3,4-DNT concentration of 6.4 mg /L of Phytagel media the plants were completely stunted without adequate tissue for analysis.

Untransformed 3,4-DNT appeared in the plants at a concentration of 0.4 mg /L of Phytagel media and continues to increase until a 3,4-DNT concentration of 4.6 mg /L of Phytagel media reaching a concentration of **170.2±13.34 ng /g dry wt**. Therefore, the concentration of untransformed 3,4-DNT increases with the increase in concentration of 3,4-DNT in the media.

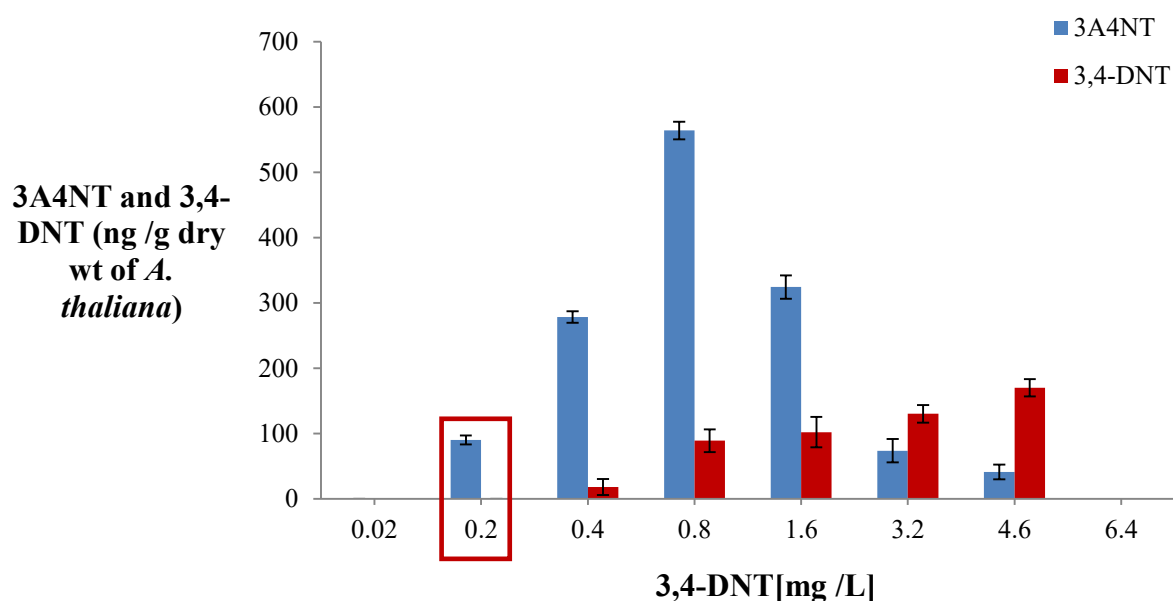


Figure 3.10: 3,4-DNT (■) and 3A4NT (■) concentrations recovered from 18 day (post cultivation) old *A. thaliana* plants grown in different concentrations of 3,4-DNT in sterile Phytigel media and analysed by GC-MS (growth temperature was $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as a mean of three replicates (plants from three pots were combined, freeze dried and homogenized to give one replicate). The red box indicates the IC_{50} as calculated from root growth analysis (Chapter 2; section 2.3). The control and 0.25% mock control treated plants were analysed with exactly the same procedures as DNT treated plants and the values of all results are shown in Table 13 of Appendix II.

2.3.2.4.2 Analysis using whole single plants

The results showed that the individual *A. thaliana* plants (mean of data from four plants analysed individually) grown in media amended with a 3,4-DNT concentration of 0.8 mg /L of Phytigel had the greatest capacity to metabolise 3,4-DNT producing 3A4NT at a concentration of 203.72 ± 10.12 ng /single dried plant (Figure 3.10).

This concentration is higher than the IC_{50} value (0.2 mg /L of Phytigel media). This shows that at concentrations higher than the IC_{50} value plants can detoxify 3,4-DNT to its reduced product. No transformed or untransformed products were observed at the lowest

concentration of 3,4-DNT (0.02 mg /L of Phytagel media). However, at a 3,4-DNT concentration of 0.2 mg/L of Phytagel media 3A4NT was observed but no untransformed 3,4-DNT.

The concentration of 3A4NT increased to a maximum (**203.72 $\pm$ 10.12 ng /single dried plant**) at a 3,4-DNT concentration of 0.8 mg /L of Phytagel media after which it decreased to **14.88 $\pm$ 8.78 ng /single dried plant** at 3,4-DNT concentration of 4.6 mg /L of Phytagel media. At a 3,4-DNT concentration of 6.4 mg /L of Phytagel media the plants were completely stunted without adequate tissue for analysis.

Untransformed 3,4-DNT appears in plants at a concentration of 0.8 mg /L of Phytagel media and continues to increase until a 3,4-DNT concentration of 4.6 mg /L of Phytagel media reaching a concentration of **22.32 $\pm$ 13.34 ng /single dried plant**. This indicates that the ability of the plant to detoxify 3,4-DNT is impaired as the concentration of 3,4-DNT in the media increases.

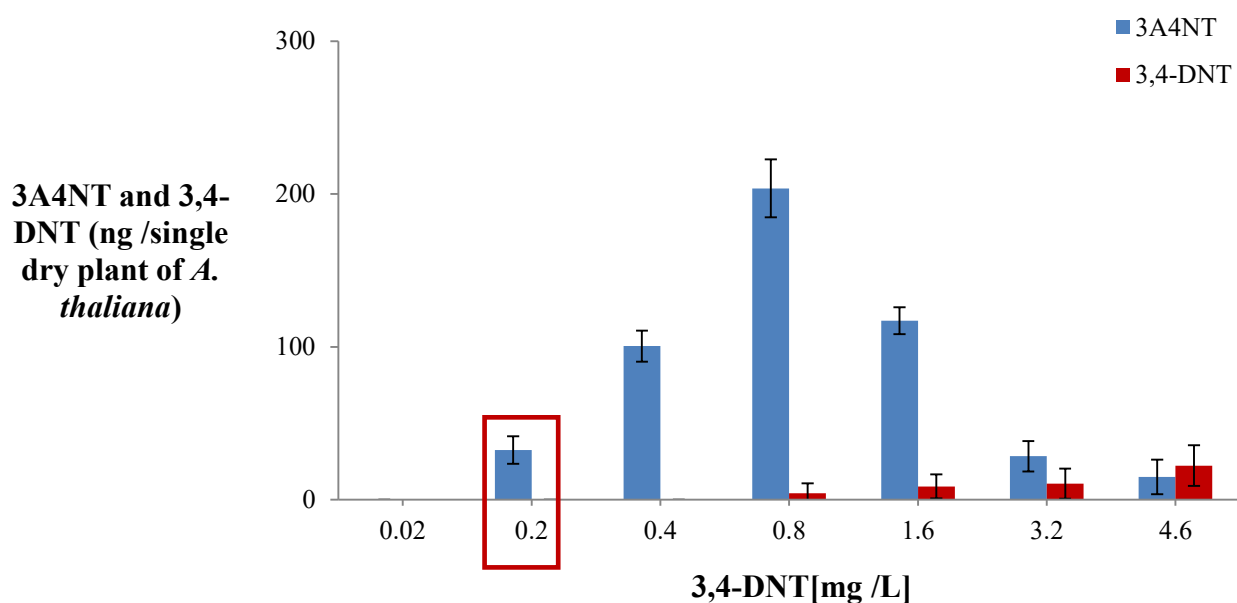


Figure 3.11: 3,4-DNT (■) and 3A4NT (■) concentrations recovered from a single 18 days (post cultivation) old *A. thaliana* plant grown in different concentrations of 3,4-DNT in sterile Phytigel media and analysed by GC-MS (growth temperature was $20\pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as mean of four replicates (only one plant from one pot was freeze dried and homogenized to give one replicate). The red box indicates the IC_{50} as calculated from root growth analysis (Chapter 2; section 2.3.1). The control and 0.25% mock control treated plants were analysed with exactly the same procedures as DNT treated plants and the values of all results are shown in Table 13 of Appendix II.

3.3.3. DNT and its metabolites from *A. thaliana* grown in hydroponic media (time-course study)

The analysis of *A. thaliana* plants treated with DNTs showed that there are variations in the concentrations of metabolites and rate of uptakes as observed from the treated plants in the hydroponic medium and Phytigel media.

3.3.3.1 DNT and its metabolites from *A. thaliana* shoots

3.3.3.1.1 Uptake and metabolism of 2,6-DNT in *A. thaliana*

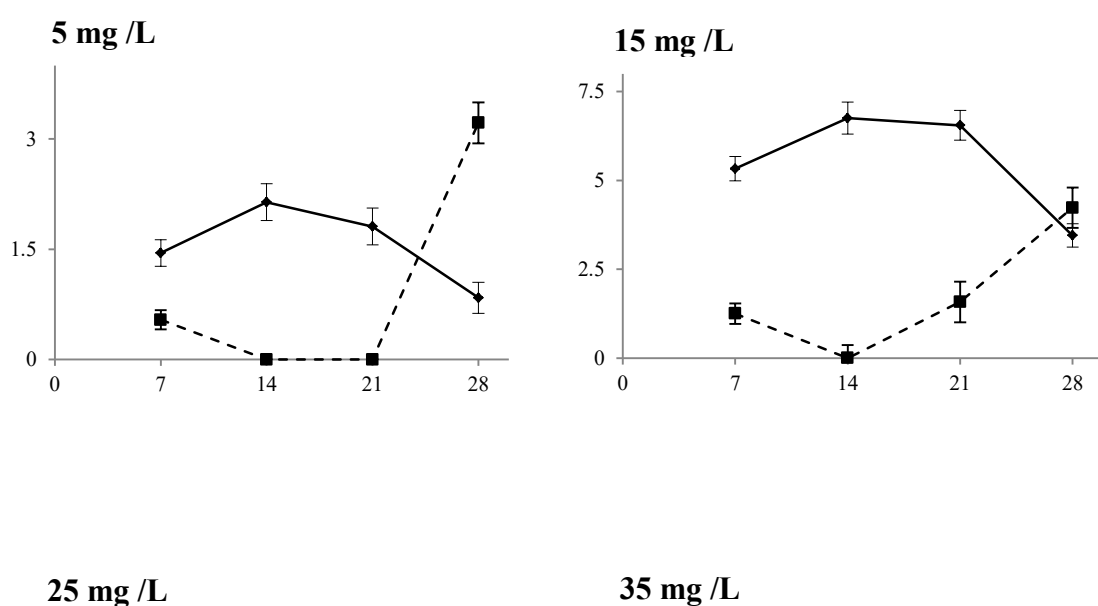
For all considered concentrations of 2,6-DNT, the maximum metabolites were obtained on 14th day of exposure and minimum on 28th day of exposure (Figure 3.12 A). According to the results the highest concentration of 2A6NT, $6.75\pm 0.27 \mu\text{g /g dry wt}$ of *A. thaliana* shoots was obtained from the plants treated with a 2,6-DNT concentration of 15 mg /L hydroponic media after 14 days of exposure, however the highest untransformed 2,6-DNT was $12.75\pm 0.22 \mu\text{g /g dry wt}$ of *A. thaliana* were obtained from the plants treated with 2,6-DNT at concentration of 55 mg /L of hydroponic media on 28th day of exposure.

After reaching the maximum concentration of 2A6NT obtained from the plants exposed to 2,6-DNT concentration of 15 mg /L of hydroponic media on the 14th day of exposure, it decreased to $6.55\pm 0.24 \mu\text{g /g dry wt}$ on 21st day and $3.45\pm 0.17 \mu\text{g /g dry wt}$ on 28th day of exposure. This shows that with the increase in time of exposure, the plant's ability to detoxify

2,6-DNT is impaired. No 2A6NT was obtained from the plants treated with 45 and 55 mg /L of hydroponic media. This shows that with the increase in concentration of 2,6-DNT the ability of detoxification is also reduced.

The results showed that the concentrations of untransformed 2,6-DNT first decreased on 14th day of exposure to 2,6-DNT and then increased until 28th day of exposure of *A. thaliana* to different concentrations of 2,6-DNT (Figure 3.12 B). The maximum metabolites were obtained on 28th day of exposure and minimum on 14th day of exposure by all considered concentrations of 2,6-DNT. With the increase in 2,6-DNT concentration in the media, the rate of untransformed 2,6-DNT also increased i.e on 28th day the maximum concentration of untransformed 2,6-DNT (**22.23±0.25 µg /g dry wt**) was obtained from the plants exposed to 2,6-DNT concentration of 55 mg /L of hydroponic media and minimum concentration (3.25±0.26 µg /g dry wt) was obtained from plants exposed to 2,6-DNT concentration of 5 mg /L in the media. No untransformed 2,6-DNT was obtained for 2,6-DNT concentration of 5 mg /L of hydroponic media on 7th 14th and 21st day of exposure, which shows that at such low concentrations of 2,6-DNT plant's are capable of efficiently detoxifying 2,6-DNT.

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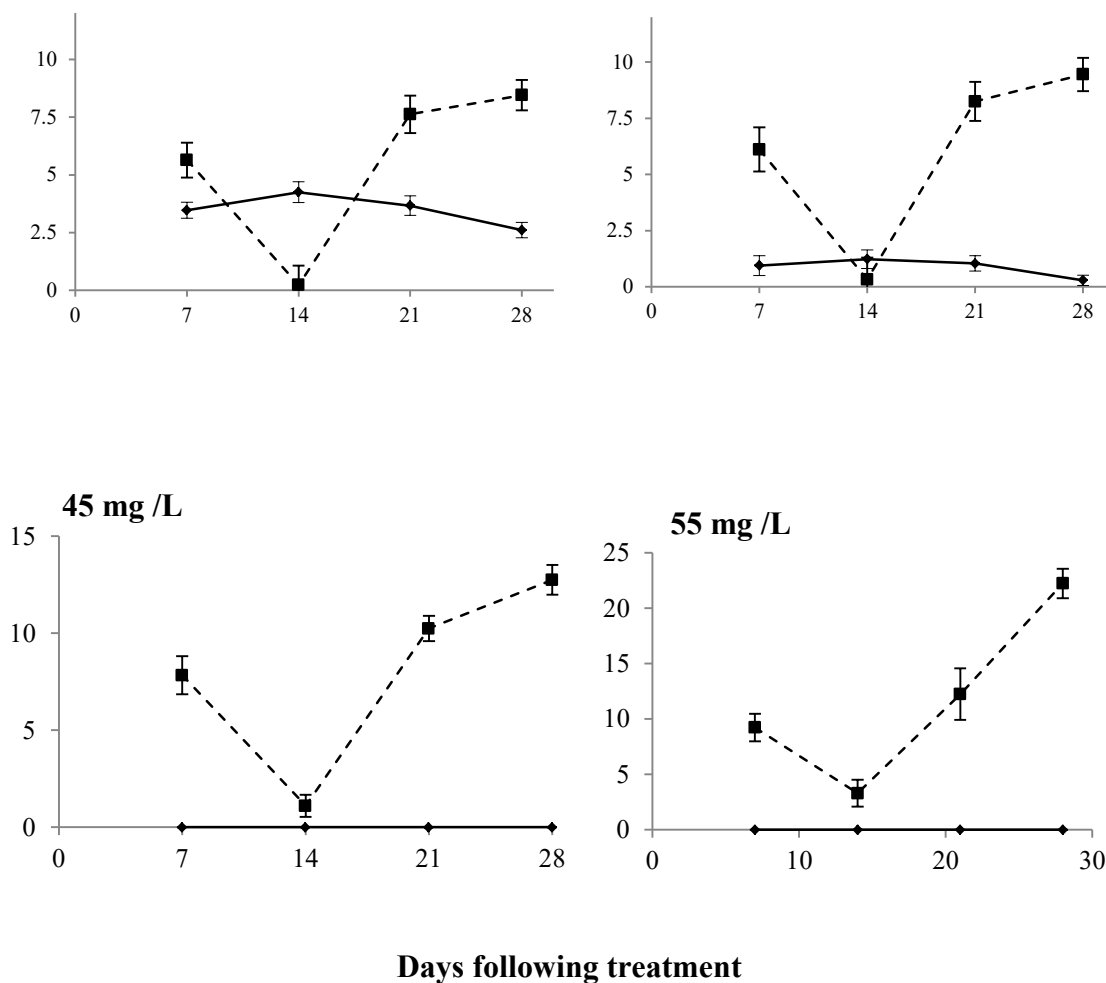


Figure 3.12: (A) 2A6NT and (B) untransformed 2,6-DNT concentrations recovered from shoots of 28 day (post cultivation) old *A. thaliana* plants grown in different concentrations of 2,6-DNT in hydroponic media and analysed by GC-MS (growth temperature was $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as a mean of four replicates (eight plants from one container were freeze dried and homogenized to give one replicate). (C) Individual variations in 2A6NT ($\blacklozenge$) and untransformed 2,6-DNT ($\blacksquare$) against different concentrations of 2,6-DNT during a time course of exposure (data in Table 14 of Appendix II).

3.3.3.1.2 Uptake and metabolism of 2,4-DNT in *A. thaliana*

The analytical data in Figure 3.13 shows that *A. thaliana* can metabolise 2,4-DNT to 2A4NT and 4A2NT with the highest concentration of metabolites recovered on the 14th day of treatment. The concentration of 2A4NT decreases with time of exposure of *A. thaliana* to 2,4-DNT in the hydroponic media increases (Figure 3.13A). The concentration of 2A4NT in

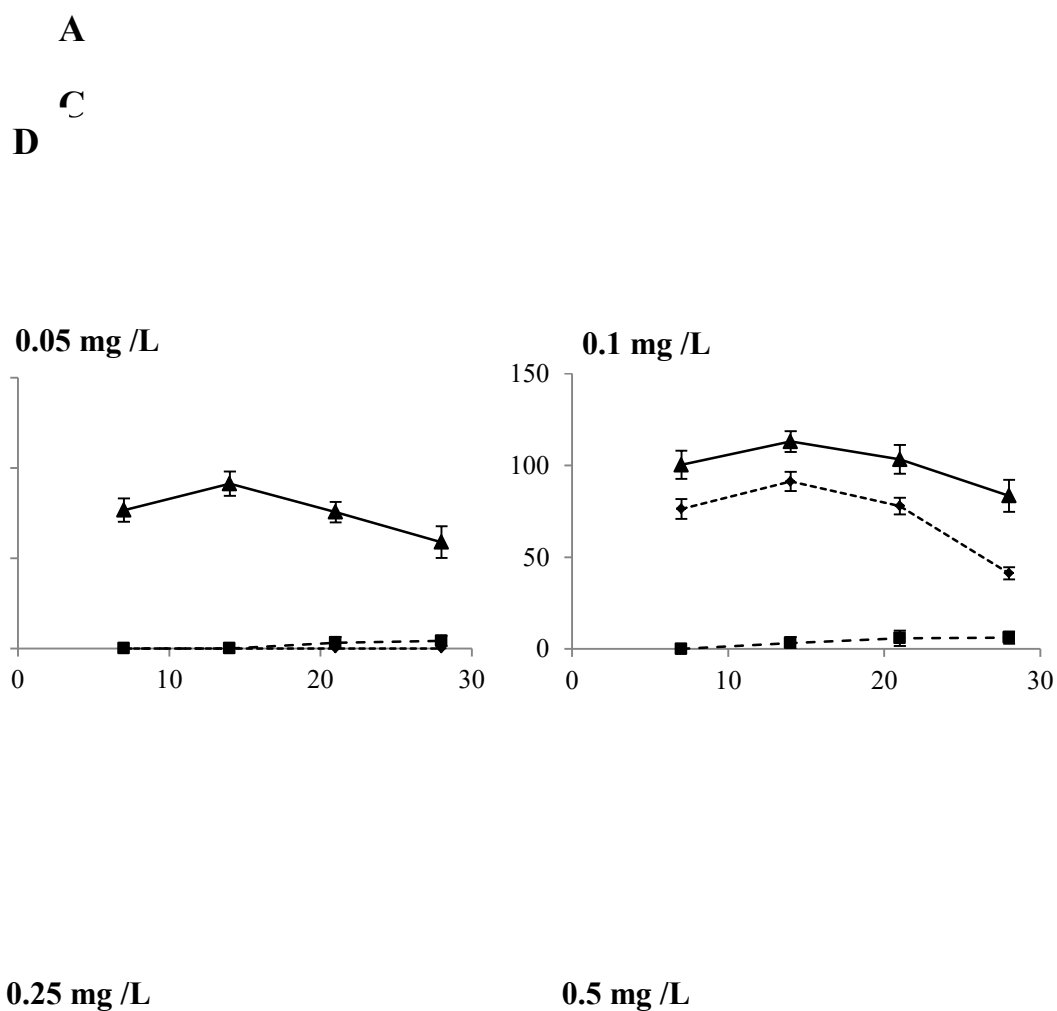
A. thaliana (Figure 3.13A) in media at a 2,4-DNT concentration of 0.5 mg /L reaches a maximum (**137.34 $\pm$ 4.25 ng /g dry wt**) on day 14 of exposure and a minimum (**98.74 $\pm$ 4.54 ng /g dry wt**) on day 28. This shows that with the increase in time of exposure, the plants lose their ability to detoxify 2,4-DNT. No 2A4NT was obtained from the plants treated with 2,4-DNT concentration of 0.05 mg /L of hydroponic media where presence of metabolites below detectable limits could be possible.

Figure 3.13B indicates that the maximum concentrations of 4A2NT was obtained on 14th day of exposure and minimum on 28th day of exposure from the plants at all considered concentrations of 2,4-DNT. The highest concentration of 4A2NT obtained was **183.45 $\pm$ 6.78 ng /g dry wt** observed on 14th day of exposure of *A. thaliana* to 2,4-DNT concentration of 0.5 mg /L of hydroponic media. After reaching this concentration, 4A2NT decreased to a concentration of **172.33 $\pm$ 8.44 ng /g dry wt** on 21st day and **112.38 $\pm$ 9.45 ng /g dry wt** on 28th day of exposure. This shows that with the increase in time of exposure, the plants lose their ability to detoxify 2,4-DNT. The minimum concentration of 4A2NT obtained was **45.66 $\pm$ 6.55 ng /g dry wt**.

When comparing the concentrations of 2A4NT and 4A2NT from 2,4-DNT treated plants, it was observed that 4A2NT has been obtained at comparatively higher concentrations compared to 2A4NT for all considered concentrations of 2,4-DNT. This shows *A. thaliana*'s preference for metabolism of nitro group at a specific position of benzene ring.

As the concentration of 2,4-DNT in the media increases the concentration of untransformed 2,4-DNT increases. The highest untransformed 2,4-DNT was observed to be **113.41 $\pm$ 6.87 ng /g dry wt** of *A. thaliana* recovered from the plants treated with 2,4-DNT concentration of 5 mg /L of hydroponic media after 28 days of exposure. This shows that with the increase in 2,4-DNT concentration in the media the plant's efficiency to detoxify 2,4-DNT decreases.

The least concentration of untransformed 2,4-DNT was observed to be 4.27 ± 5.66 ng /g dry wt of *A. thaliana* recovered from the plants on 7th day of exposure 2,4-DNT concentration of 2.5 mg /L of hydroponic media.



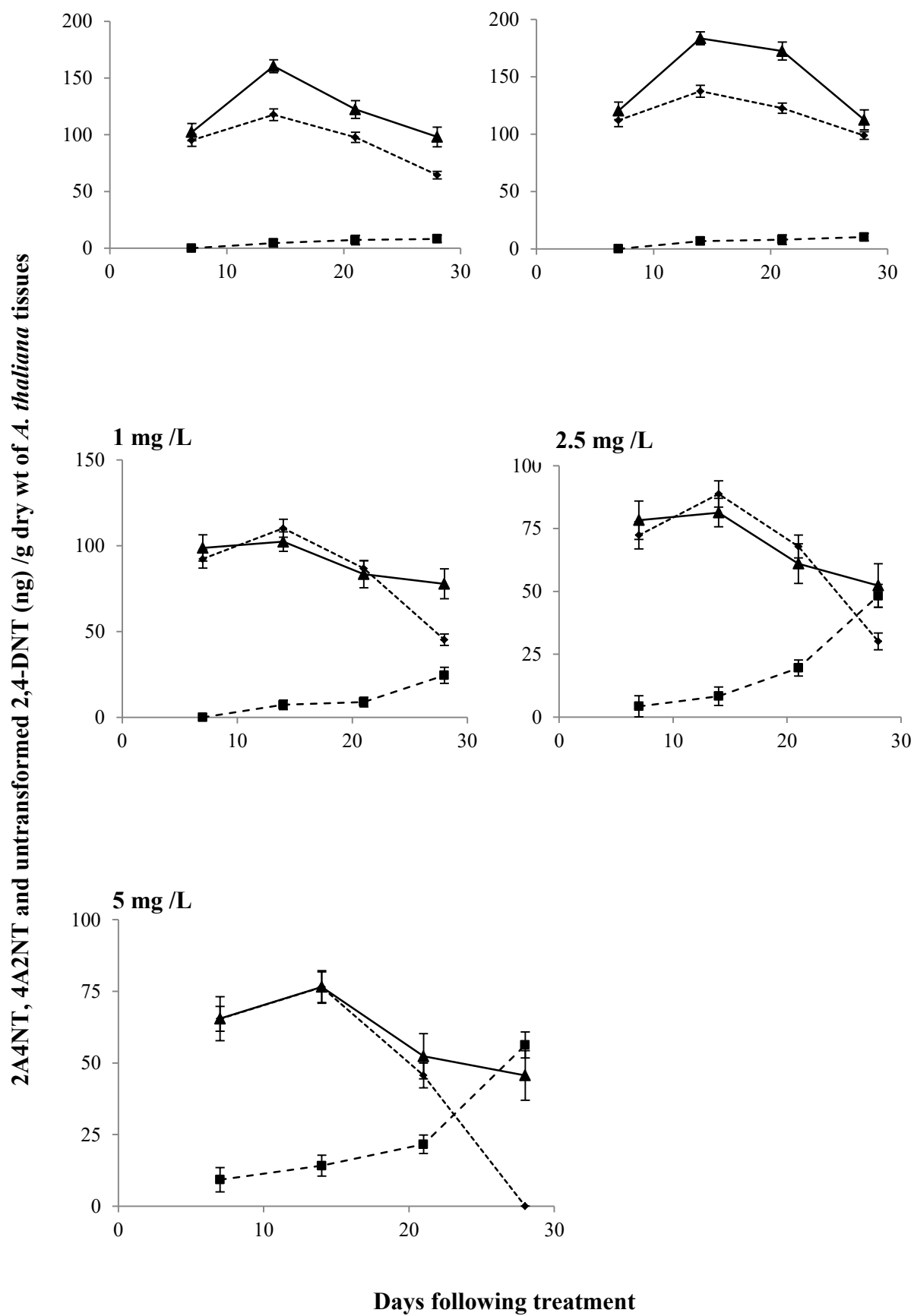
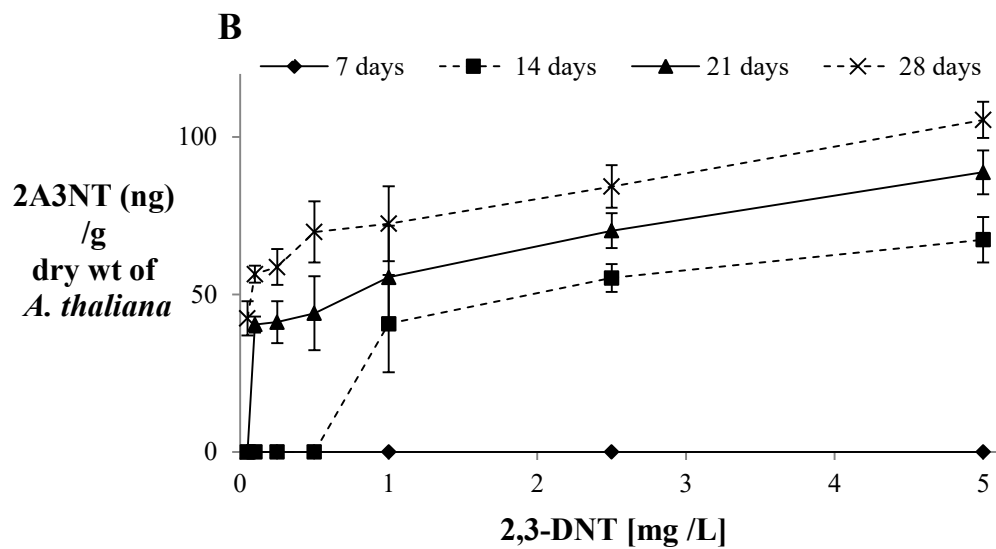
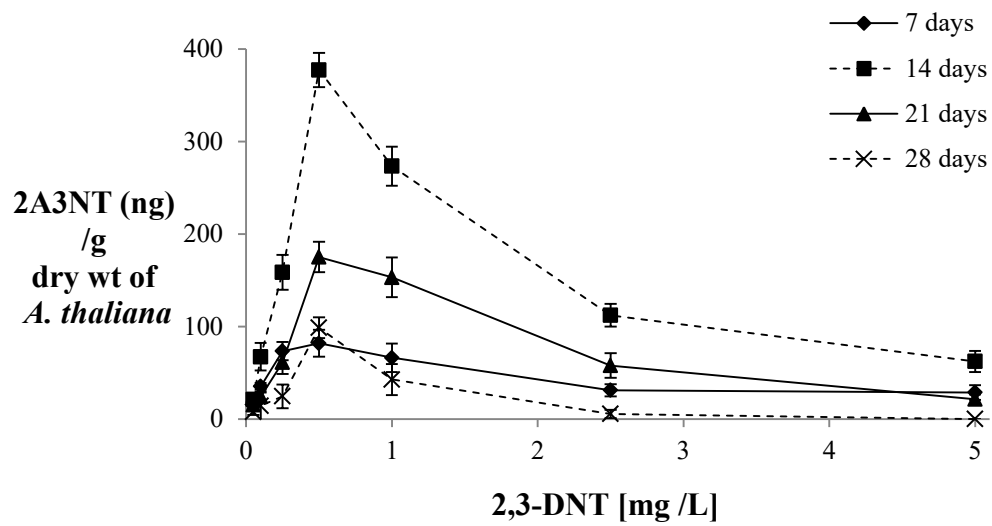


Figure 3.13: (A) 2A4NT, (B) 4A2NT and (C) 2,4-DNT concentrations recovered from shoot of 28 days (post cultivation) old *A. thaliana* plant grown in different concentrations of 2,4-DNT in hydroponic media and analysed by GC-MS (growth temperature was $20\pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as mean of four replicates (eight plants from one container were freeze dried and homogenized to give one replicate). (D) Individual variations in 2A4NT (—◆—), 4A2NT (—▲—) and untransformed 2,4-DNT (—■—) against different concentrations of DNT during a time course of exposure (data in Table 15, 16 and 17 of Appendix II).

3.3.3.1.3 Uptake and metabolism of 2,3-DNT in *A. thaliana*

The data of Figure 3.14A indicates that *A. thaliana* can metabolise 2,3-DNT to 2A3NT with the highest concentrations of metabolites recovered on the 14th day of exposure and minimum on 28th day of exposure by all the considered concentrations of 2,3-DNT. The highest concentration of 2A3NT obtained was 377.48 ± 18.56 ng /g dry wt respectively at 2,3-DNT concentration of 0.5 mg /L of hydroponic media after 14 days of exposure, which decreased to 175.27 ± 16.54 ng /g dry wt on 21st day and further decreased to 98.74 ± 11.32 ng /g dry wt on 28th day of exposure. This shows that *A. thaliana* efficiency to metabolise 2,3-DNT to 2A3NT reduces as the time of exposure increases.

The data in Figure 3.14B indicates that like other DNT isomers, as the concentration of 2,3-DNT in the media increases the rate of metabolism of DNT to ANTs decreases. The highest untransformed 2,3-DNT obtained was 105.45 ± 5.75 ng /g dry wt of *A. thaliana* from the plants exposed to 2,3-DNT concentration of 5 mg /L of hydroponic media on 28th day of exposure and no untransformed 2,3-DNT was observed for 2,3-DNT concentrations lower than 2.5 mg /L of hydroponic media on 7th day of exposure. This also shows that as the time of exposure to 2,3-DNT increases the rate of metabolism decreases.

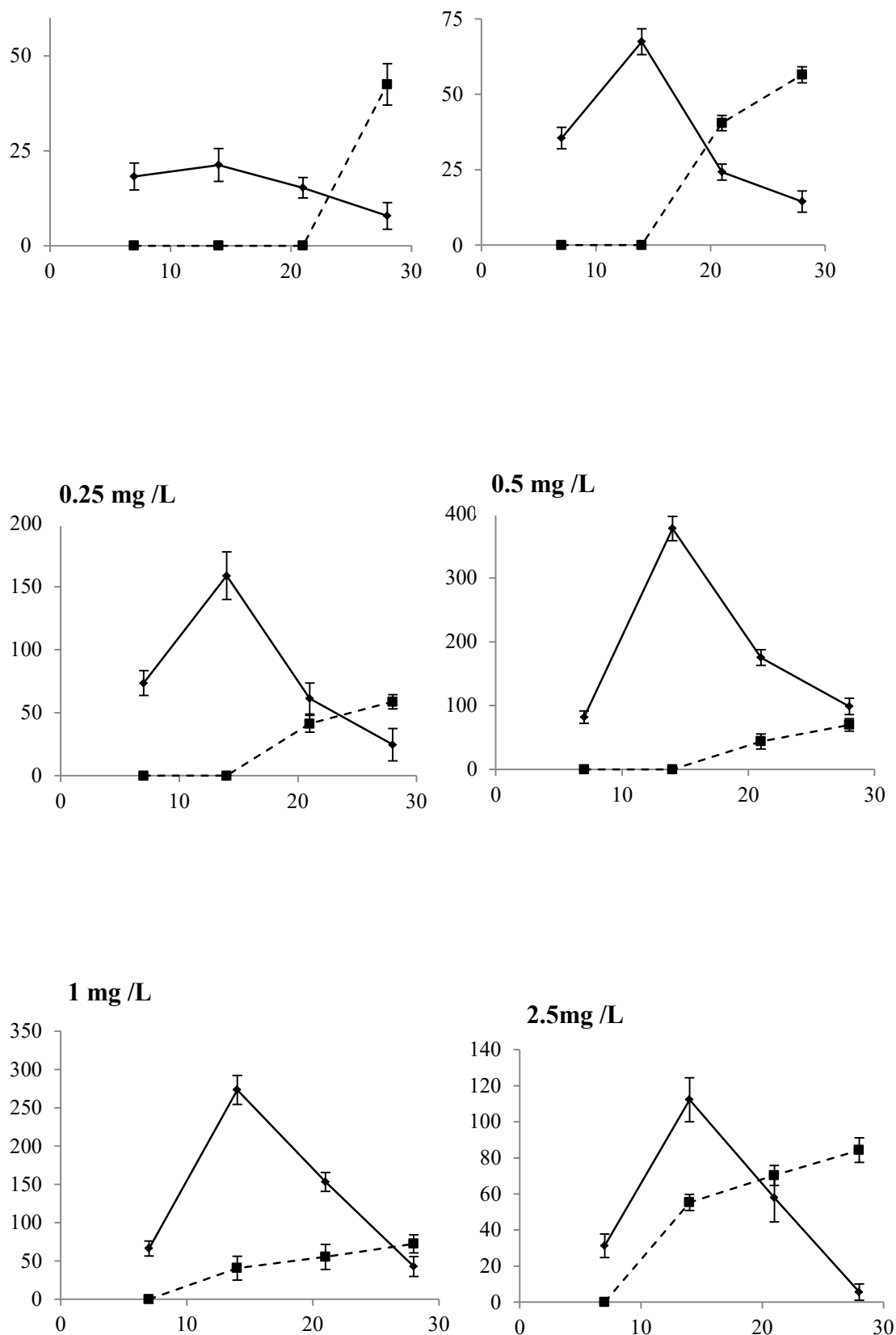


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0.05 mg/L

0.1 mg/L

2A3NT and untransformed 2,3-DNT (ng) /g dry wt of *A. thaliana* tissues



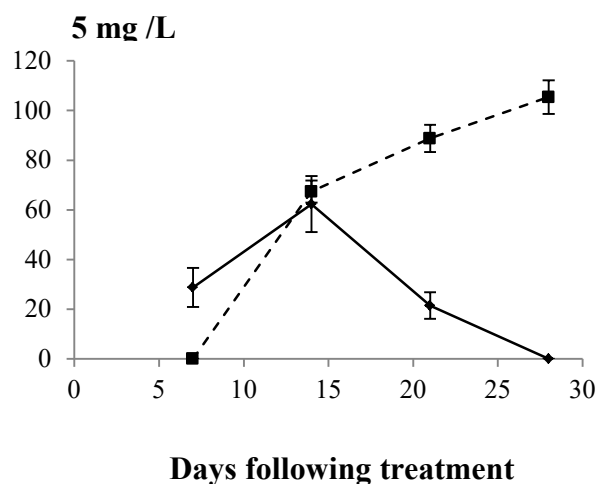
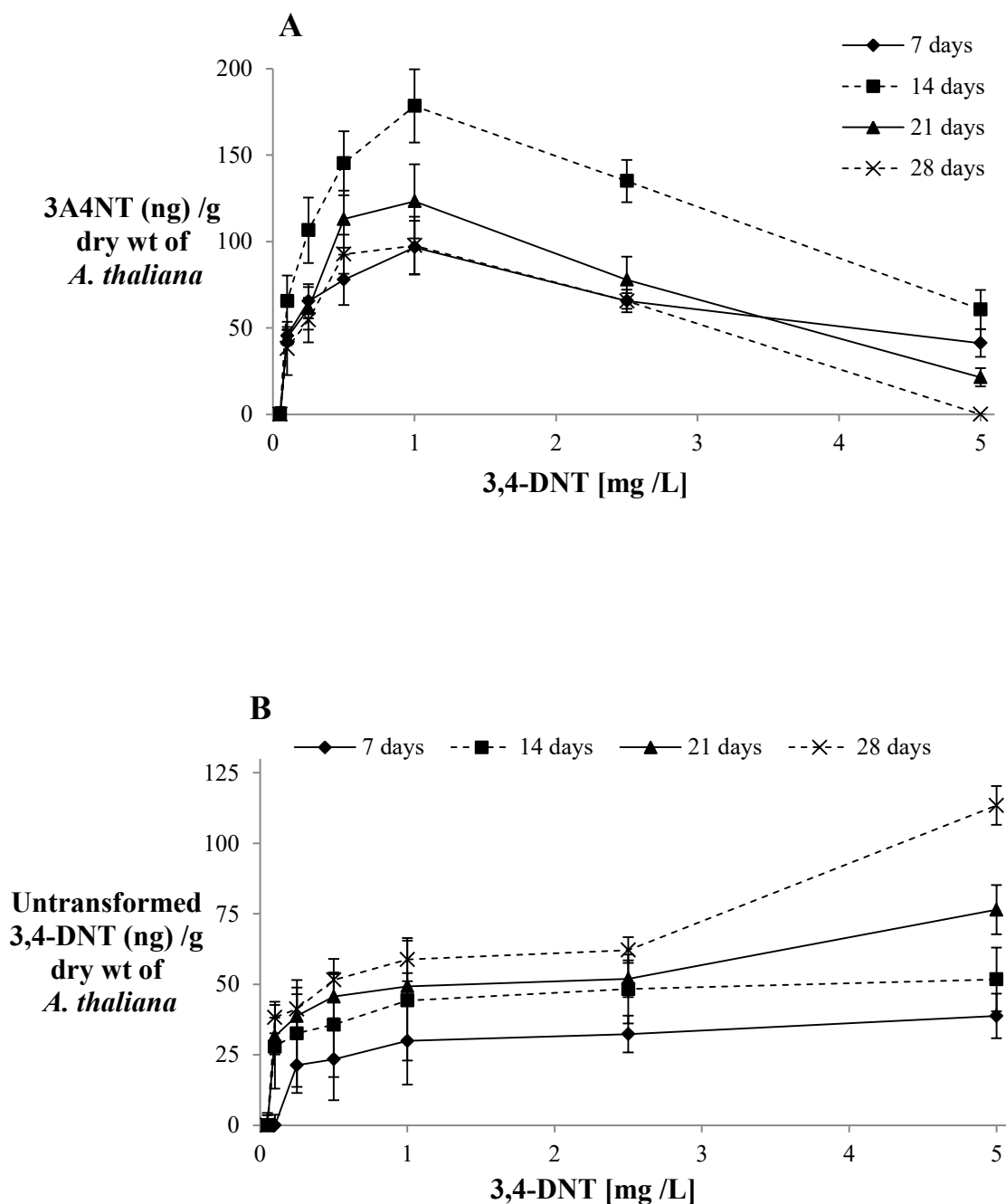


Figure 3.14: (A) 2A3NT and (B) 2,3-DNT concentrations recovered from shoot of 28 days (post cultivation) old *A. thaliana* plants grown in different concentrations of 2,3-DNT in hydroponic media and analysed by GC-MS (growth temperature was $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as mean of four replicates (eight plants from one container were freeze dried and homogenized to give one replicate). (C) Individual variations in 2A3NT ($\blacklozenge$) and untransformed 2,3-DNT ($-\blacksquare--$) against different concentrations of DNT during a time course of exposure (data in Table 18 and 19 of Appendix II).

3.3.3.1.4 Uptake and metabolism of 3,4-DNT in *A. thaliana*

The results as shown in Figure 3.15A indicates that *A. thaliana* plants can efficiently reduce the absorbed 3,4-DNT to 3A4NT on 14th day of exposure. The highest concentration of 3A4NT obtained was 178.44 ± 7.87 ng /g dry wt of *A. thaliana* from the plants exposed to 3,4-DNT concentration of 1 mg /L of hydroponic media after 14 days of exposure. This concentration then reduced to 97.66 ± 8.56 ng /g dry wt of *A. thaliana* on 28th day of exposure. This shows that the ability of *A. thaliana* to reduce 3,4-DNT to 3A4NT decreases as the time of exposure to 3,4-DNT increases. The highest metabolites were observed on 14th day of exposure and least on 28th day of exposure of plants to all considered concentrations of 3,4-DNT. At low concentrations like 0.05 mg /L of hydroponic media, no metabolites were observed. However, concentration of metabolites could also be below detectable limits at this concentration of 3,4-DNT.

The Figure 3.15B indicates that as the concentration and time of exposure of 3,4-DNT increases the metabolism of DNT to ANT's decreases. The highest recovery of untransformed 3,4-DNT was observed to be 281.41 ± 12.32 ng/g dry wt of *A. thaliana* recovered at 3,4-DNT concentration of 5 mg /L of hydroponic media on 28th day of exposure.



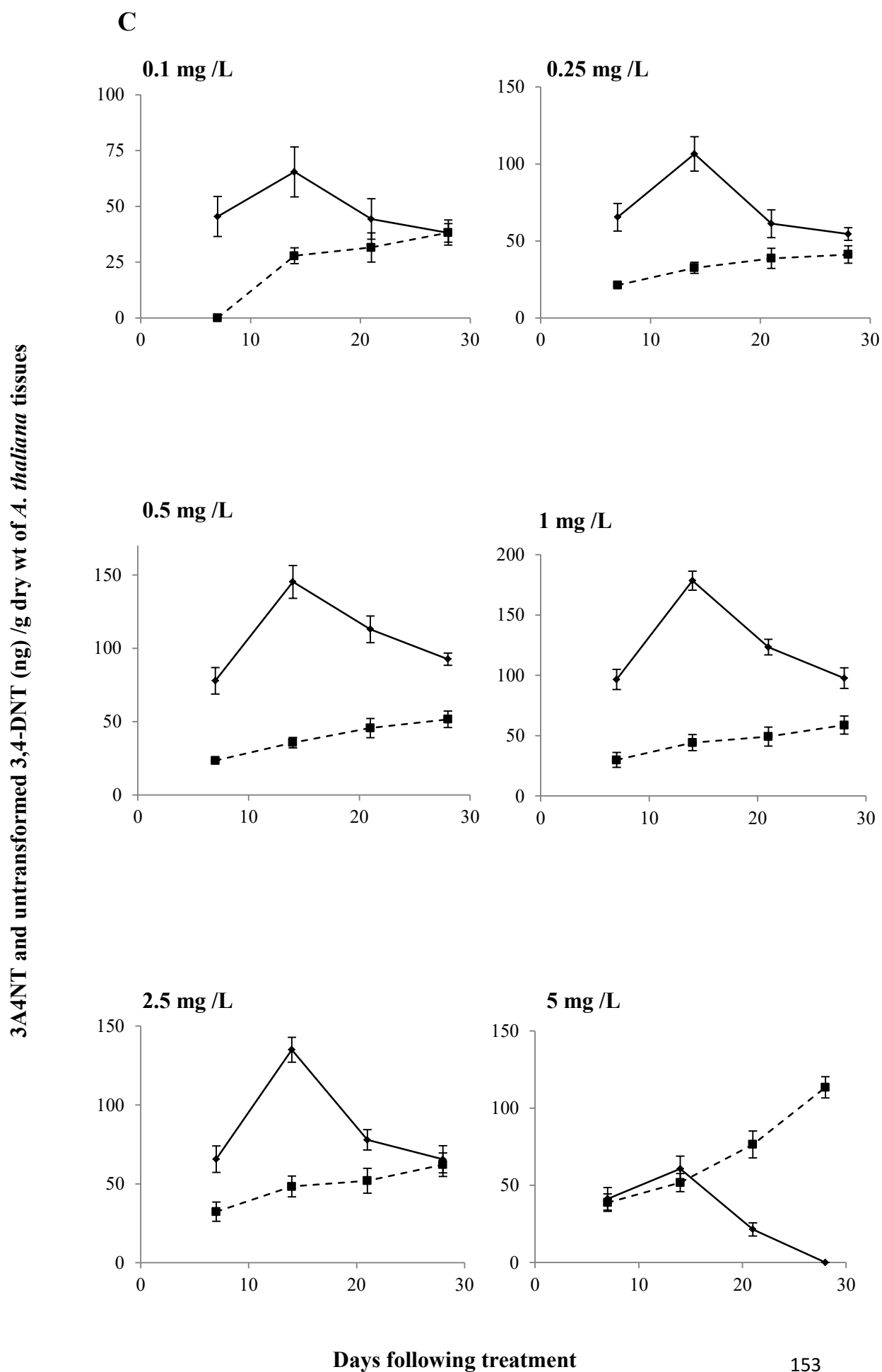


Figure 3.15: (A) 3A4NT and (B) 3,4-DNT concentrations recovered from shoot of 28 days (post cultivation) old *A. thaliana* plants grown in different concentrations of 3,4-DNT in hydroponic media and analysed by GC-MS (growth temperature was $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as mean of four replicates (eight plants from one container were freeze dried and homogenized to give one replicate). (C) Individual variations in 3A4NT (—◆—) and untransformed 3,4-DNT (-■-) against different concentrations of DNT during a time course of exposure. (data in Table 20 and 21 of Appendix II).

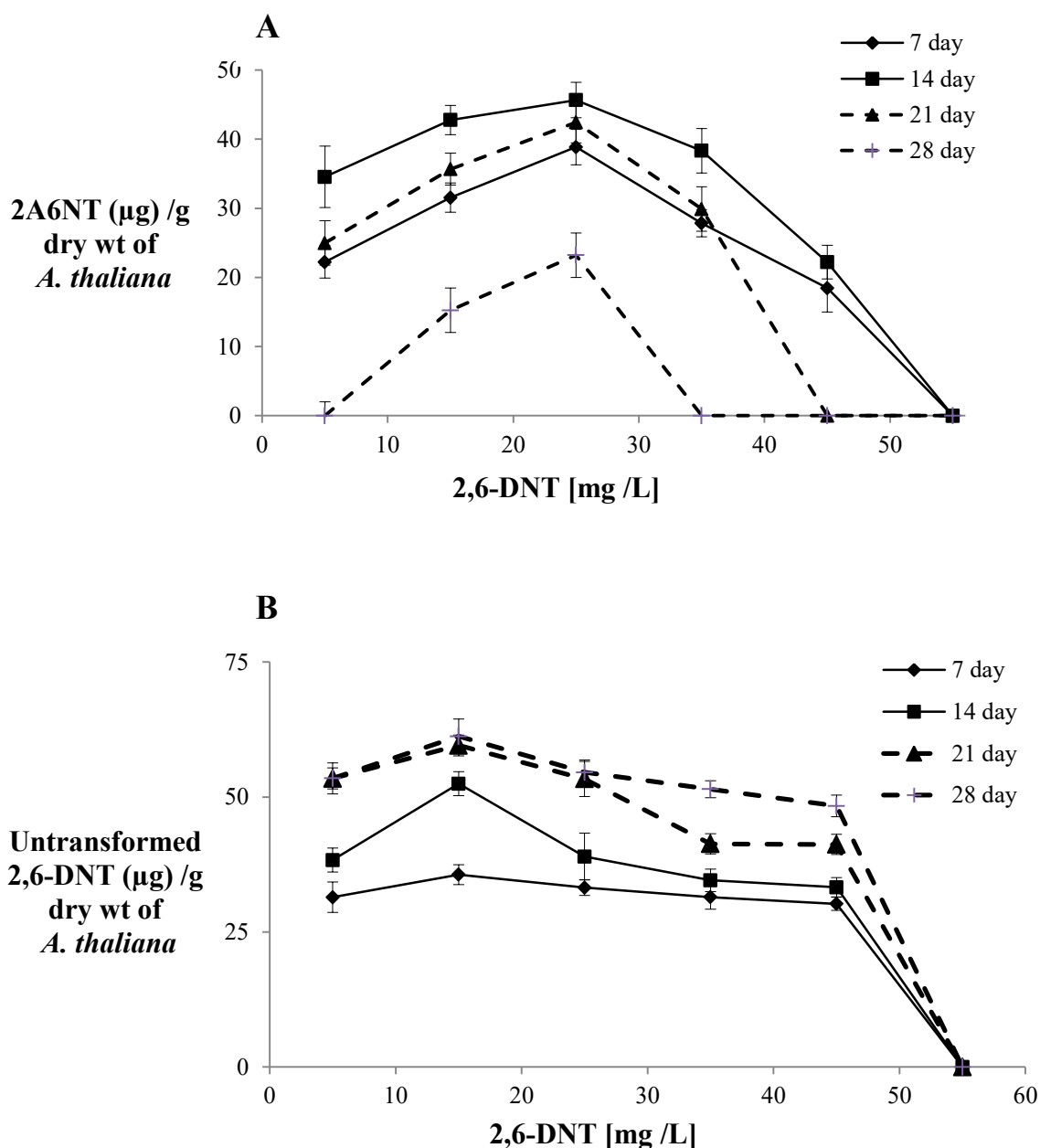
3.3.3.2 DNT and its metabolites from *A. thaliana* roots

3.3.3.2.1 Uptake and metabolism of 2,6-DNT in *A. thaliana*

Figure 3.16 shows that *A. thaliana* roots can metabolise 2,6-DNT to 2A6NT, however, this rate of this reduction also varies with the concentration of 2,6-DNT in the media.

The highest concentrations of 2A6NT were obtained from the *A. thaliana* roots exposed to 2,6-DNT on 14th day of exposure, which was observed to decrease with the increase in time of exposure. According to Figure 3.16A, the highest concentration of 2A6NT was observed to be $45.66 \pm 2.31 \mu\text{g/g dry wt}$ of *A. thaliana* roots recovered from the plants treated with 2,6-DNT concentration of 25 mg /L hydroponic media after 14 days of exposure, however it was observed to reduce to $23.22 \pm 2.88 \mu\text{g/g dry wt}$ of *A. thaliana* on 28th day. No metabolites were observed by the least considered concentration of 2,6-DNT (5 mg /L of hydroponic media) on 28th day of exposure to 2,6-DNT. At 2,6-DNT concentration of 45 mg /L of hydroponic media the concentration of metabolites obtained was very low on 7th and 14th day of exposure with no metabolites obtained on 21st and 14th day of exposure, which shows that this concentration is very toxic for the plants and they were struggling to detoxify the compounds. At highest considered concentration of 2,6-DNT (55 mg /L of hydroponic media) no metabolites were observed which indicates that this concentration is very toxic and roots were unable to metabolise 2,6-DNT to 2A6NT at this concentration.

Overall, the highest untransformed 2,6-DNT concentrations were obtained from the roots after 28 days of exposure to different concentrations of 2,6-DNT (Figure 3.16 B). Among the untransformed 2,6-DNT concentrations, the highest concentration was **61.22±3.21 mg /g dry wt** measured from the plants treated with 2,6-DNT at concentration of 15 mg /L of hydroponic media on 28th day of exposure. This shows that as the concentration of 2,6-DNT in the media increases, it impacts the plants ability to detoxify 2,6-DNT. The root mass obtained from the plants exposed to the concentration of 55 mg /L of hydroponic media was not sufficient to be analysed therefore no data can be obtained.



2A6NT and untransformed 2,6-DNT (μg) /g dry wt of *A. thaliana* tissues

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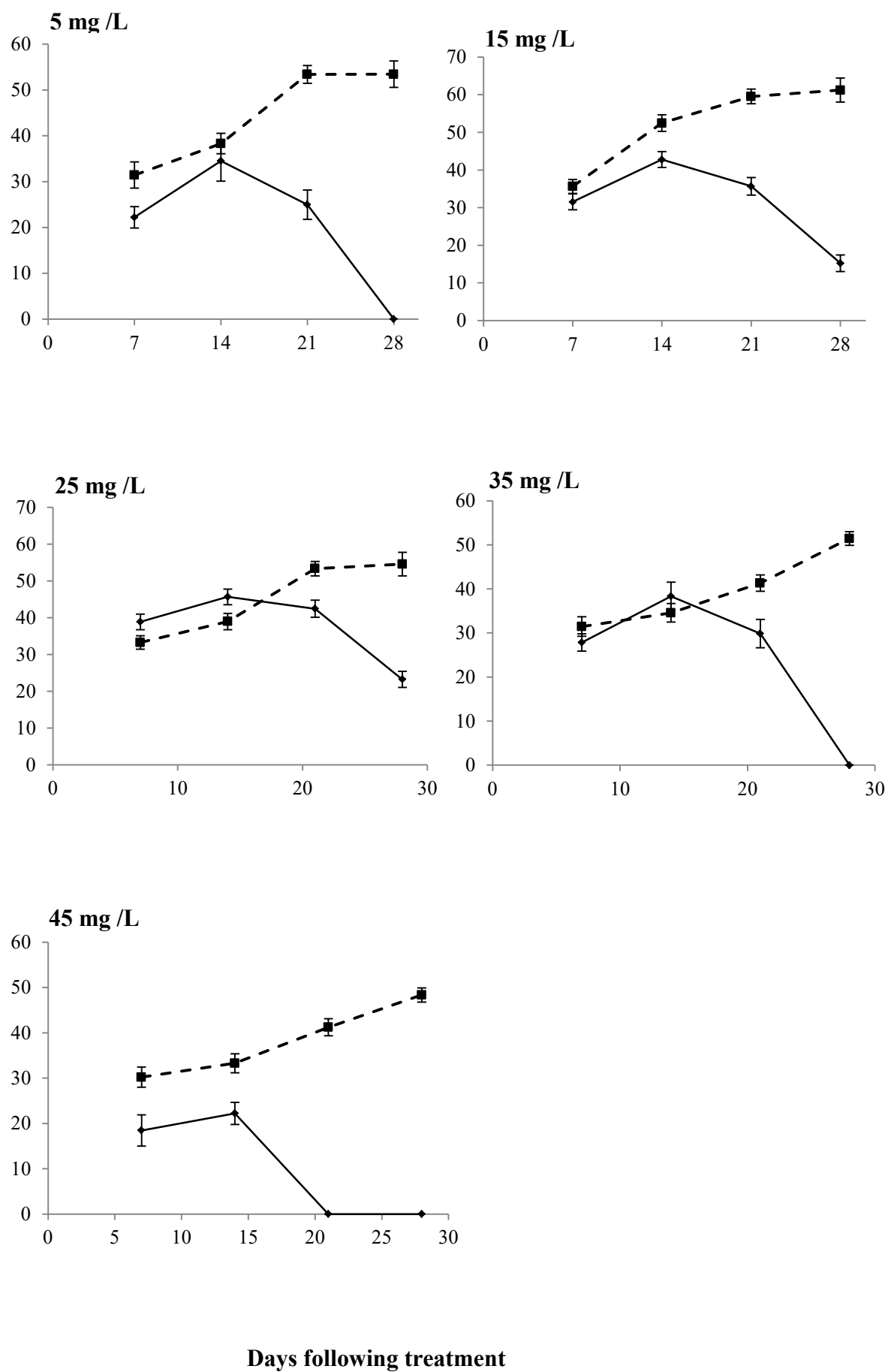


Figure 3.16: (A) 2A6NT and (B) untransformed 2,6-DNT concentrations recovered from roots of 28 days (post cultivation) old *A. thaliana* plants grown in different concentrations of 2,6-DNT in hydroponic media and analysed by GC-MS (growth temperature was $20\pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as mean of four replicates (roots from eight plants from one container were freeze dried and homogenized to give one replicate) (C) Individual variations in 2A6NT (—◆—) and untransformed 2,6-DNT (—■—) against different concentrations of DNT during a time course of exposure (data is in Table 22 and 23 of Appendix II).

3.3.3.2.2 Uptake and metabolism of 2,4-DNT in *A. thaliana*

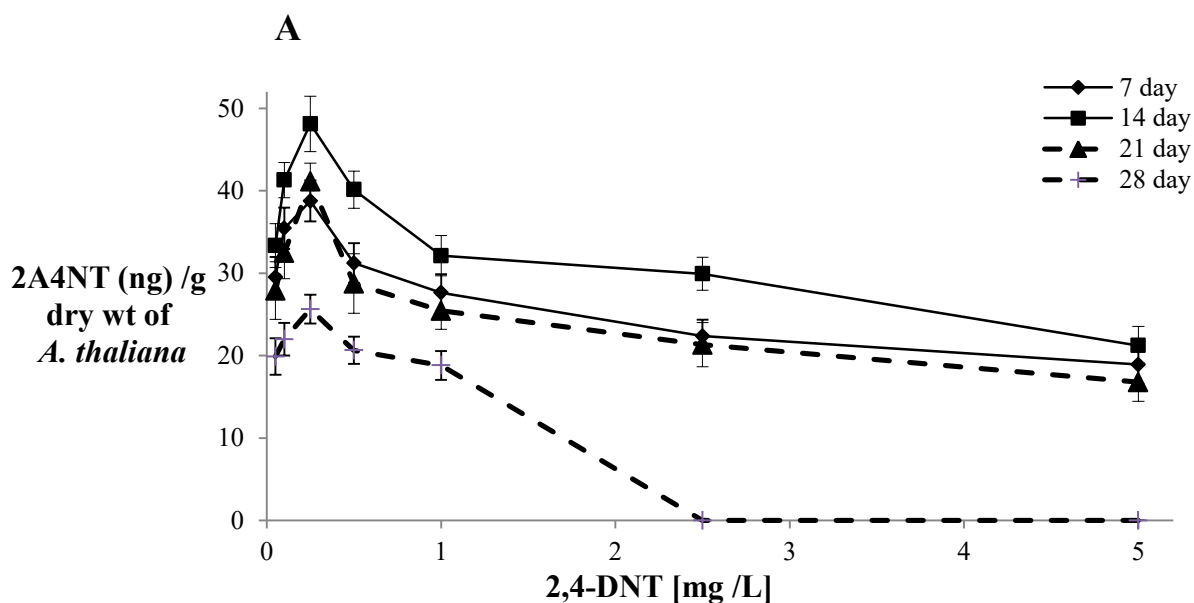
Figure 3.17 shows that *A. thaliana* roots can metabolise 2,4-DNT to 2A4NT and 4A2NT the concentrations of metabolites, however, varied with the concentration of 2,4-DNT in the media.

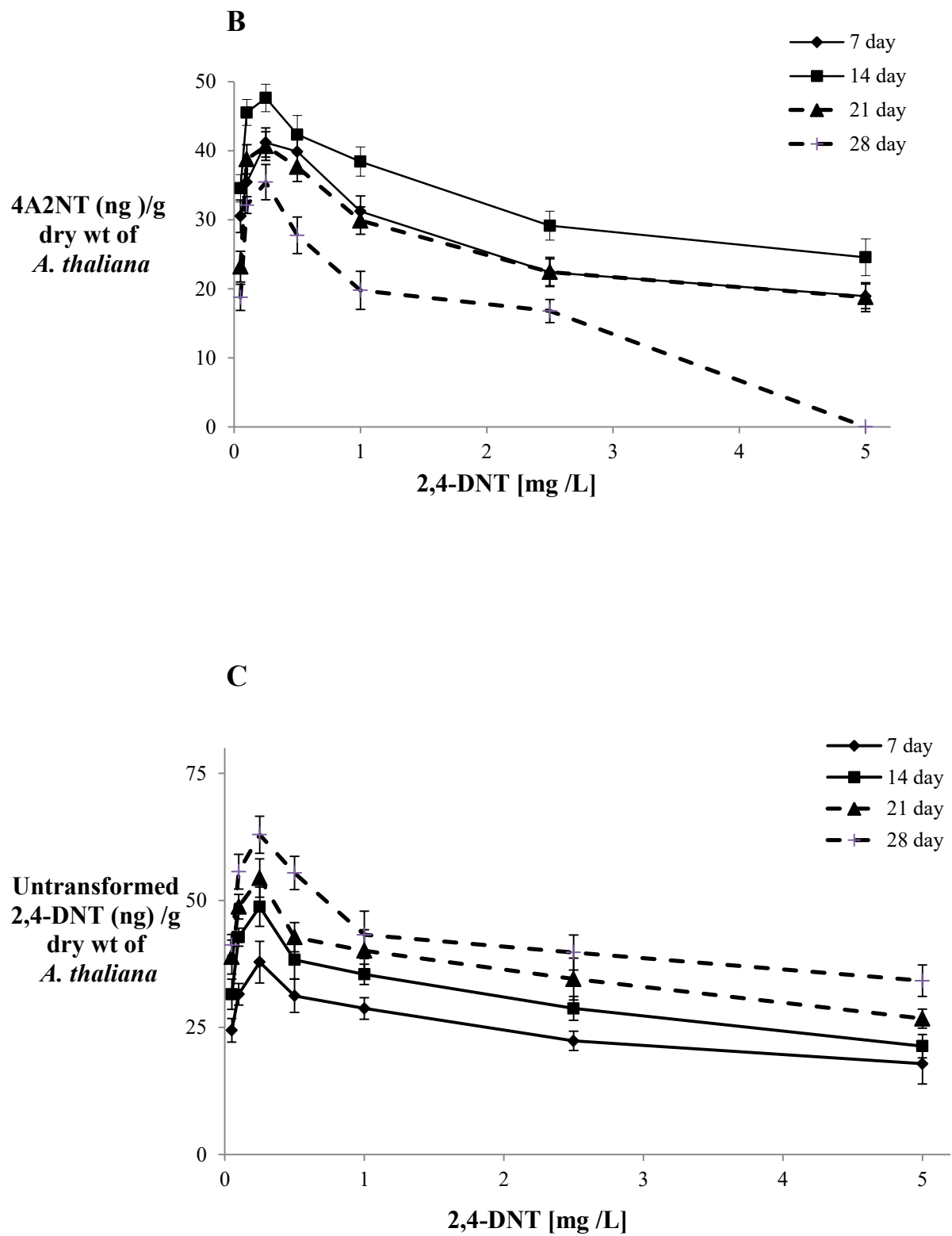
The highest concentrations of 2A4NT were obtained from the *A. thaliana* roots exposed to 2,4-DNT on 14th day of exposure, which was observed to decrease with the increase in time of exposure. According to Figure 3.17A the highest concentration of 2A4NT was observed to be 48.11 ± 3.36 ng /g dry wt of *A. thaliana* roots recovered from the plants treated with 2,4-DNT concentration of 0.25 mg /L hydroponic media after 14 days of exposure, however it was observed to reduce to 25.64 ± 3.34 ng /g dry wt of *A. thaliana* on 28th day. For higher concentrations of 2,4-DNT like 2.5 and 5 mg /L of hydroponic media, no metabolites were observed at 28th day of exposure, which indicates that these concentrations of 2,4-DNT are enough toxic to disable plant's ability to detoxify the compounds.

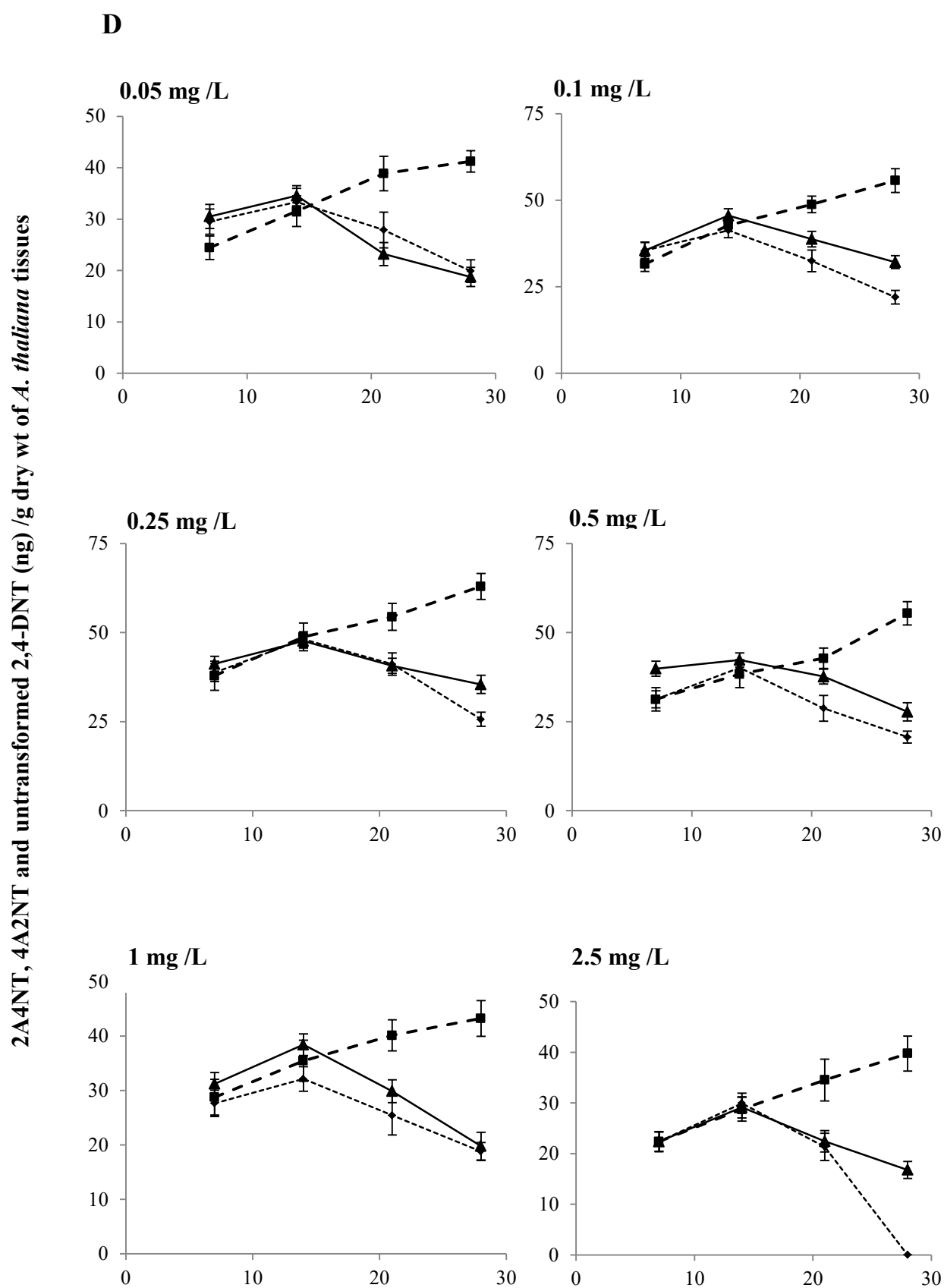
The highest concentrations of 4A2NT were obtained from the *A. thaliana* roots exposed to 2,6-DNT on 14th day of exposure, which was observed to decrease with the increase in time of exposure. According to Figure 3.17B the highest concentration of 2A4NT was observed to be 47.64 ± 3.45 ng /g dry wt of *A. thaliana* roots recovered from the plants treated with 2,4-DNT concentration of 0.25 mg /L hydroponic media after 14 days of exposure, however it was observed to reduce to 35.44 ± 3.76 ng /g dry wt of *A. thaliana* on 28th day. For higher

concentration of 2,4-DNT like 5 mg /L of hydroponic media, no metabolites were observed at 28th day of exposure, which indicates that this concentration of 2,4-DNT is toxic enough to limit detoxification.

Overall, the highest untransformed 2,4-DNT concentrations was obtained from the roots after 28 days of exposure to different concentrations of 2,4-DNT (Figure 3.17C). Among the untransformed 2,4-DNT concentrations, the highest concentration was **62.94±4.11 ng /g dry wt** measured from the plants treated with 2,4-DNT at concentration of 0.25 mg /L of hydroponic media on 28th day of exposure. This shows that as the concentration of 2,4-DNT in the media increases, it impacts the plants ability to detoxify 2,4-DNT. The least untransformed 2,4-DNT concentration was obtained from the roots exposed to 5 mg /L of hydroponic media on 7th day of exposure, which indicates that this concentration is toxic that is why roots have limited absorption for 2,4-DNT.







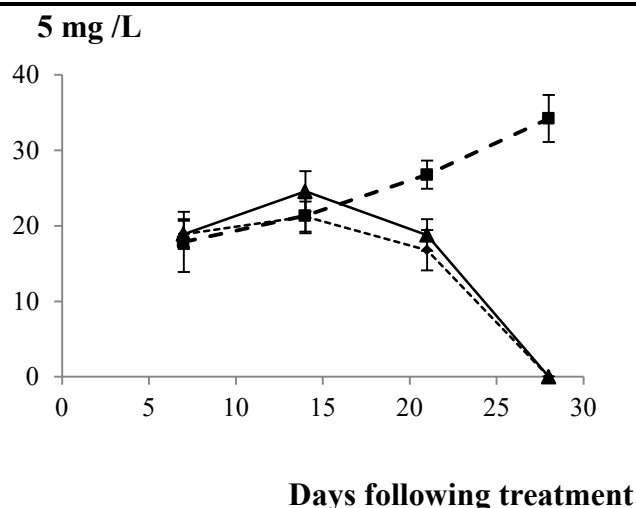
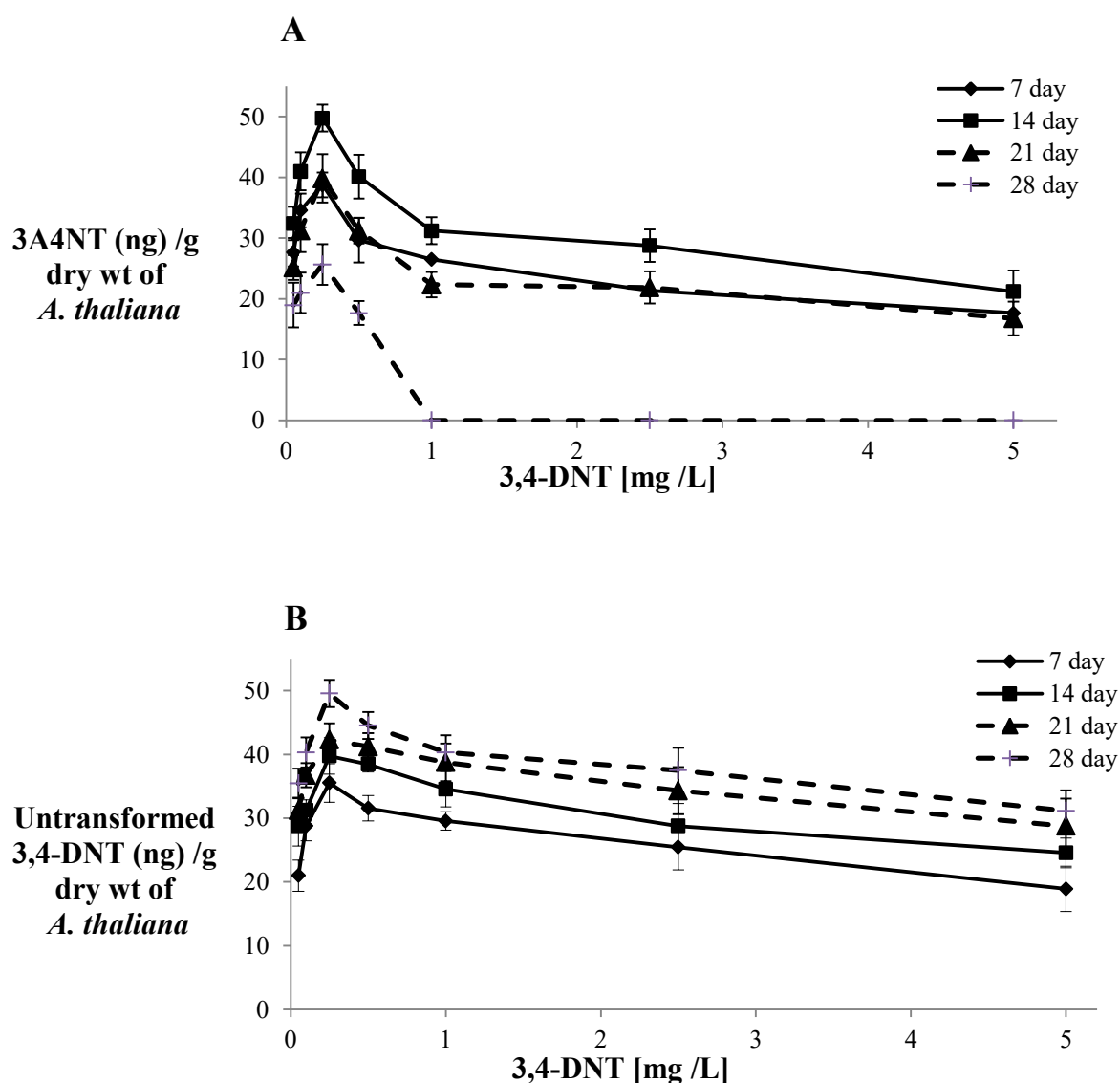


Figure 3.17: (A) 2A4NT and (B) 4A2NT and (C) 2,4-DNT concentrations recovered from 28 day (post cultivation) old *A. thaliana* plant grown in different concentrations of 2,4-DNT in hydroponic media and analysed by GC-MS (growth temperature was $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as a mean of four replicates (rots from eight plants from one container were freeze dried and homogenized to give one replicate) (D) Individual variations in 2A4NT (—◆—), 4A2NT (—▲—) and untransformed 2,4-DNT (—■—) against different concentrations of DNT during a time course of exposure (data is in Table 24, 25 and 26 of Appendix II).

3.3.3.2.3 Uptake and metabolism of 3,4-DNT in *A. thaliana*

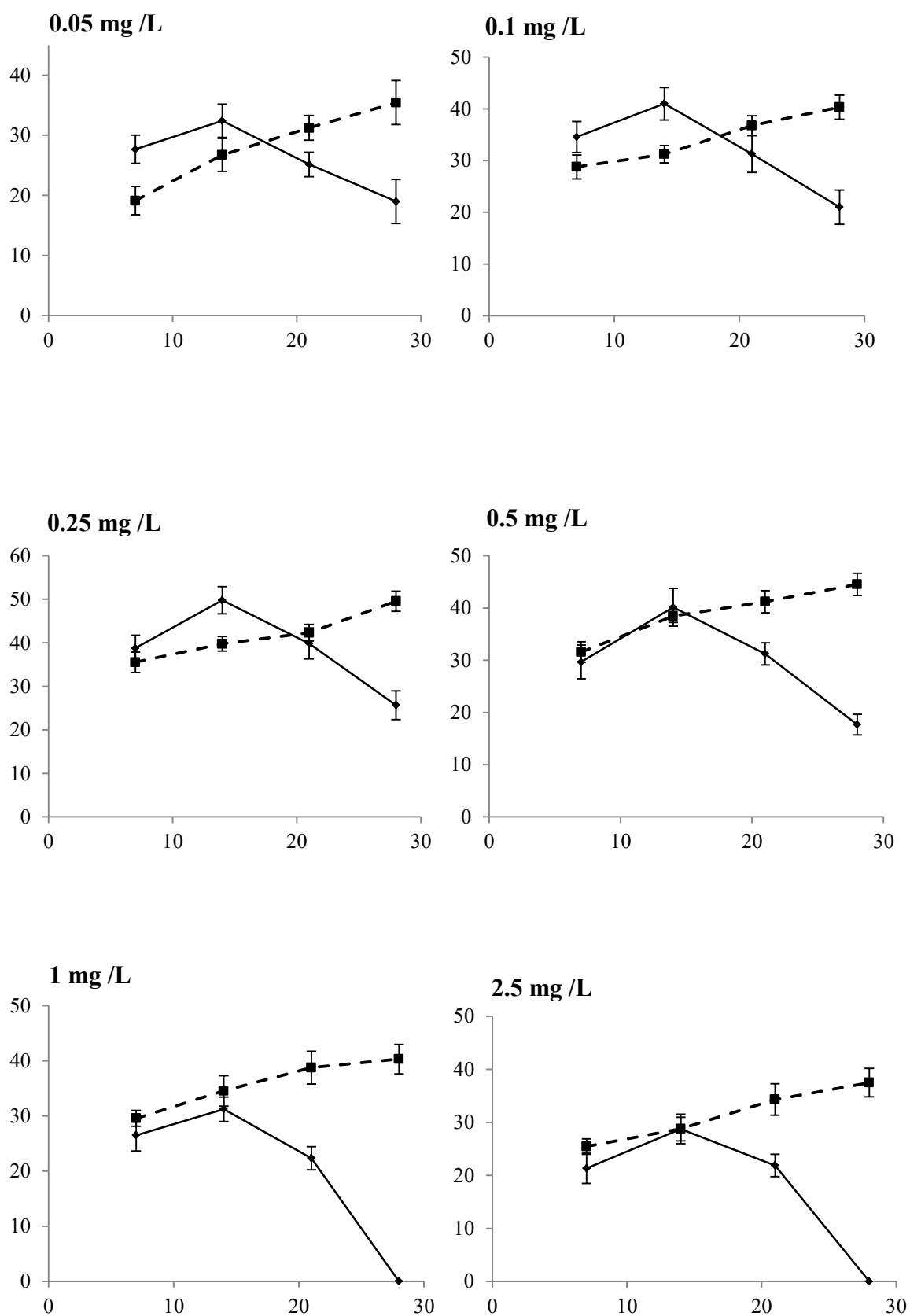
Figure 3.18A shows that *A. thaliana* roots can metabolise 3,4-DNT to 3A4NT with the highest concentrations of 3A4NT were obtained from the *A. thaliana* roots exposed to 3,4-DNT on 14th day of exposure, which was observed to decrease with the increase in time of exposure. According to Figure 3.18A the highest concentration of 3A4NT was observed to be 49.76 ± 4.32 ng /g dry wt of *A. thaliana* roots recovered from the plants treated with 3,4-DNT concentration of 0.25 mg /L hydroponic media after 14 days of exposure, however it was observed to reduce to 25.66 ± 2.89 ng /g dry wt of *A. thaliana* on 28th day. For higher concentrations of 3,4-DNT like 1, 2.5 and 5 mg /L of hydroponic media, no metabolites were observed at 28th day of exposure, which indicates that these concentrations of 3,4-DNT are enough toxic to disable plant's ability to detoxify the compounds.

The highest untransformed 3,4-DNT concentrations were obtained from the roots after 28 days of exposure to different concentrations of 3,4-DNT (Figure 13.8B). Among the untransformed 3,4-DNT concentrations, the highest concentration was 49.55 ± 2.81 ng /g dry wt measured from the plants treated with 3,4-DNT at concentration of 0.25 mg /L of hydroponic media on 28th day of exposure. This shows that as the time of exposure to 3,4-DNT increases, the rate metabolism decreases. The concentrations of untransformed 2,4-DNT obtained tend to lower with the increase in concentration of 3,4-DNT in the media. Very low concentrations (18.91 ± 3.38 ng /L of hydroponic media) of untransformed 3,4-DNT was observed at 7th day corresponding to the concentration of 5 mg /L of hydroponic media.



3A4NT and untransformed 3,4-DNT (ng) /g dry wt of *A. thaliana* tissues

C



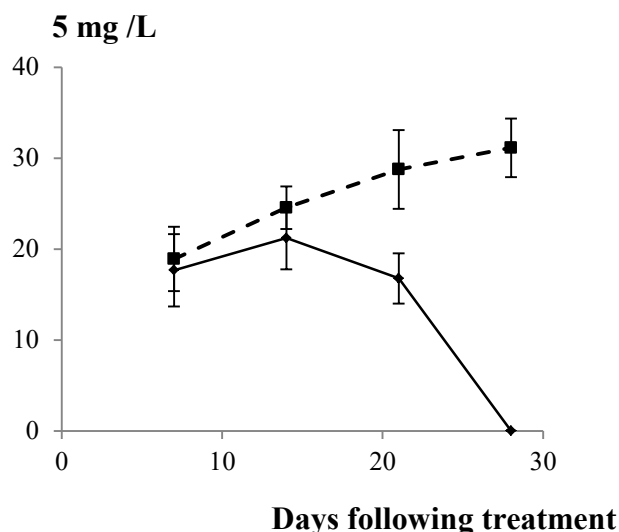
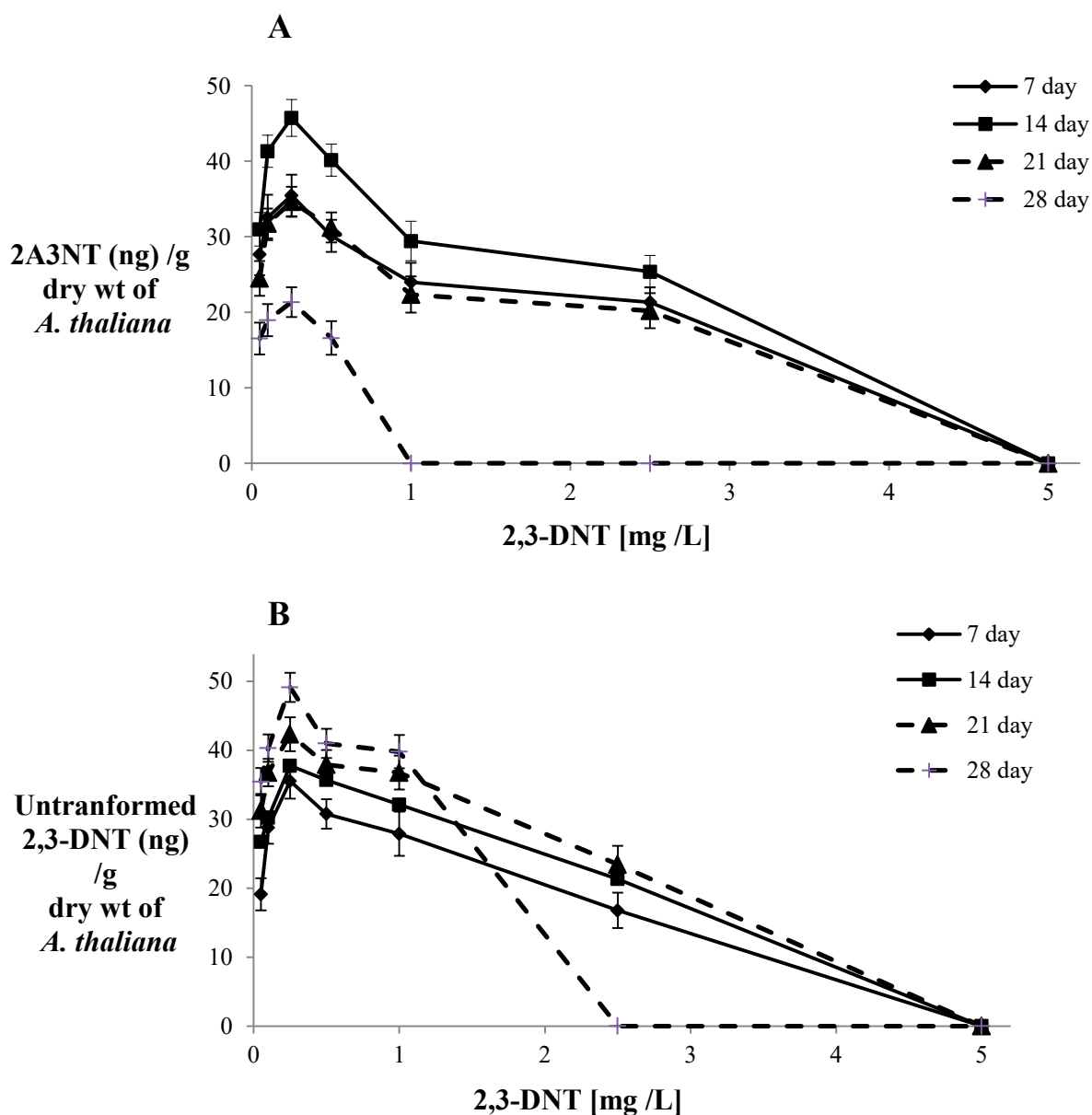


Figure 3.18: (A) 3A4NT and (B) 3,4-DNT concentrations recovered from 28 day (post cultivation) old *A. thaliana* plants grown in different concentrations of 3,4-DNT in hydroponic media and analysed by GC-MS (growth temperature was $20\pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as a mean of four replicates (roots from eight plants from one container were freeze dried and homogenized to give one replicate) (C) Individual variations in 3A4NT ($-\diamond-$) and untransformed 3,4-DNT ($- \blacksquare - -$) against different concentrations of DNT during a time course of exposure (data is in Table 27 and 28 of Appendix II).

3.3.3.2.4 Uptake and metabolism of 2,3-DNT in *A. thaliana*

Figure 3.19 shows that *A. thaliana* roots can metabolise 2,3-DNT to 2A3NT with the highest concentrations of 2A3NT were obtained from the *A. thaliana* roots exposed to 2,3-DNT on 14th day of exposure, which was observed to decrease with the increase in time of exposure. According to Figure the highest concentration of 2A3NT was observed to be 45.71 ± 3.45 ng /g dry wt of *A. thaliana* roots recovered from the plants treated with 2,3-DNT concentration of 0.25 mg /L hydroponic media after 14 days of exposure, however it was observed to reduce to 21.34 ± 3.34 ng /g dry wt of *A. thaliana* on 28th day. For higher concentration of 2,3-DNT like 5 mg /L of hydroponic media, not enough biomass was recovered to carry out analysis, which indicates that these concentrations of 2,3-DNT is toxic to limit the plant growth.

The highest untransformed 2,3-DNT concentrations were obtained from the roots after 28 days of exposure to different concentrations of 2,3-DNT. The highest concentration of untransformed 2,3-DNT was 49.11 ± 3.34 ng/g dry wt measured from the plants treated with 2,3-DNT at concentration of 0.25 mg/L of hydroponic media on 28th day of exposure. This shows that the rate of metabolism decreases as the time of exposure to 2,3-DNT increases. The concentrations of untransformed 2,3-DNT obtained tend to decrease with the increase in concentration of 2,3-DNT in the media. At 2,3-DNT concentration of 5 mg/L of hydroponic media, not enough tissues was recovered to carry out the analysis.



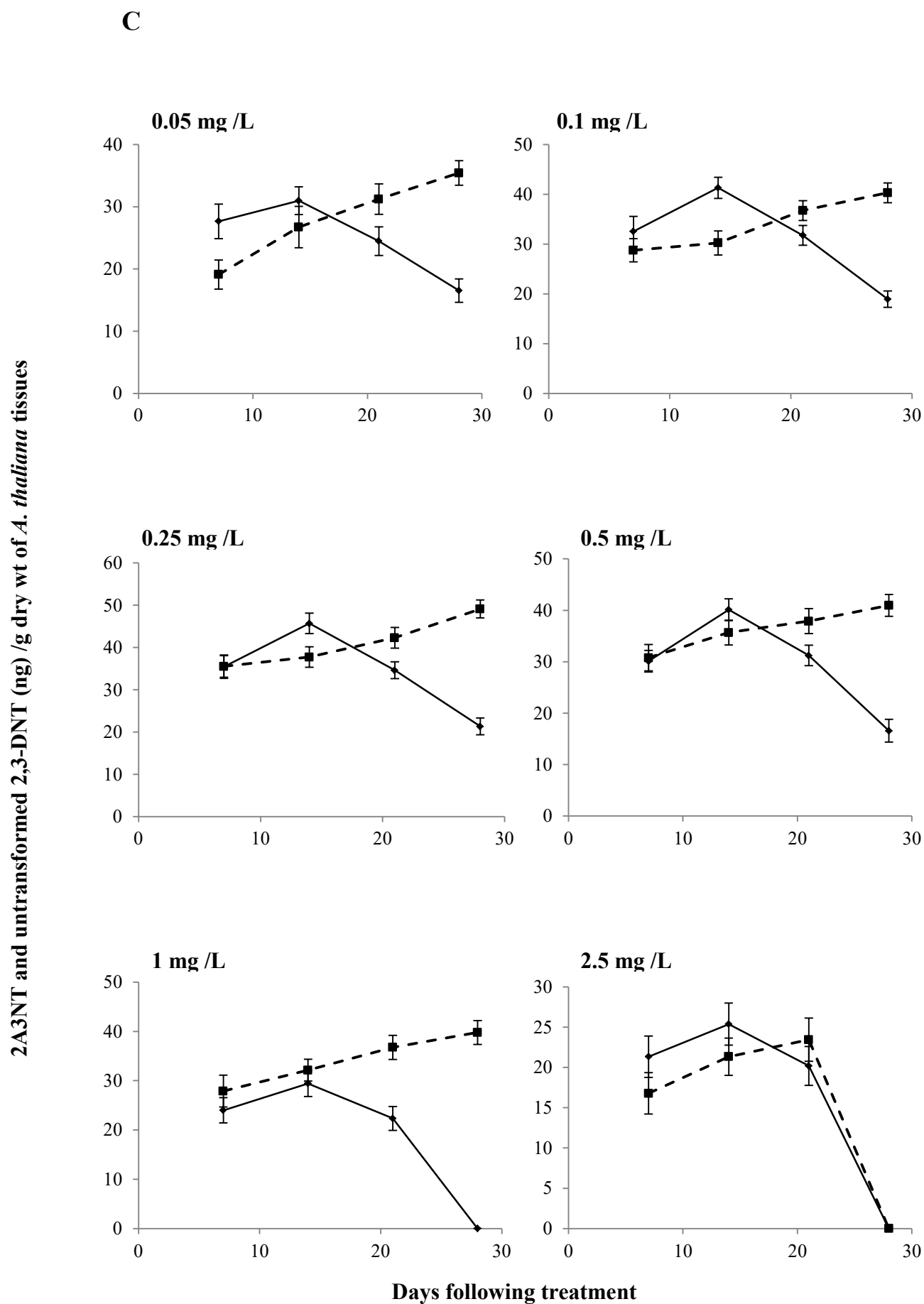


Figure 3.19: (A) 2A3NT and (B) 2,3-DNT concentrations recovered from a single 28 days (post cultivation) old *A. thaliana* plant grown in different concentrations of 2,6-DNT in hydroponic media and analysed by GC-MS (growth temperature was $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period). Data was plotted as mean of four replicates (eight plants from one container were freeze dried and homogenized to give one replicate) (C) Individual variations in 2A3NT (—◆—) and untransformed 2,3-DNT (-■-) against different concentrations of DNT during a time course of exposure (data is in Table 29 and 30 of Appendix II).

Overall distribution of metabolites in roots and shoots of *A. thaliana* exposed to DNT isomers in hydroponic media (time-course study):

Overall for all isomers of DNT, the highest metabolites were observed on 14th day of exposure and lowest on 28th day of exposure for all considered concentrations. In case of shoots higher concentrations of reduced metabolites were obtained compared to untransformed DNTs. However, in case of roots analyses, high concentrations of untransformed DNTs were obtained compared to their respective metabolites.

3.4 Discussion:

Transformation of 2,4-DNT and 2,6-DNT to their respective amines has been demonstrated in several studies (Schneider et al., 1996; Yoon et al., 2006 and 2007; Tognetti et al., 2007; Rocheleau et al., 2010; Doken and Davis 2011). To advance the knowledge about efficient phytoremediation, the metabolites of two major (2,4-DNT and 2,6-DNT) (highly researched in plants) and two minor (2,3-DNT and 3,4-DNT) isomers (never researched in plants) in *A. thaliana* were considered in the present study. The results showed that *A. thaliana* reduced 2,6-DNT to 2A6NT, 2,4-DNT into 2A4NT and 4A2NT, 2,3-DNT to 2A3NT and 3,4-DNT into 3A4NT. The rationale behind the reduction of DNTs to ANTs could be explained by the ‘Phase I’ reaction of the principal degradation process for all xenobiotics in which the molecule undergoes reduction or oxidation (Mezzari et al., 2005; Komives and Gullner, 2005; Beynon et al., 2009; Chung et al., 2012). The electron with-drawing property of the

nitro-groups attached to DNT allows the molecule to get reduced (Sens et al., 1998; Mantha et al., 2002; Beynon et al., 2009). This reduction is further assisted in biological systems in the presence of several enzymes and reducing agents (such as ascorbic acids) (Esteve-Nunez et al., 2001; Oms-Oliu et al., 2012). The high amount of aminotoluenes obtained from the DNT treated *A. thaliana* indicates that the reduction has followed double electron transfer. In case of single electron transfer a nitrosoderivative is usually first putative intermediate, which was not isolated in any of the DNT treatments. Similar results have been found by a study (Tognetti et al., 2007) when tobacco plants reduced 2,4-DNT to 2A4NT.

The analysis of transformed DNT products indicated that unlike other DNT isomers, two reduced products (2A4NT and 4A2NT) were obtained when *A. thaliana* was treated with 2,4-DNT (Figure 3.6-3.7). The concentrations of 4A2NT obtained against different concentrations of 2,4-DNT in the media, were more compared to 2A4NT from same plants (Figure 3.7). The partial positive charge on nitro-groups attached to the benzene ring creates an electron delocalization over the whole ring. The strong deactivating effect at the nitro group of *para*-position (Ikotun et al., 2012) allows it to get reduced easily compared to the nitrogroups at other positions. This behaviour of nitro group at *para*-position with reference to methyl group has been explained in several studies (Wang et al., 2011; Uchimiya, 2010; Tognetti et al., 2007; Yoon et al., 2006)

Analysis of ANTs obtained against different concentrations of DNTs in the media indicated that transformation of DNT is dose dependent. The ANTs obtained against different concentrations of DNTs in the media decreased after reaching a maximum concentration (Figure 3.4-3.11). Since DNTs are toxic to enzymes and biomass at high concentrations (Yoon et al., 2006 and 2007). This suggests a bottleneck phenomenon where metabolic flux is decreasing with increasing concentrations of DNT due to the loss of enzyme efficiency and biomass becoming the limiting factor in DNT transformation. Several studies supported the

idea that increasing concentrations of DNTs in growth media decreased their metabolism in plants (Dokken and Davis, 2011; Yoon et al., 2006; Nepovim et al., 2003). Since DNTs are toxic for living cells and likely cause of DNA damage. It is highly likely that after a specific concentration the enzymes to reduce DNTs are highly damaged (may be due to increased concentration of reactive oxygen species- ROS) and their activity is severely inhibited resulting in reduced transformation (Zu and Jing, 2012; Oliveira et al., 2010). The influx of DNTs from the media is through roots via xylem. The rate of this influx varies with the evapo-transpiration rate from shoot (Su and Liang, 2011; Dokken et al., 2005). As the concentration of DNTs in the media increased, the higher concentrations transported in the xylem tissues overloaded the plant's metabolic machinery. Therefore a high level untransformed DNT was observed as the concentrations of DNT in the media increased (Figure 3.4-3.11) and the transformation is compromised.

The analysis of data obtained against different concentrations of DNT indicated that high concentrations of metabolites can be obtained from *A. thaliana* corresponding to DNT concentrations higher than EC_{50} (obtained from Chapter 2). The highest transformed 2,6-DNT was obtained corresponding to a 2,6-DNT concentration of 23 mg /L of Phytigel media, however EC_{50} for 2,6-DNT was 13 mg /L of Phytigel media (Figure 3.4). One of the reasons for this transformation could be that the tissues were slightly damaged at concentrations higher than EC_{50} . The metabolic machinery was still capable enough to carry out the maximum transformation. Higher concentrations than this caused severe tissue damage and the biotransformation of DNTs was compromised with likely interference of biochemical and detoxification pathways. However, most of the toxicology studies for DNTs (Yoon et al., 2007; Rocheleau et al, 2010; Tognetti et al., 2007) and other nitroaromatics and xenobiotics reported higher inhibition in metabolism rate after a certain value in the growth media (Hannink et al., 2001; Gong et al., 1994). Growth of *Pseudokirchneriella subcapitata* and

Scenedesmus obliquus had been shown to be reduced at concentrations than the EC₅₀ value of melamine in the growth media (Li et al., 2010).

The analysis of compounds from whole *A. thaliana* plant treated with DNT isomers in hydroponic media showed that the transformed products (ANTs) were present in both roots and shoots. The time course analysis of *A. thaliana* treated with DNTs in the hydroponic media showed that ANTs prevailed over DNTs on 14th day of exposure (Figure 3.12). However, the transformed products disappeared after the passage of time and prolonged exposure caused untransformed DNTs concentrations to prevail. For example at 2,6-DNT concentration of 15 mg /L of hydroponic media, the 2A6NT concentrations of 6.75 µg /g dry tissue wt obtained on 14th day of exposure reduced to 3.45 µg /g dry tissue wt on 28th day of exposure (Figure 3.12). These observations suggest that the ANTs obtained were most likely to be the transient intermediates undergoing further transformation (Hannink et al., 2001; Tognetti et al., 2007; Dixit et al., 2011). The products are either unextractable, or sequestration has limited their availability for plants. This shows processes to overcome the toxicity of the compounds by transforming and then binding them up in the plant material. This performance of plants favours phytoextraction phenomenon during phytoremediation (Tognetti et al., 2007). The decline in plants' ability to absorb and transform DNT in wild type, *pfld* and *cfld* lines of tobacco after 30-40 h of incubation was shown in a study which indicates the load over enzymatic activity to metabolise DNT (Tognetti et al., (2007). The reduction of TNT metabolism by aquatic plants (*Myriophyllum spicatum*) and terrestrial plants after a specific time of exposure has also been shown in a study (Pavlostathis et al., 2000; Vila et al., 2007). This shows that plants have certain capacity and limit to absorb and metabolise nitroaromatics.

A variation in rate of tolerance of DNTs was observed when metabolites obtained from Phytigel media grown *A. thaliana* were compared with the metabolites from the plants grown

in hydroponic media. Availability of DNTs and therefore phytotoxicity highly varies with the nature of medium (Rocheleau et al, 2010 and 2006). The water/octanol partition coefficient (Kow) for 2,4-DNT is 1.98 and for 2,6-DNT is 1.72 to 2.1, which shows the sorption hydrophilic nature of DNTs (diethyl ether with Kow 0.83 is hydrophilic and pentachlorobiphenyl with Kow 6.41 is hydrophobic). According to results the *A. thaliana* tolerance for DNTs has been observed to high in hydroponics compared to Phytigel medium. One of the reasons could be *A. thaliana* is a cress and propagates very well in water. The nature of growth medium could be one reason to combat the toxicity of DNTs in the medium (Rocheleau et al., 2010).

As the exposure to DNTs increased in hydroponic media, the rate of transformation also appeared to decrease. For example the untransformed 2,6-DNT concentration of 0.33 $\mu\text{g/g}$ dry tissue wt obtained corresponding to 35 mg /L of 2,6-DNT in the media on 14th day of exposure increased to 9.45 $\mu\text{g/g}$ dry tissue wt on 28th day of exposure. This trend suggests the bottleneck phenomenon, where the metabolic machinery of plants is overloaded and continual influx of DNTs and the transformation is compromised due to lack of enzymes to assist in degradation (Makris et al., 2010). This can likely affect enzymatic efficiency to reduce DNTs.

The analysis of data obtained for DNT treated *A. thaliana* roots indicated that more untransformed DNTs were obtained compared to transformed ANT. Since roots are continuously exposed to DNT containing media, therefore there is a likelihood of their accumulation of compounds more than their capacity to transform (Dokken and Davis, 2011). Plant roots lack the proper metabolic machinery to degrade and transform contaminants; instead the enzymes involved in cell wall modification become abundant in roots in the conditions of stress (Landa et al., 2010; Vila et al., 2008). Therefore, in the present study

roots were observed to have more untransformed DNTs compared to ANTs for all considered concentrations.

In conclusion, the concentration of reduced metabolites of DNT varies with the concentration in the media. The concentrations of these metabolites as obtained in the present study show that most of the metabolites have been transformed to some other form (possibly conjugation) limiting their availability during analysis. For example, only 0.87µg of 2A6NT /single dry plant of *A. thaliana* was obtained when plants were treated with 2,6-DNT concentration of 23 mg /L of Phtagel media (0.0037 % 2A6NT was recovered from the plants). This shows that very less amount is obtained.

Chapter 4

Conjugation of dinitrotoluene (DNT) metabolites in *Arabidopsis thaliana*

4.1 Introduction

From the findings of previous chapter (Chapter 3) it was observed that dinitrotoluene (DNT) metabolites in *Arabidopsis thaliana* decreased over time. This indicates that the reduced metabolites of DNTs [aminonitrotoluenes (ANTs)] are transformed into other metabolites i.e. phase II conjugates and/or are tightly bound to the cell wall components and are not extractable under the conditions used.

Like other xenobiotics, DNTs follow the three phases of the putative route of detoxification once they gain entry into the plants. The reduction products of DNTs have been identified and isolated in *A. thaliana* and other plants (Schneider et al., 1996; Yoon et al., 2006 and 2007; Tognetti et al., 2007; Rocheleau et al., 2010; Doken and Davis 2011). The up-regulation of glutathione-*S*-transferases (GSTs) in response to *A. thaliana* exposure to 2,4-DNT and 2,6-DNT has been reported (Yoon et al., 2006 and 2007). Therefore the lack of availability of DNTs (as described in Chapter 3) may be due to their conjugation with certain plant based molecules. The present study is designed to show the formation of conjugated products of reduced DNT metabolites [aminonitrotoluenes (ANTs) or hydroxylaminonitrotoluenes (HANTs)].

The conjugation of xenobiotics can take place with a number of different moieties such as malonyl, glutathione and glycosides. The nature of reduced products determines the preference of their conjugation. Dimethomorph (tetramethylthioperoxydicarbonic diamide),

which is an organic fungicide, has been shown to be metabolised by glucosyltransferases (GTs) instead of GSTs where pre-incubation resulted in a high activity of *O*- and *N*- GTs (Dosnon-Olette et al., 2011). The reduced TNT intermediates have been shown to conjugate both with glycosides and glutathione, however, glucosylation was more prevalent. *A. thaliana* harbours 121 GTs some of which play an important role in the detoxification of xenobiotics (others are involved in biosynthesis such as glucosinolate biosynthesis), which seems to be the preferred metabolism over glutathione conjugation (Khater et al., 2012).

GTs catalyses the glucosylation of xenobiotics via glucose transfer from uridine 5'-diphosphoglucose (UDPG) to the phase I transformed product acting as an acceptor molecule. GTs are a large multigene family in plants where the total GTs in *A. thaliana*, isolated so far, are subdivided into 12 classes. These GTs have been classified on the basis of their glycosylation activity and role in metabolism of xenobiotics (Khater et al., 2012; Schrick et al., 2012). One of the most important families is family 1 of GTs also known as uracil glucosyltransferases (UGTs). These enzymes act as biocatalysts for detoxification of aromatic organic compounds through *O*-, *S*- and *N*- glycosylation or gluco-conjugation using sugar donors obtained from nucleoside diphosphate (NDP)-activation (Hall et al., 2011; Zheng et al., 2011). This enzyme family has been shown to conjugate persistent pollutants such as 3,4-dichloroaniline, 2,4,5-trichlorophenol and TNTs with glucose through *O*- and *N*-glucosylation. The UGTs structure has two parts, an *N*-terminal aglycone domain that binds to substrate and a *C*-terminal that binds to the UDP-glycoside (Bazier-Hicks et al., 2007). In animals the UGTs are membrane bound and are located in the endoplasmic reticulum; however, in plants the soluble cytosolic UGTs have been identified to play an important role in detoxification. The bound reduced xenobiotics initiate UGTs to start cleaving UDP and release glucose. The glucose thus obtained is conjugated to a specific atom of the transformed xenobiotics (Figure 4.1) (Ahn et al., 2012).

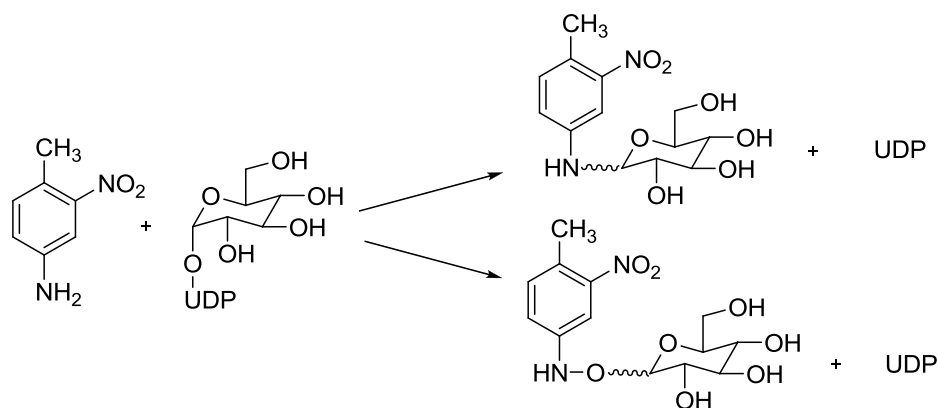


Figure 4.1: A possible reaction for glycosylation of 4-amino-2-nitrotoluene.

The conjugation of transformed metabolites has initially been reported for TNT, where both 2- and 4- ADNTs have been shown to be attached to a six-carbon unit revealed by acid-hydrolysis (Harvey et al., 1990) but the compounds could not be fully identified i.e. the position of glucose in the conjugate was not specified. It was assumed previously that due to the low chemical stability of these compounds and a lack of sensitive analytical techniques that they could not be detected (Hannink et al., 2002). The conjugation processes have been shown to take place in cytosol of the cell and undergo a phase III activation step during which the compounds thus formed are compartmentalized within the vacuole or are bound to the plant cell wall matrix (Gandia-Herrero et al., 2008).

During the phase I reaction DNTs form hydroxyamino-nitrotoluene (HANT) and amino-nitrotoluenes (ANT) (Schneider et al., 1996; Yoon et al., 2006 and 2007; Tognetti et al., 2007). These compounds are considered favourable for glycoside conjugation since previous studies with TNT and dichloroaniline having more or less similar reduced intermediates have been shown to undergo glycosylation (Pascal-Lorber et al., 2010; Brazier-Hicks et al., 2007). The present study is focused on the isolation and quantification of glycoside conjugates (phase II products) of the DNT phase I reaction products (ANTs and HANTs) from *A. thaliana* treated with DNT using LC-MS as an analytical technique. In addition the origin of

the DNT derived conjugates was confirmed by feeding ring deuterated 2,6-dinitrotoluene to *A. thaliana*.

The high sensitivity, increased specificity and low detection limit (Meyer et al., 2012; Wang et al., 2011) makes LC-MS a suitable technique for the detection of DNT-reduced conjugates. The detection through MS/MS increases the reliability of the results through multiple reaction monitoring (MRM) and enhanced product ion scans (EPI) (Li et al., 2012; Forcat et al., 2008). The advantage of LC-MS over other techniques is that it can accurately analyse thermally labile analytes and polar compounds can be detected without derivatization. Here MS/MS (operated in negative mode) coupled with the analyser can give structural elucidation and accuracy.

The current study utilizes electrospray ionisation (ESI) for generating ions. This is the most suitable ion formation technique for highly polar compounds with high molecular weight and thermal instability. A very high voltage is usually applied to the capillary tip present within the ion formation source of mass spectrometer. The strong electric field thus generated makes the samples emerge in the form of an aerosol of charged droplets. Here a nebulising gas directs the spray towards the mass spectrometer. The drying gas evaporates the solvent molecules and only charged sample ions can pass through the sampling cone for further analysis. The negatively charged deprotonated molecular ions $[M-H]^-$ are then processed in the negative mode (Figure 4.2).

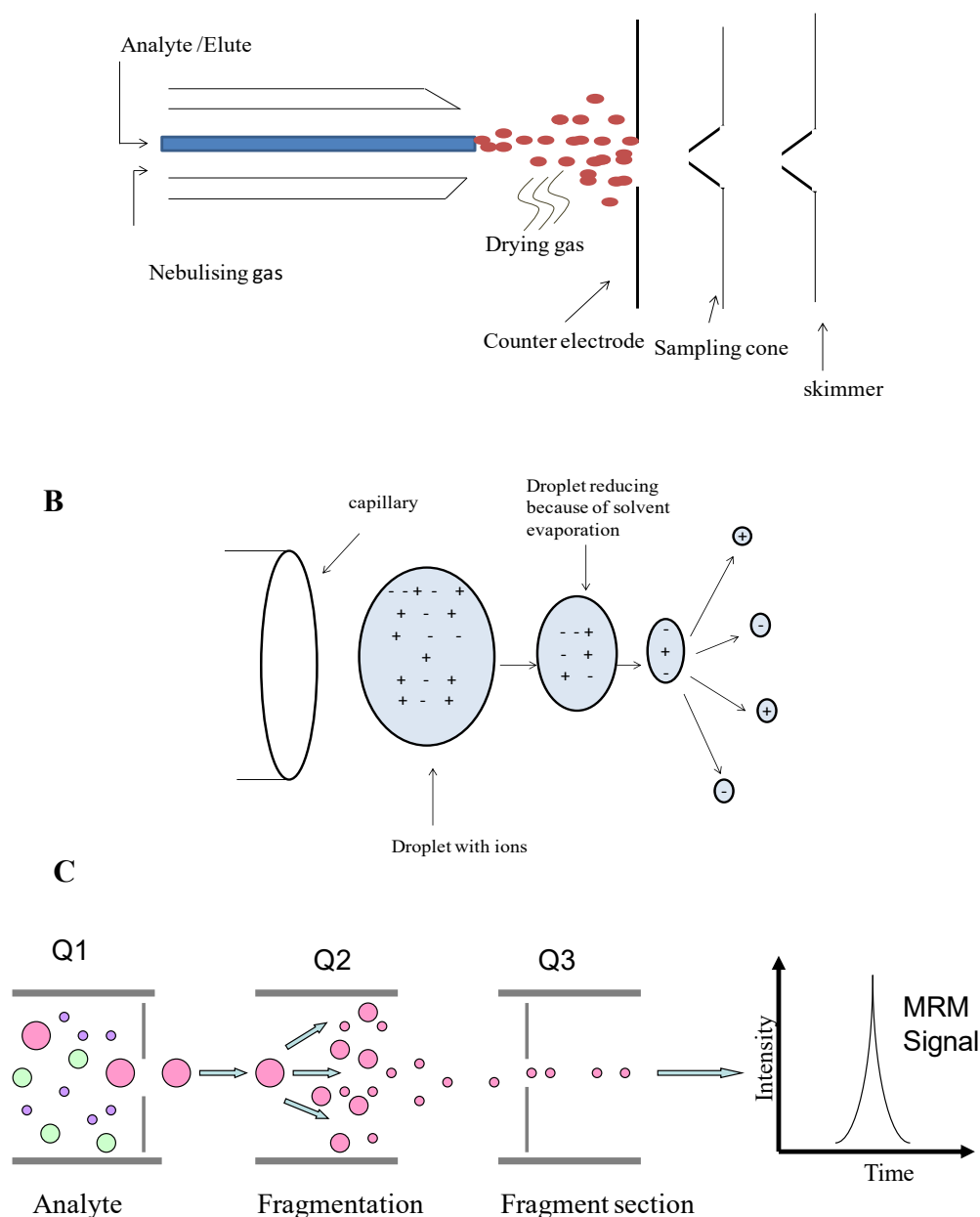


Figure 4.2: Schematic diagram of Electrospray Ionization (ESI). (A) Overview of instrument (B) Production of ions (C) MRM analysis through triple quadrupole mass spectrometer.

The triple quadrupole of the Q-trap makes the system extremely specific and importantly where Q1 and Q3 are standard mass filter quadrupoles they can sequentially scan masses and focus on a single mass. Q2 is a gas filled collision cell where fragmentation occurs, however, it does not filter the ions and is based on ions sent by Q1 and passes them to Q3 for sorting

(Figure 4.2). The ions are trapped to further sensitize the analysis (Wang et al., 2011; Wolschin et al., 2007).

4.1.1 Hypotheses:

The present study was based on following hypotheses:

1. The reduced forms of all four isomers of DNTs form conjugates with glucose and other polar metabolites in *A. thaliana*.
2. The concentration and type of these conjugates varies with the position of the nitro-group attached to the benzene ring.

4.1.2 Aims:

Based on the above hypothesis **the aims** of this study are therefore:

- I. To develop methods to analyse the conjugates of four different isomers of DNT by LC-MS/MS in *A. thaliana*.
- II. To confirm the origin of these conjugates by feeding deuterated DNT to *A. thaliana* and then comparing the products.
- III. To analyse the formation of conjugates in plants treated with different concentrations for all isomers of DNT.
- IV. To estimate the structural variation of conjugates for different isomers of DNT.

4.2 Materials and Methods

4.2.1 Chemicals

2,4-DNT (purity $\geq 98\%$), 2,3-DNT (purity $\geq 99\%$), 2,6-DNT (purity $\geq 99\%$), 3,4-DNT (purity $\geq 99\%$) and 4-Nitrophenyl β -D-glucopyranoside (purity $\geq 98\%$) were purchased from Sigma

Aldrich (UK). Acetonitrile (purity $\geq 97.8\%$, HPLC grade) and methanol (purity $\geq 97\%$) were reagent grade and obtained from VWR, UK. Phytigel (P8169), Murashige and Skoog salts (MS), 2-(*N*-morpholino) ethanesulfonic acid (MES) and sucrose (99.8%) for plant growth were also purchased from Sigma Aldrich (UK). Deuterated d_3 -2,6DNT (2,6-dinitrotoluene- α,α,α - d_3) (98 atom % D) was purchased from C/D/N Isotopes Inc. Canada.

4.2.2 Cultivation of the plants

4.2.2.1 DNT stock solutions for the plant growth media

The concentrations of DNT isomers were selected as described in the Chapter 3 (section 3.2.2.2; Table 3.2). The stock solutions were prepared in 100% ethanol and were further diluted accordingly, such that each growth media (except the control) contained 0.25% ethanol. Pipettes were calibrated once every week for accuracy.

4.2.2.2 Cultivation of the plants

The *A. thaliana* Col 0 seeds were surface sterilized and allowed to grow in sterilized Phytigel media stored in petri plates as explained in previous chapter (Chapter 3; section 3.2.2.2). The plates were placed vertically in the growth rooms under conditions as described in Chapter 3 (section 3.2.2.2).

After four days, ten seedlings of similar root lengths were carefully transferred to 500 ml conical flask with 100 ml of half strength MS, 1% sucrose and 0.025% MES adjusted to pH 5.7 with 1M KOH amended with a specific concentration of the DNT isomer. The procedure was repeated for all concentrations of DNT isomers (Table 4.1) with three replicates. The plants were allowed to grow for 14 days in the growth room under light conditions of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Hanstech Quantitherm light meter) on a shaker with constant shaking at 130 rpm. The temperature of $20 \pm 3^\circ\text{C}$ for 16:8 h of day:night period was maintained for the

growth. These plants were processed further and analysed to calculate the concentration of DNT-reduced conjugates per gram dry weight of DNT treated *A. thaliana* tissues.

4.2.2.3 Extraction of metabolites from DNT treated *A. thaliana*

After 14 days plants were removed from the liquid MS media and washed with deionised water, patted dry on blotting paper and their fresh weights were recorded. The plants were wrapped in aluminium foil and flash frozen in liquid nitrogen. These were then freeze dried (HETO Dry Winner) for 48 h. Precautions were taken while handling plants to avoid cross contamination. After freeze drying, the plants from each conical flask of the same treatment were combined together and ground in a coffee grinder (Wahl James Martin ZX595 coffee grinder) to obtain a homogeneous mixture i.e. one replicate is an homogenate of 10 plants in one conical flask. Sample (20 mg) was taken from the homogenous mixture of freeze dried treated *A. thaliana* plants and placed into 2 ml Eppendorf tubes. A stainless steel ball (5/32") and 10% methanol (1ml) and 5 µl of 2 mg /ml of 4-nitrophenyl β-D-glucopyranoside as an internal standard was added to the tubes and they were processed using a QIAGEN Retsch Tissue Lyser at a frequency of 30.0 Hz/sec for 2 min. The extracts were vortexed for 1 min and centrifuged in an Eppendorf centrifuge 5415D (13.2×1000 rpm, 15 min). The solvent phase was decanted and collected in HP GC vials (clear glass; 2 ml). The samples were re-extracted in 10% methanol and the solvent phase was combined with the previous extraction. The resulting 2ml extract was then analysed by LC-MS/MS. The process was repeated for further two replicates and data was plotted as a mean of these three replicates.

4.2.3 Method Development

Methods were developed for the analysis of glucosylated reduced metabolites of DNTs in *A. thaliana*. Qualitative and quantitative analysis of DNT based glucoside conjugates was carried out to confirm the origin of conjugates. This was achieved by feeding deuterated d₃-2,6DNT and non-deuterated 2,6-DNT to *A. thaliana* in separate experiments The conjugates

thus extracted and analysed on LCMS were compared to confirm the origin i.e. exogenously applied DNT. The masses obtained for precursor and product ions and their mass fragmentation patterns were compared to predict the possible conjugates. Following qualitative confirmation of the structures by examination of molecular and fragmentation ions, a quantitative assessment was made by including an internal standard, 4-nitrophenyl β -D-glucopyranoside. The areas of the peaks with and without standard were compared to show the reliability of the extraction procedure. After reliable results, further analysis was carried out.

4.2.3.1 Analytical technique (LC-MS/MS)

A full scan acquisition was performed on test sample (DNT treated freeze dried tissues extracted in 10% methanol). This gave the total ion current (TIC) plot and intense peaks were identified and ions of interest were selected through analysis of multiple reaction monitoring (MRM) and enhanced product ion (EPI) scan. Further tests were performed to show reproducibility and accuracy of the method and ions of interest.

To obtain the sample, plants were grown in DNT amended liquid MS media (strength MS, 1% sucrose and 0.025% MES adjusted to pH 5.7 with 1M KOH) for 14 days (post cultivation) on a shaker at 130 rpm under light conditions of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Hanstech Quantitherm light meter) and temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h of day:night period. After 14 days the plants were flash frozen in liquid nitrogen and processed as described in the section 4.2.3.2. The pooled supernatant (sample) was stored in HP GC vials for LCMS analysis.

Samples (40 μl) were analysed by HPLC-electrospray ionisation/MS-MS (Table 4.2). This was carried out on an Agilent 1100 HPLC coupled with Applied Biosystems Q-TRAP 2000 (Applied Biosystems). Chromatographic separation was carried out using on a 3 μm C18 (100 mm $\times$ 2) Phenomenex Luna at 35°C (www.phenomenex.com) fitted with Phenomenex

Luna Guard column. The details are listed in Table 4.2. The analysis and confirmation of compounds was based on fragments by EPI and MRM of ion pairs for DNT-reduced glucose conjugates using the mass transitions (explanation given in the results section).

For all samples, the monitoring of column elute was carried out using a channel electron multiplier (CEM) detector (amplification occurs by collision of electrons with the wall of detector). The ions were generated in the negative mode using Turbo-IonsprayTM as the ion source. The optimum conditions for the analysis were determined by the Quantitative Optimisation feature of the software (Analyst 1.4.2) through preliminary sample runs. The optimized conditions are given in Table 4.1.

To minimize dimerization, declustering potential (DP) of -30 V was applied. Ions were fragmented in the ion trap by collisionally induced data-dependent fragmentation. The intense ions of interest are selected from co-eluting mass spectrum and allowed to pass through MS1 (Q1). These ions are then generated in the collision cell. Only particular fragment ions are allowed to pass through MS2 (Q3) quadrupole. The parent ions and qualifier ions are then used to identify the peaks.

Table 4.1: LC-MS conditions to determine dinitrotoluene isomers and their metabolites.

Instrument	Agilent 1100 HPLC coupled to an Applied Biosystems Q-TRAP 2000
Agilent Pump	Agilent 1100 LC Binary Pump
Maximum pressure (psi)	4000
Maximum pressure ramp (psi/sec)	290
Chromatographic separation	
Column	Phenomenex Luna 3 µm C18(2)

Guard Column	Security guard Phenomenex Luna
Dimension	100 mm × 2.0 mm column
Temperature of column	35°C
Mobile phase A	94.9% H ₂ O 5% CH ₃ CN 0.1% CHOOH
Mobile phase B	5% H ₂ O 94.9% CH ₃ CN 0.1% CHOOH
Gradient (ESI- negative)	100% A to 100% B over first 20 min 100% B held for next 8 min 100% A returned for 2 min for equilibration
Solvent flow rate	200 µl /min
Sample volume used	40 µl
Mass Spectrometer	
Analysis	Multiple Reaction Monitoring (MRM) of ion pairs and Enhanced Product Ion (EPI)
Component	Linear Ion Trap Quadrupole LC/MS/MS Mass Spectrometer
MS mode	Negative
Software	Analyst 1.4.2 software (Applied Biosystems)
Ion Source	
Ion-source	Turbo-Ionspray™
Optimized temperature	400°C
Ion source gas 1	40 psi

Ion source gas 2	60 psi
Ion spray voltage	-4500 V
Curtain gas	40 psi
Collision Activation dissociation (CAD)	
gas setting	2
Constants	Declustering Potential (DP = -30 V)
	Entrance Potential (EP = -10 V)
	Collision Cell Entrance Potential (CEP = -2V)
Collision energies	-25
Dwell time	30 m sec

4.2.4 Calculations:

(i) For calculating the recovery, average area (n=3) of selected precursor ion peaks (MRM scan) was obtained and processed using the following equation

$$\% \text{ recovery (for 48 h)} = \frac{[\text{Area of single conjugated glucoside peak at 48 h storage}]}{[\text{Area of single conjugated glucoside peak at point of storage}]} \times 100$$

(ii) The concentration of conjugates was then calculated as relative response to the internal standard using the formula as described below:

Internal standard, 4-nitrophenyl β -D-glucopyranoside = 10 μ g

Amount of sample = 20 mg

Molecular mass of internal standard = 301.25

Relative concentration of conjugates = X $\mu\text{mol/g}$ = (Area of sample peak/Area of internal standard peak) $\times$ (10/301.25) $\times$ (1000/20)

4.3 RESULTS

4.3.1 Method development

LC-MS methods were developed for the analysis of DNT-reduced conjugates extracted from the plants, grown in DNT treated liquid MS media. Peaks were selected through MRM scans and confirmed with EPI scans. The stability of conjugates and reliability of the analysis were determined.

4.3.1.1 Selection of Ions

4.3.1.1.1 Selection of Ions

Initially MRM chromatograms obtained from LC-MS/MS analysis of different DNT treated samples and untreated controls were overlaid. Highly intense peaks (Figure 4.3) (parent ions) thus obtained (provided they were not present in control samples) were screened and subjected to MS/MS fragmentation. The EPI scan was then conducted for these intense peaks to determine the masses (m/z ratio). These peaks were further fragmented to predict the composition of the parent ions (peaks obtained through initial MRM scan). This yielded product ion mass spectra at pre-selected collision energies. The resulting spectra with product ions and neutral loss atoms were identified through matching with the existing mass spectral library and available literature (compound structure archived through PubChem–NCBI, 2012 and SciFi Scholar, 2009). The MRM and EPI scans confirmed the presence of ten different reduced DNT-based conjugates in repeated preliminary experiments.

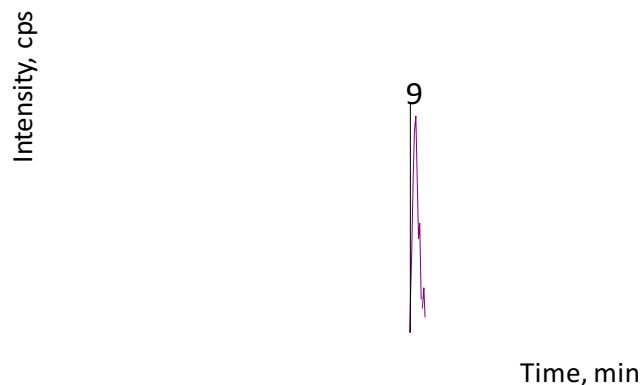


Figure 4.3: MRM scan for possible glucosylated DNT metabolites precursor ions extracted from *A. thaliana* treated with 5 mg /L of 2,6-DNT after screening the peaks absent in control (untreated *A. thaliana* extract). The plants were treated with 2,6-DNT for 14 days in liquid MS media under growth temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period. The analysis was carried out on 20 mg of freeze dried tissue. The peak numbers are given randomly. The retention times are 1=11.5, 2= 8.2, 3=12.66, 4=14.2, 5=10.4, 6=11.19, 7=10.3, 8=7.2, 9=10.2, 10= 11.5.

Further confirmation of these compounds to be DNT based was done by analysing deuterated d_3 -2,6-DNT treated *A. thaliana* tissues (treatment and extraction carried out exactly as for non deuterated 2,6-DNT).

4.3.1.1.2 Quantitative and Qualitative analysis of DNT conjugates:

2,6-DNT fed to *A. thaliana* produced potential conjugates. To confirm the origin of these conjugates, *A. thaliana* was fed with deuterated d_3 -2,6DNT and conjugates extracted and analysed by LC-MS/MS and were compared with the non deuterated DNT conjugate profile

(this would show that the metabolites are derived from DNT and are not constitutive plant metabolites).

To determine whether the conjugates are DNT based, 4 day old seedling were placed in 100 ml mixture of half strength MS, 1% sucrose, 0.025% MES (Sigma UK) and adjusted to pH 5.7 with 1M KOH in 500 ml conical flask. The liquid media then received 15 mg /L of deuterated d_3 -2,6-DNT and plants were allowed to grow for 14 days. *A. thaliana* plants were then harvested and washed twice with deionised water and patted dry on blotting paper. The plants were flash frozen in liquid nitrogen after placing them in aluminium foil and freeze dried for 48 h. These freeze dried tissues were extracted in 10% methanol following same procedure as described above. The extract was analysed by LC-MS.

The results (Figure 4.4) from MS/MS analysis of deuterated d_3 -2,6DNT fed *A. thaliana* showed that the peaks of conjugates were DNT based and were not constitutive plant metabolites. This shows the validity of the LC-MS/MS methodology.

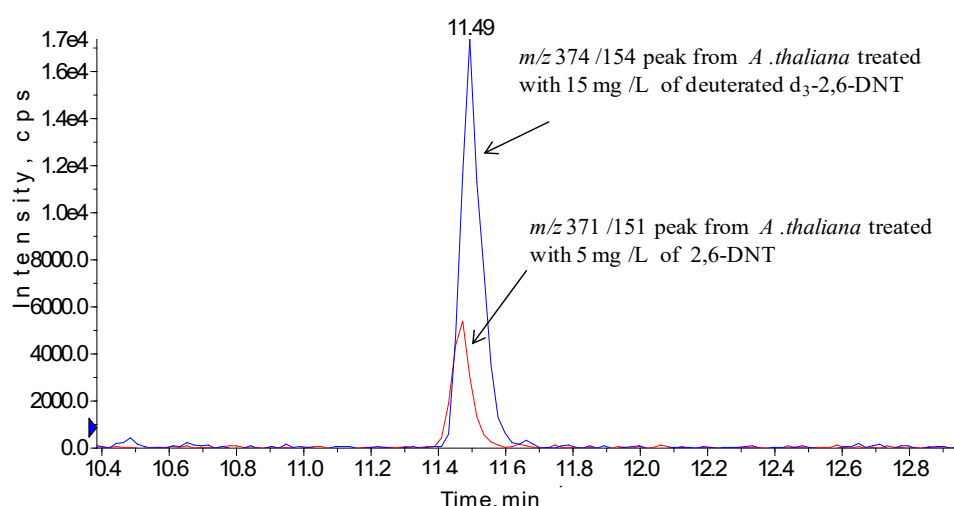


Figure 4.4: MRM scan for precursor ion /product ion m/z 374 /154 extracted from *A. thaliana* treated with 15 mg /L of deuterated d_3 -2,6-DNT and m/z 371 /151 *A. thaliana* treated with 5 mg /L of 2,6-DNT . The different concentrations of treatments were

maintained to avoid peak overlapping and enhance clarity. The plants were treated with d_3 -2,6-DNT and 2,6-DNT (separately) for 14 days in liquid MS media under growth temperature of $20 \pm 3^\circ\text{C}$ for 16:8 h day:night period. The analysis was carried out on 20 mg of freeze dried tissue.

The EPI scan of the peaks showed a mass increase of m/z 3 due to deuterated compounds (Figure 4.5). This confirmed the peaks originated from DNT.

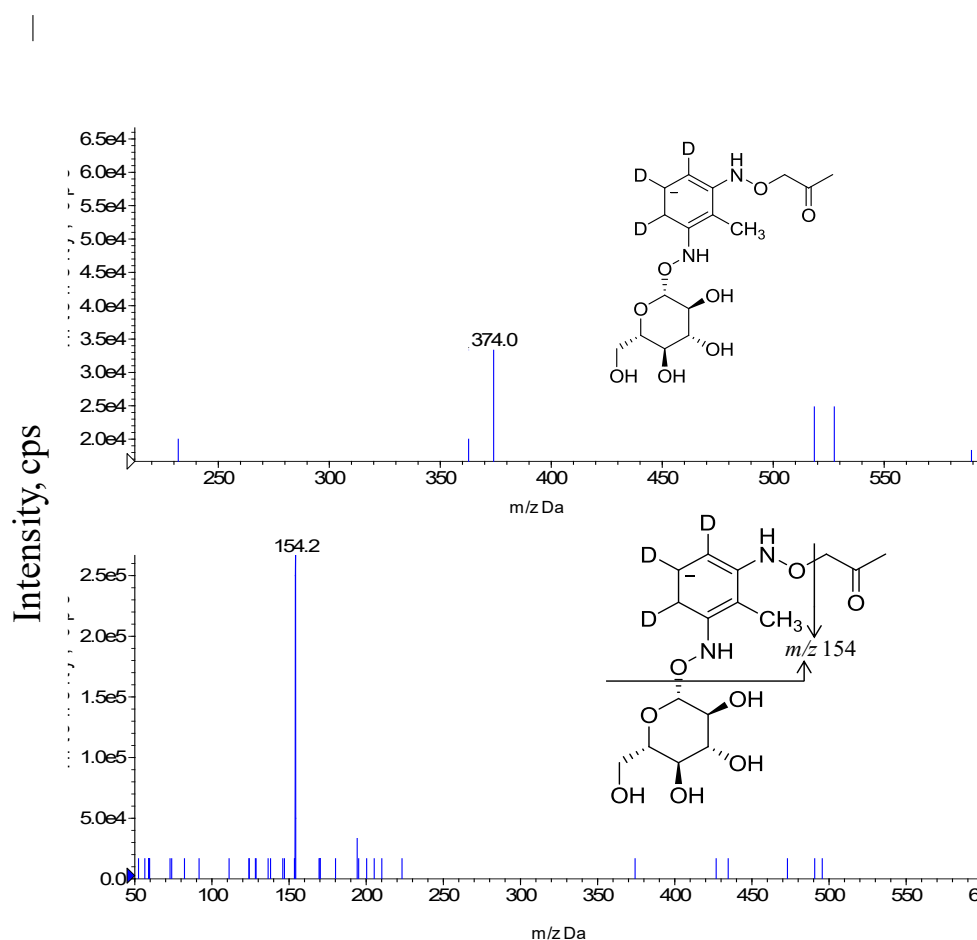


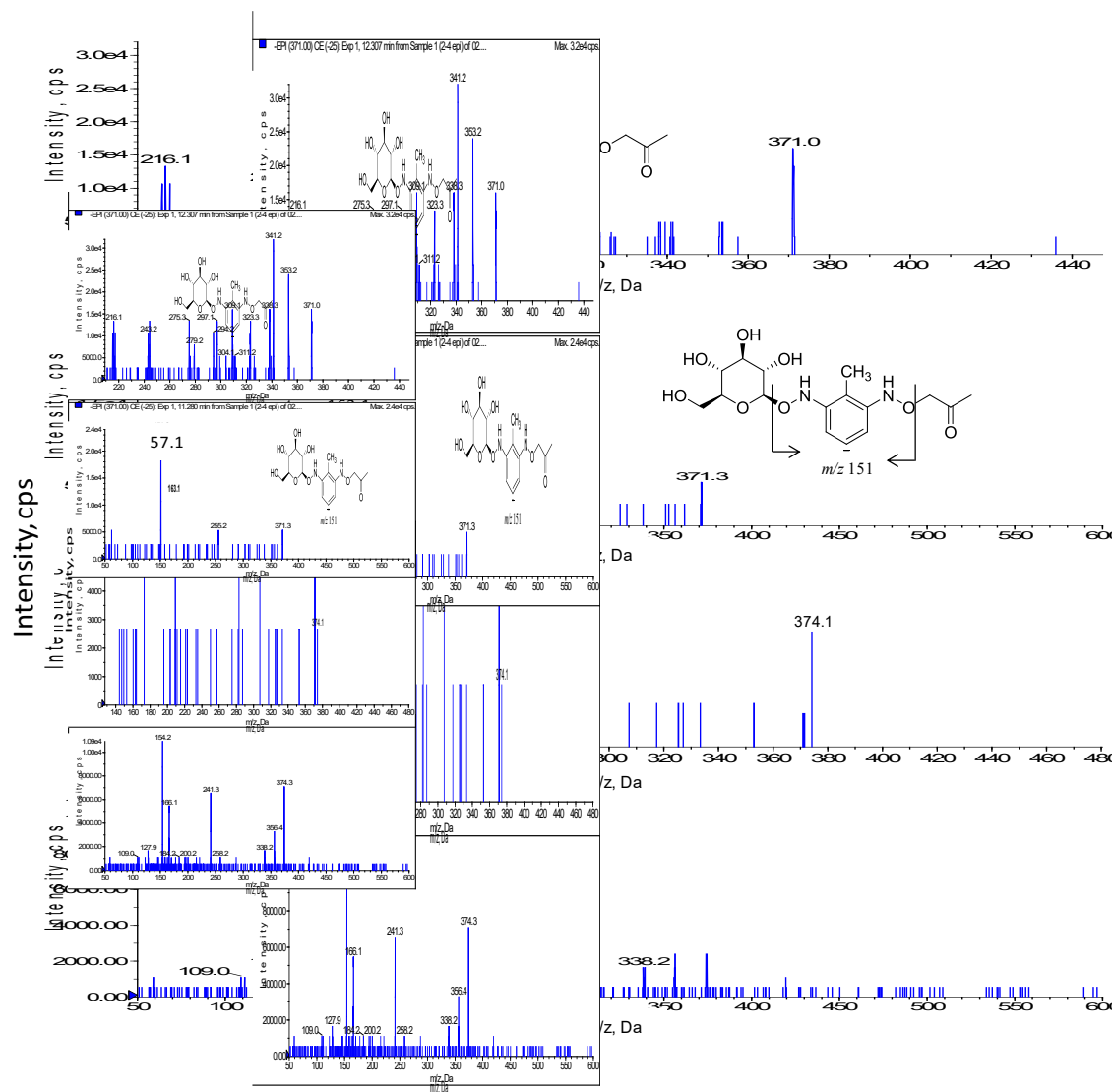
Figure 4.5: EPI scan for precursor ion /product ion m/z 374 /154 extracted from *A. thaliana* treated with 15 mg /L of deuterated d_3 -2,6-DNT from 20 mg of freeze dried tissue mass. The plants were treated with d_3 -2,6-DNT for 14 days in liquid MS media under growth temperature was $20 \pm 3^\circ\text{C}$ for 16:8 h day:night period.

The EPI scan of rest of the glucosylated compounds were compared with the EPI scan obtained from deuterated d_3 -26DNT originated glucosylated compounds. The mass fragmentation patterns were interpreted to show the possible glucoside conjugates.

Compound name: DHAT-*O*-MG-*N*-OP (1)

Precursor ion /product ion (m/z): 371 /151

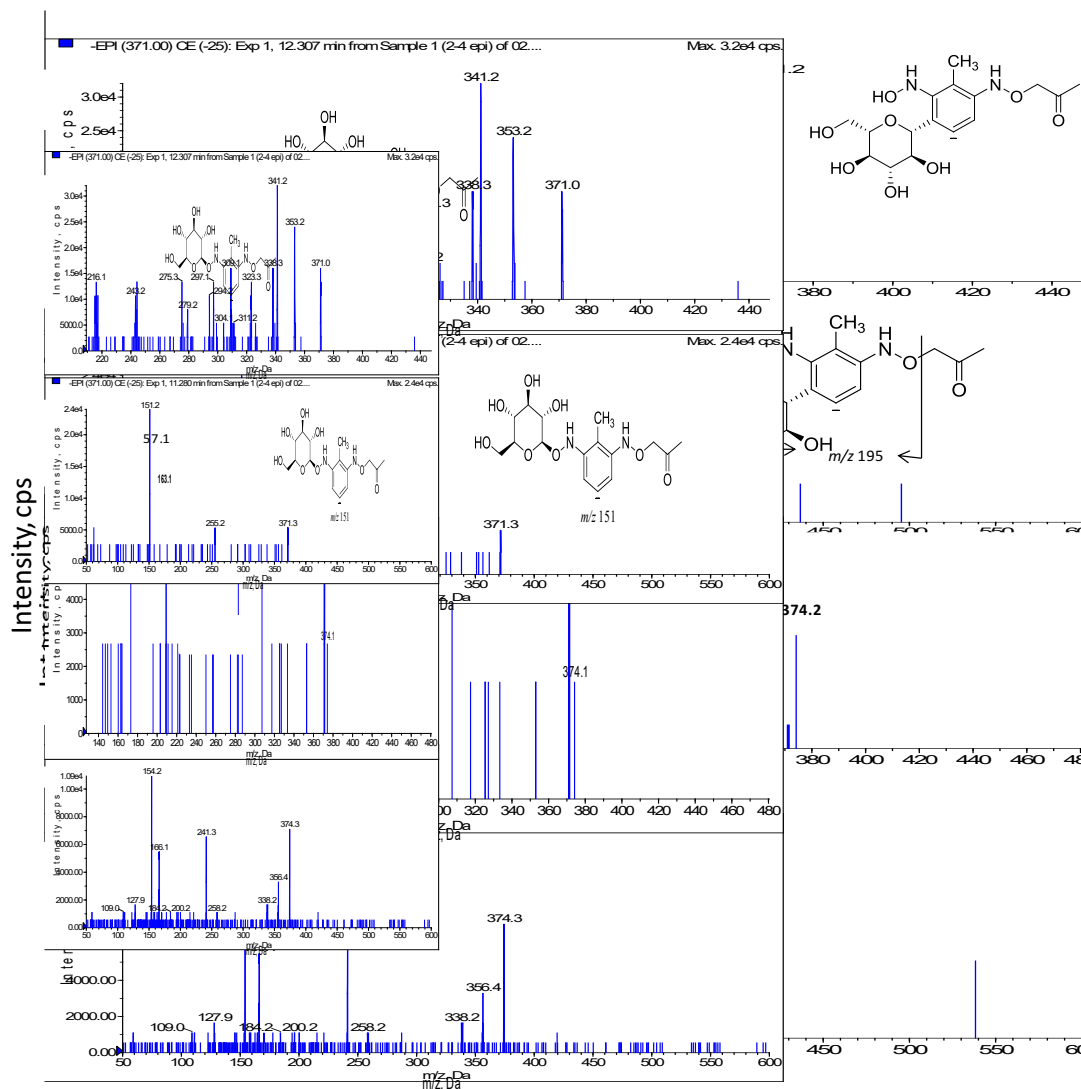
Basis of structure: The precursor ion 371 was 374 in deuterated d_3 -DNT extract. Product fragmentation gave m/z 151.2 = HANT; m/z 163.1= glucose; m/z 58.1= propanone; the structure below supports major m/z 151 fragment.



Compound name: DHAT-C-MG-N-OP (2)

Precursor ion /product ion (m/z): 371 /195

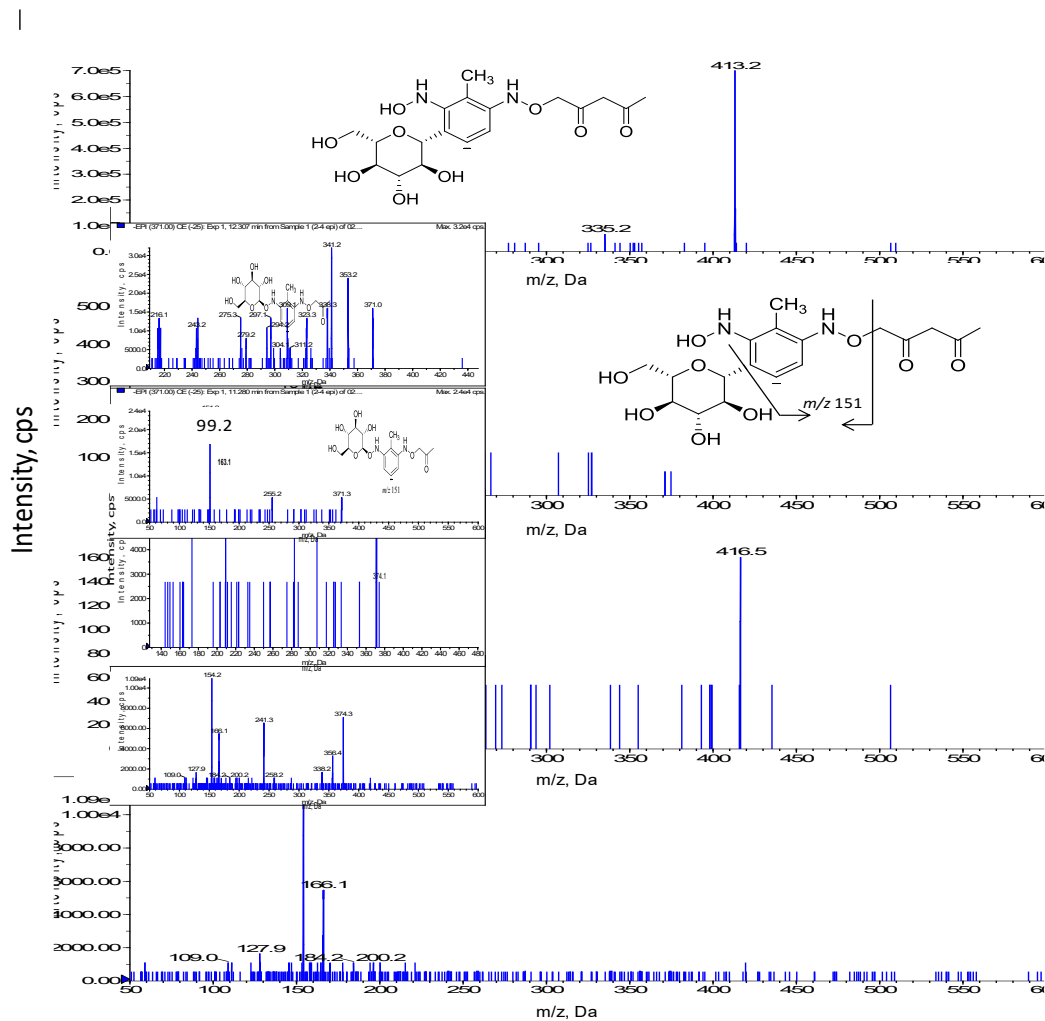
Basis of structure: The precursor ion m/z 371.2 corresponds to that of m/z 374.2 for deuterated d₃-2,6DNT glucosylated conjugate. This fragments into m/z 195.1= HANT-C-ethanol; m/z 120.1= C₄H₈O₄ glucose fragment; m/z 58.1= propanone; the structure support m/z 195 fragment.



Compound name: HANT-C-MG-N-OB (3)

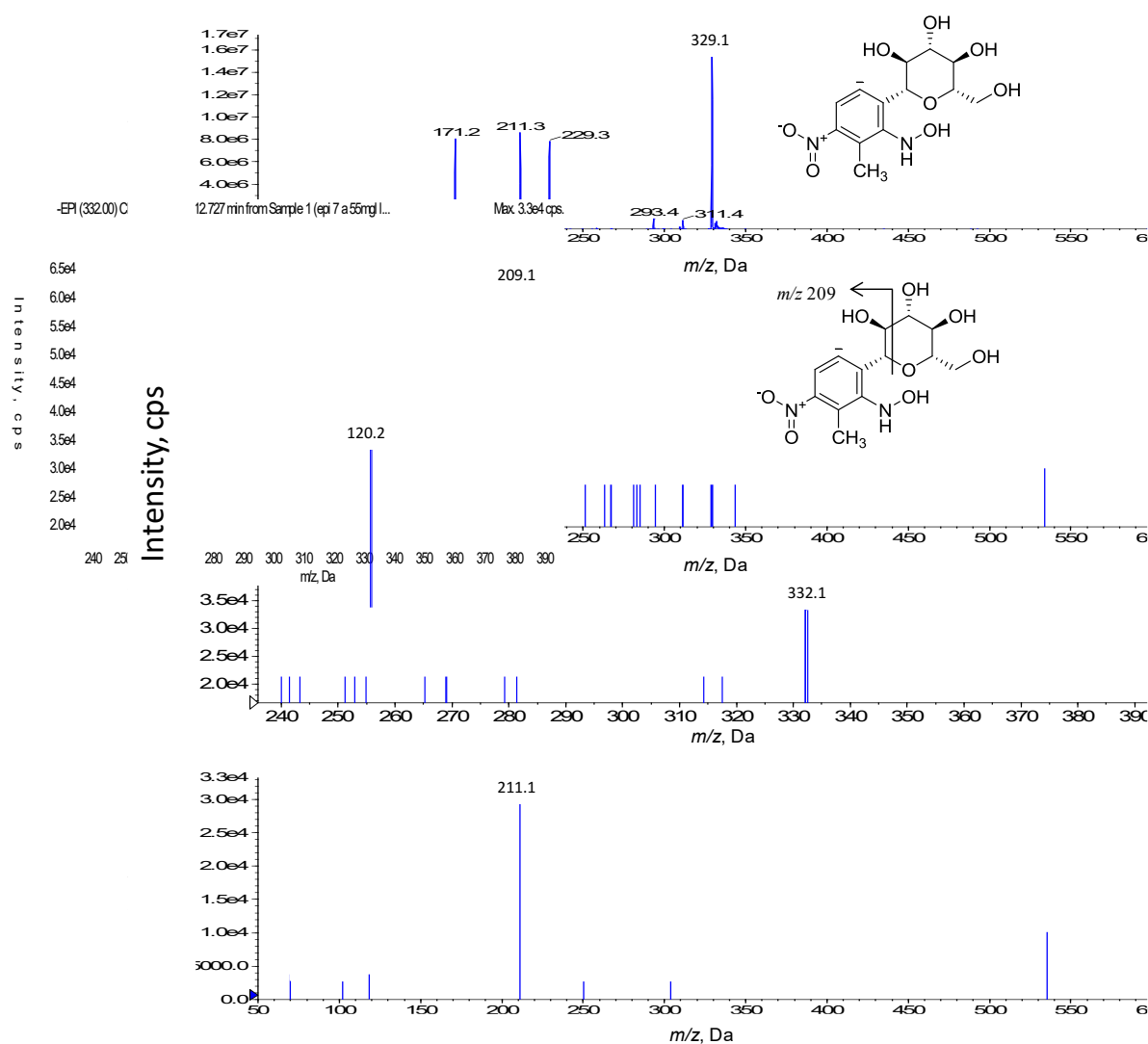
Precursor ion /product ion (m/z): 413 /151

Basis of structure: The precursor ion m/z 413 corresponds to that of m/z 416 for deuterated d_3 -2,6DNT glucosylated conjugate. This fragments into m/z 151= HANT; m/z 163= glucose; m/z 99 = butanone. The structure below supports m/z 151 fragment.



Compound name: HANT-C-MG (4)**Precursor ion /product ion (m/z):** 329 /209

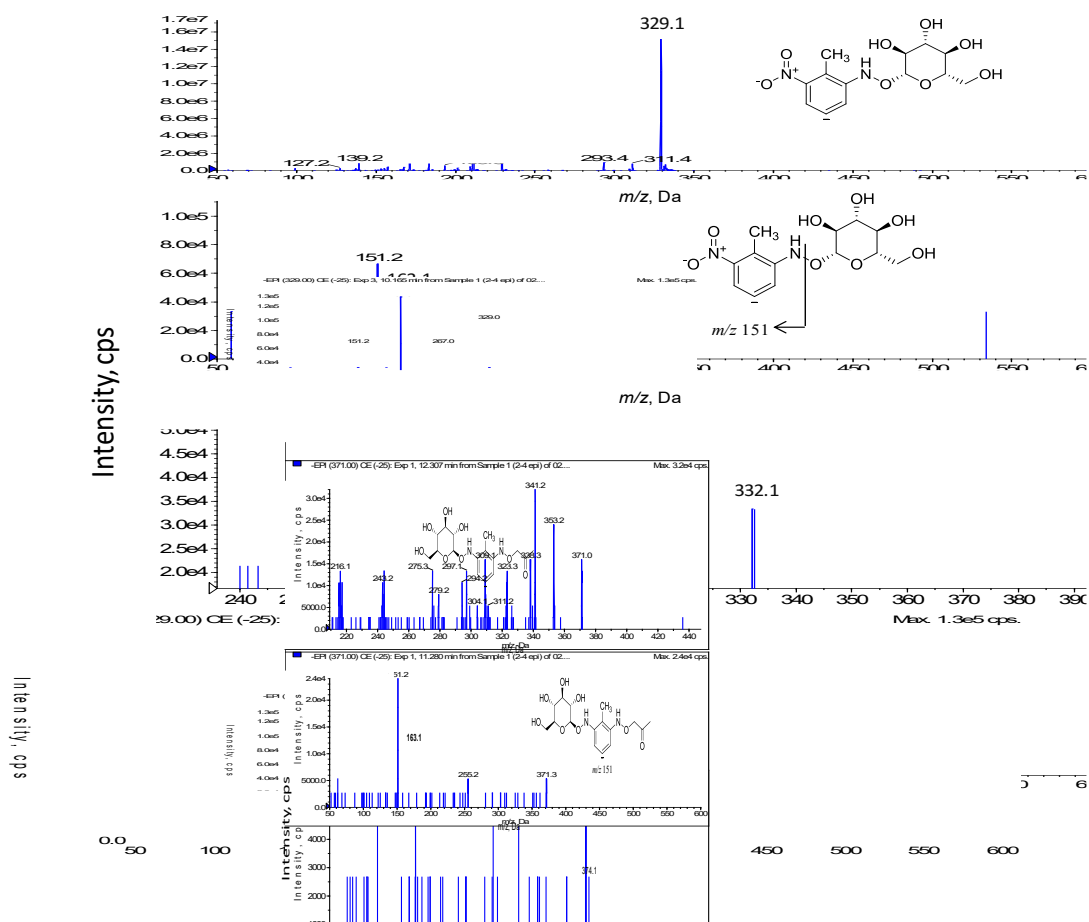
Basis of the structure: The major precursor ion is m/z 329.1 corresponds to that of m/z 332.1 for deuterated d_3 -2,6DNT glucosylated conjugate. This fragments into m/z 209.1= HANT-C-ethanol; m/z 120.1= $C_4H_8O_4$ glucose fragment; the structure drawn below support m/z 209.1 fragment.



Compound name: HANT-O-MG (5)

Precursor ion /product ion (m/z): 329 /151

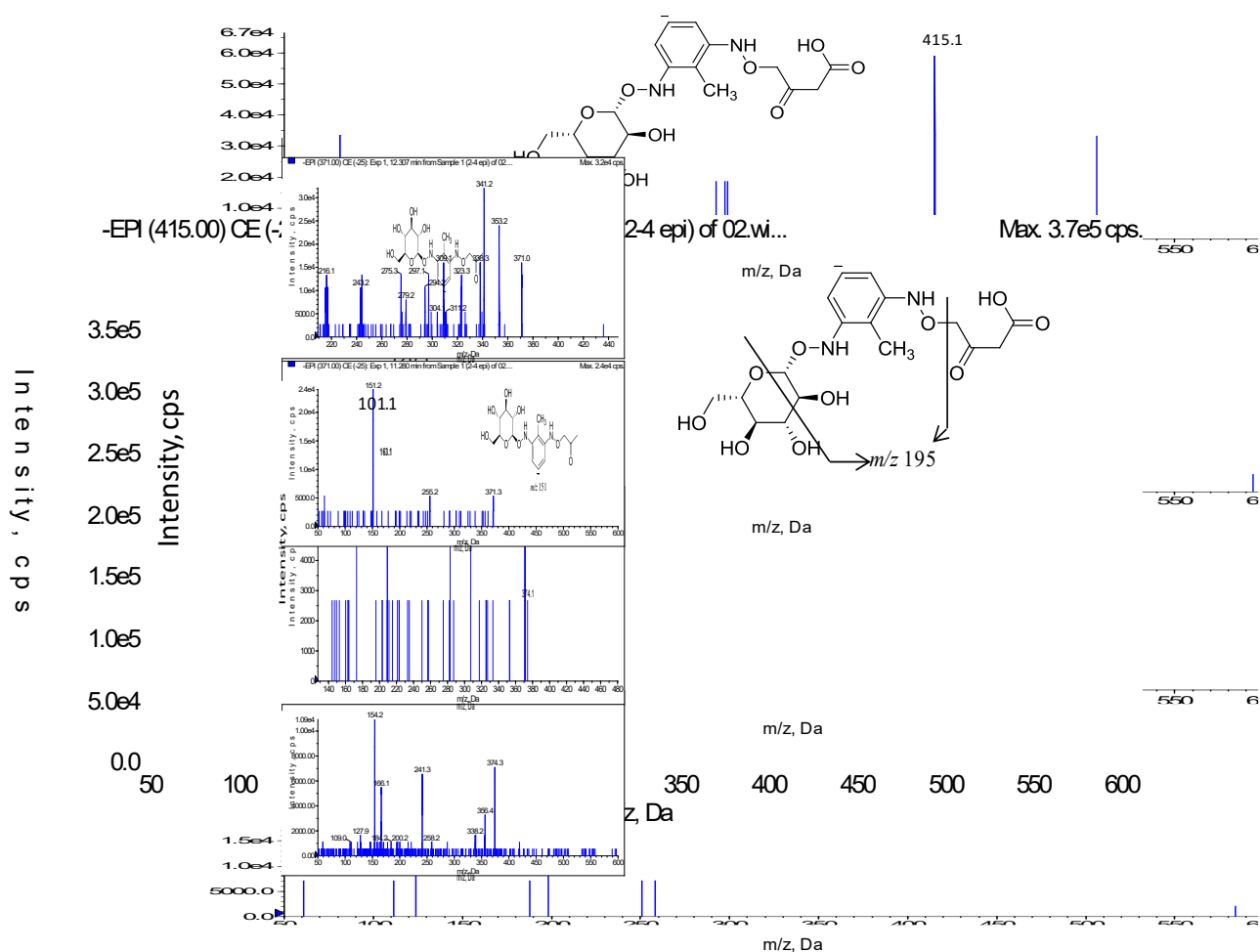
Basis of the structure: The major precursor ion is m/z 329.1 corresponds to that of m/z 332.1 for deuterated d_3 -2,6DNT glucosylated conjugate. This fragments into m/z 151.2= HANT; m/z 163.1= glucose fragment; the structure drawn below support m/z 151.2 fragment.



Compound name: HANT-O-MG-O-M (6)

Precursor ion /product ion (m/z): 415 /195

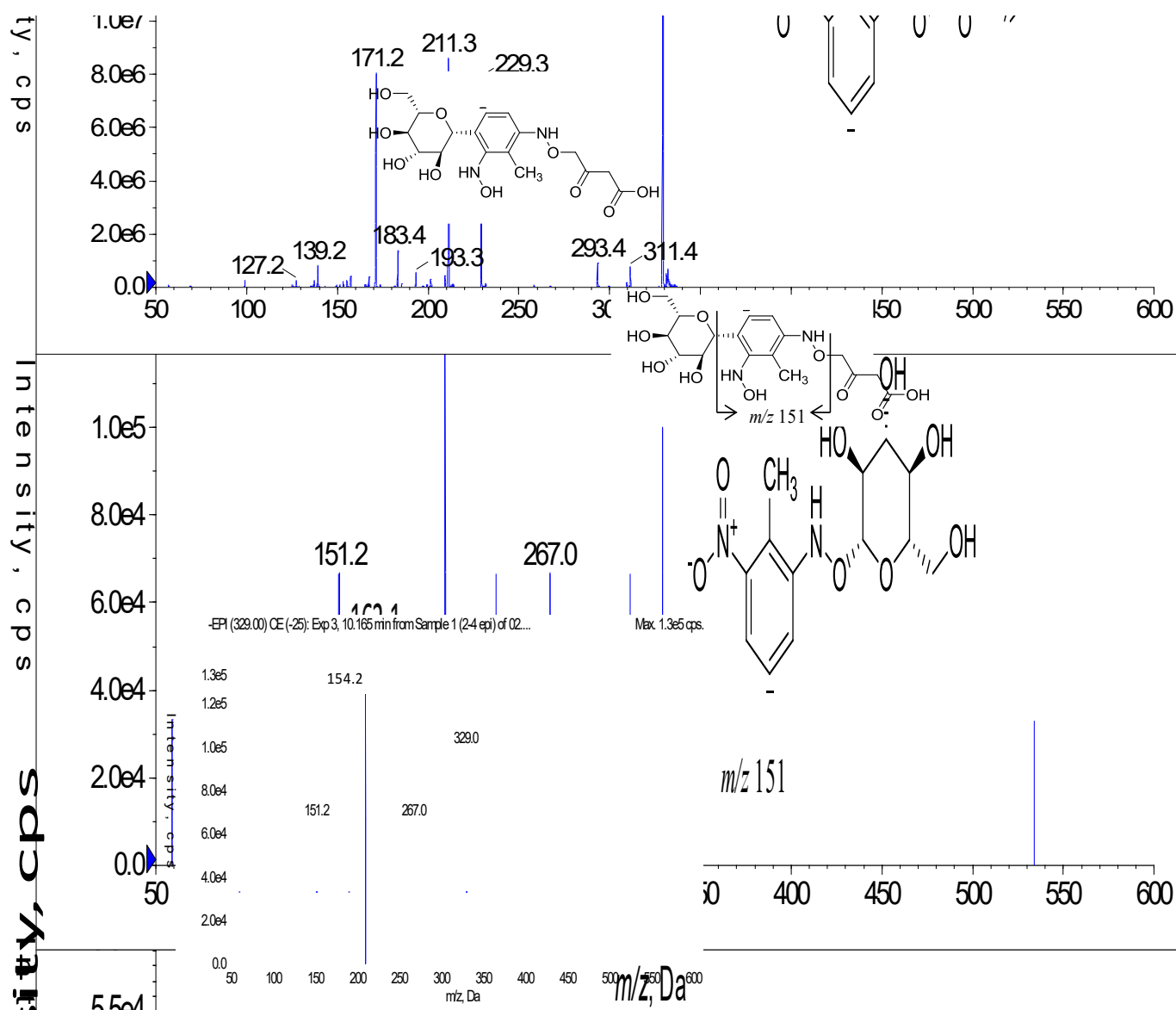
Basis of structure: The m/z 415 was m/z 418 for deuterated d3-2,6DNT glucosylated conjugate. Fragmented into m/z 195= HANT-O-ethanol; m/z 120= $C_4H_8O_4$ glucose fragment; m/z 101 = malonyl; the structure support m/z 195 fragment.



Compound name: HANT-C-MG-O-M (7)

Precursor ion /product ion (m/z): 415 /151

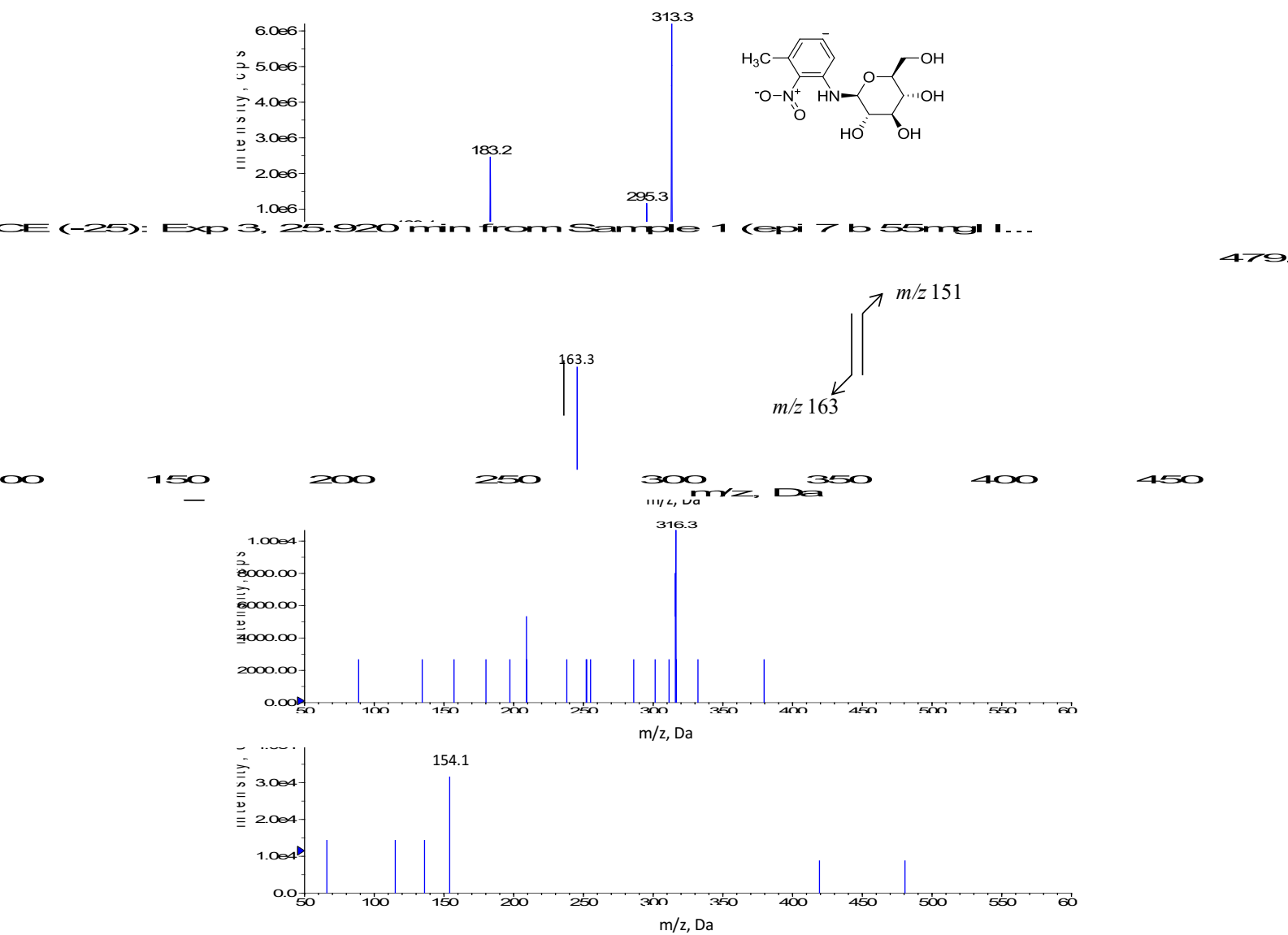
Basis of structure: The precursor ion m/z 415 was m/z 418 for the deuterated d3-2,6DNT glucosylated conjugate. This fragmented into an ion of m/z 151= HANT; m/z 163= glucose fragment; the structure supports the m/z 195 fragment.



Compound name: ANT-*N*-MG (8)

Precursor ion /product ion (m/z): 313/151

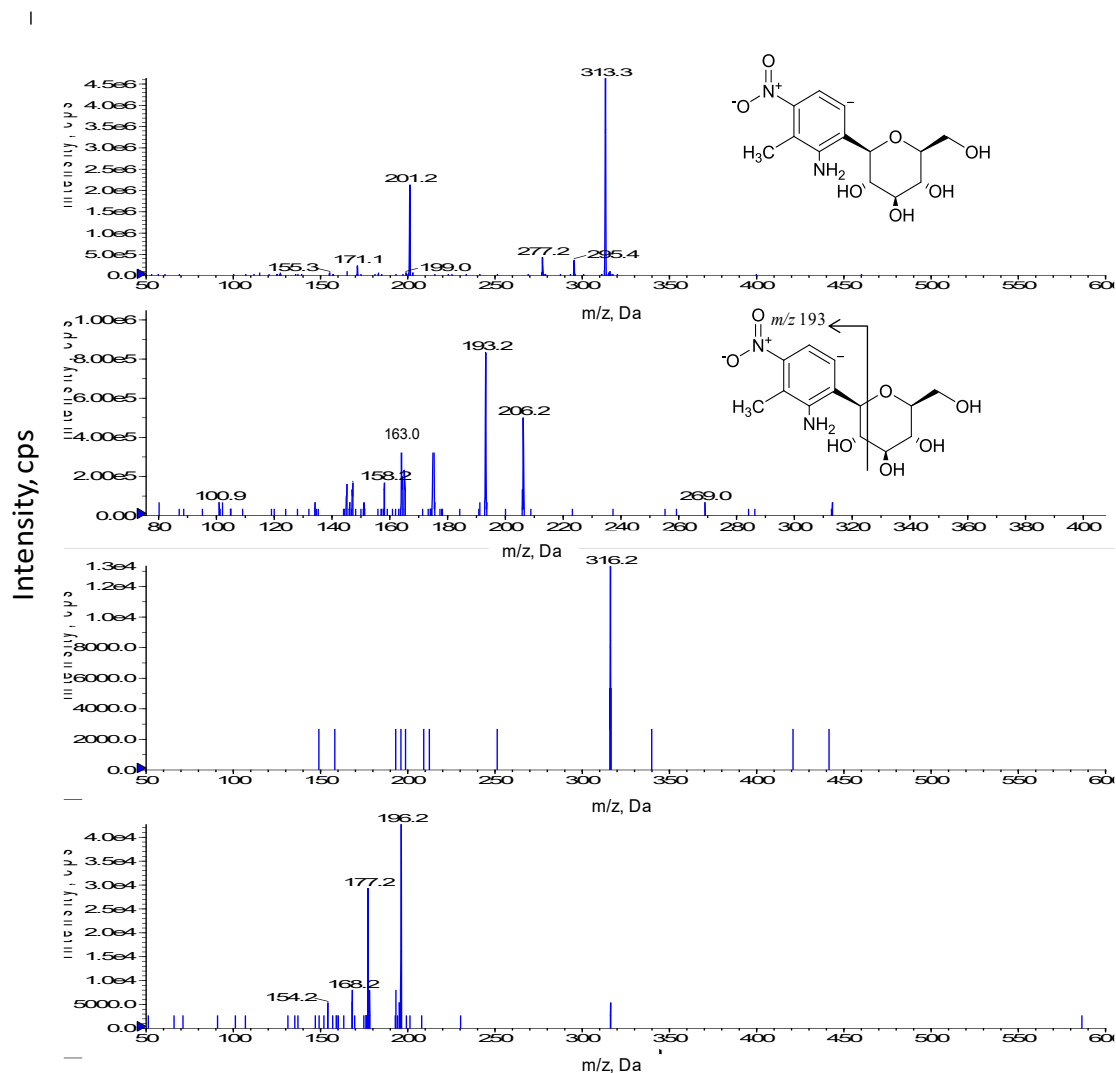
Basis of the structure: The major precursor ion is m/z 313.2. This fragments into m/z 151.1= ANT; m/z 163.1= glucose fragment; the structure drawn below support m/z 313.2 fragment.



Compound name: ANT-C-MG (9)

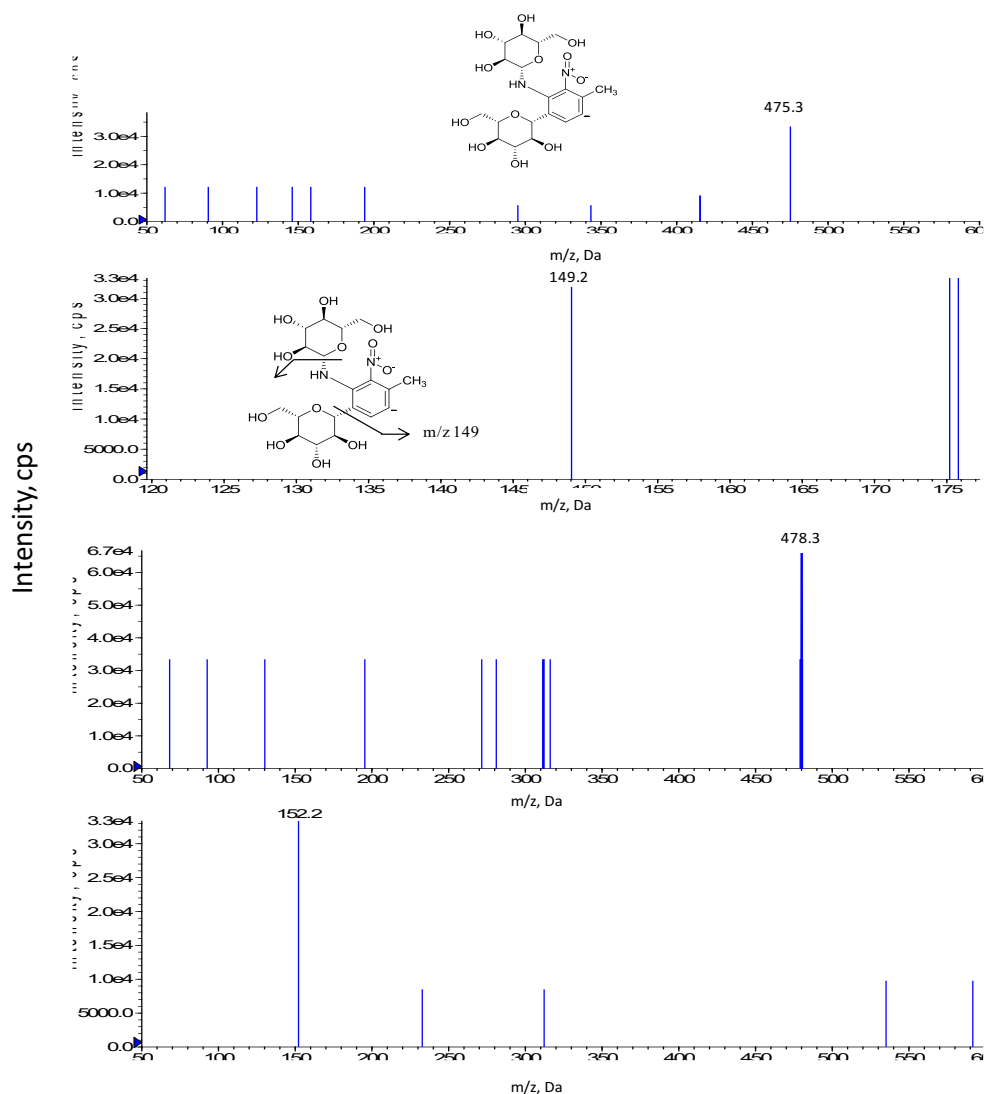
Precursor ion /product ion (m/z): 313 /193

Basis of the structure: The major precursor ion is m/z 313.3 which corresponds to m/z 316.2 for deuterated d_3 -26DNT glucosylated conjugates. This fragments into m/z 193.2= ANT-C-ethanol;; the structure drawn below support m/z 193.2 fragment.



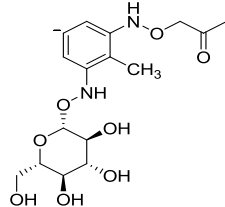
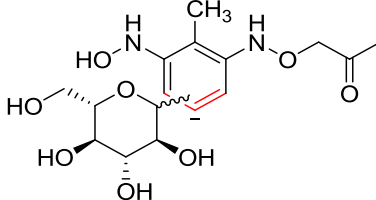
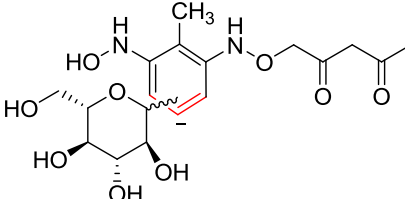
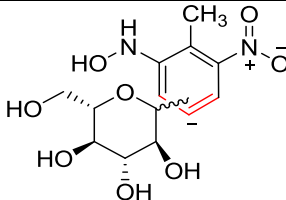
Compound name: ANT-C & N- DG (10)

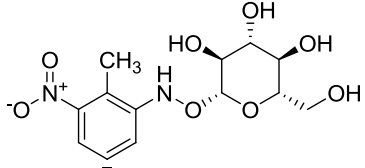
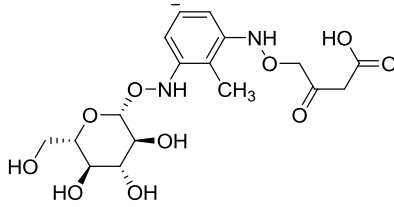
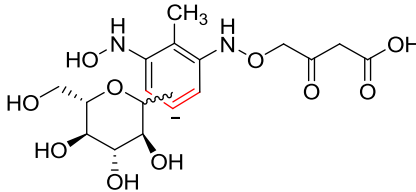
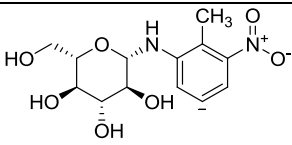
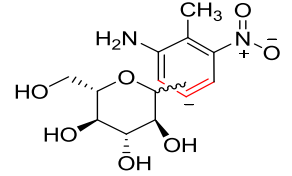
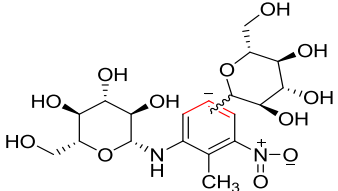
Precursor ion /product ion (m/z): 475 /149



After confirmation of the peaks and prediction of possible structures, the names and numbers were assigned to the different conjugates (Table 4.3). Due to the lack of standards, it was not possible to exactly predict the position of C-C glucosidic bonds, therefore possible conjugation sites are highlighted (red colour) in the structure (Table 4.2).

Table 4.2: Glucosyl conjugates derived from 2,6-DNT and deuterated d₃-2,6DNT metabolites with their retention times.

Compound Number	Semi-systematic name	Retention time (min)	Structure [M-H] ⁻	2,6-DNT based precursor /product ions (m/z) [M-H] ⁻	Deuterated d ₃ -2,6DNT based precursor /product ions (m/z) [M-H] ⁻
1	Aminohydroxynitro-toluene –O-monoglucoside- <i>N</i> -oxopropanone (DHAT- <i>O</i> -MG- <i>N</i> -OP)	11.5		371/151	374/154
2	Aminohydroxynitro-toluene –C-monoglucoside- <i>N</i> -oxopropanone (DHAT- <i>C</i> -MG- <i>N</i> -OP)	8.2		371/195	374/194
3	Aminohydroxynitro toluene –C-monoglucoside- <i>N</i> -oxobutanoate (HANT- <i>C</i> -MG- <i>N</i> -OB)	12.6		413/151	416/154
4	Aminohydroxy nitrotoluene –C-monoglucoside (HANT- <i>C</i> -MG)	14.11		329/209	332/212

5	Aminohydroxy nitro-toluene –O-monoglucoside (HANT-O-MG)	10.4		329/151	332/154
6	Aminohydroxy nitro-toluene –O-monoglucoside-O-malonyl (HANT-O-MG-O-M)	11.19		415/195	418/194
7	Aminohydroxy nitrotoluene –C-monoglucoside-O-malonyl (HANT-C-MG-O-M)	10.36		415/151	418/154
8	Aminonitro-toluene- N-monoglucoside (ANT-N-MG)	7.19		313/151	316/154
9	Aminonitro-toluene- C-monoglucoside (ANT-C-MG)	10.3		313/193	316/196
10	Aminonitro-toluene-C & N- diglucoside (ANT-C & N- DG)	11.5		475/149	478/152

*the red colour highlighted bonds indicates possible conjugation sites

4.3.1.2 Mass determination of the glucosides:

Since standards for all of the metabolites were not available MS/MS generated data was interpreted and fragmented to get obtain the structure of the precursor and product ions. The major peaks from the fragmentation of precursor ions were interpreted to show the relevant conjugates. There were very little mass differences between experimental and theoretical m/z (Table 4.3) indicating that the compounds were DNT based conjugates.

Table 4.3: Overview of experimental (from MS/MS scan) and theoretical masses of precursor and their relevant product ions of glucosyl conjugates.

Compound Number	Elemental composition of anions	Precursor ions		Product ions	
		Theoretical	Experimental	Theoretical	Experimental
		m/z	m/z	m/z	m/z
1	$C_{16}H_{23}N_2O_8^-$	371.15	371.0	151.06	151.2
2	$C_{16}H_{23}N_2O_8^-$	371.15	371.2	195.07	195.1
3	$C_{17}H_{21}N_2O_{10}^-$	413.16	413.2	151.06	151.2
4	$C_{13}H_{17}N_2O_8^-$	329.1	329.1	209.06	209.1
5	$C_{13}H_{17}N_2O_8^-$	329.1	329.1	151.06	151.2
6	$C_{17}H_{23}N_2O_{10}^-$	415.14	415.1	195.07	195.2
7	$C_{17}H_{23}N_2O_{10}^-$	415.14	415.2	151.06	151.2
8	$C_{13}H_{17}N_2O_7^-$	313.1	313.3	151.06	151.1
9	$C_{13}H_{17}N_2O_7^-$	313.1	313.2	193.06	193.2
10	$C_{19}H_{27}N_2O_{12}^-$	475.16	475.1	149.04	149.2

4.3.1.3 Stability of the samples:

To measure the stability of the samples under analysis, 20 mg of homogenous freeze dried 2,6-DNT (15 mg /L of liquid MS media) treated *A. thaliana* tissues were added to a 2 ml Eppendorf tube. To these tissues 1 ml of 10% methanol was added and the samples lysed using a QIAGEN Retsch Tissue Lyser with a frequency of 30.0 Hz/sec for 2 min after adding a stainless steel ball (5/32"). Samples were vortexed and centrifuged in Eppendorf centrifuge 5415D (13.2×1000 rpm) for 7 min. The supernatant was decanted and stored in a HP vial. 1 ml of 10% methanol was added again to the tissue pellet in an Eppendorf tube and after vortexing and centrifuging in an Eppendorf centrifuge 5415D (13.2×1000 rpm) for 7 min, the supernatant was decanted and combined with the previous supernatant. This 2 ml of supernatant was analysed by LC-MS. Samples were processed in four replicates. After LC-MS run two replicates were stored at room temperature and two at -20°C. The samples were analysed after 48 h and recoveries were measured. The peak areas of five conjugated glucosides (MRM scan) were calculated individually using following equation.

$$\% \text{ recovery} = \frac{[\text{Area of single conjugated glucoside peak at 48 h storage}]}{[\text{Area of single conjugated glucoside peak at point of storage}]} \times 100$$

The results (Table 4.4) indicated that the conjugates were stable over the time course of analysis and remained unaffected by temperature. As there was no significant degradation or change in peak area the this enabled multiple analysis of DNT treated plants.

Table 4.4: The % recoveries of the five randomly chosen most intense glucosyl conjugates (MRM scan) as extracted from 20 mg freeze dried plant tissues treated with 2,6-DNT (15 mg /L of liquid MS media). The data is mean from samples (n = 3) kept at room temperature and -20°C. The comparison was made for data at t = 0 and t = 48 h.

DNT based conjugates	% recovery at room temperature (Mean value)	% recovery at -20°C (Mean value)
----------------------	---	----------------------------------

DHAT-O-MG-N-OP (1)	99.82 $\pm$ 1.02	98.87 $\pm$ 0.97
HANT-C-MG (4)	98.88 $\pm$ 0.98	99.02 $\pm$ 0.82
HANT-O-MG (5)	98.85 $\pm$ 0.79	98.78 $\pm$ 0.93
HANT-C-MG-O-M (7)	99.13 $\pm$ 0.78	99.33 $\pm$ 1.16
ANT-C-MG (9)	99.22 $\pm$ 1.02	99.05 $\pm$ 0.99

4.3.1.4 Reproducibility of the extraction technique:

To analyse the reproducibility of the extraction method 20 mg of freeze dried 2,6-DNT (15 mg /L of liquid MS media) was added to 2 ml Eppendorf tubes. Six replicates were prepared and divided into two equal sets. One set (20 mg dried tissue samples in triplicate) received 5 μ l of 2 mg /ml of 4-nitrophenyl β -D-glucopyranoside as an internal standard. The samples were prepared in triplicate. The samples were extracted in 10% methanol as described previously (section 4.2.3.1.2) and analysed on LC-MS.

The results (Table 4.5) from the analysis indicated that this method gave highly reliable and reproducible quantitation of DNT based glucosides. The amount of different glucosides as obtained through two different methods in all the samples was similar. The reproducibility of the extraction procedure can also be estimated through standard errors of mean (less than 7% of mean of replicates).

Table 4.5: The peak areas of the five randomly chosen most intense glucosyl conjugates (MRM scan) as extracted from 20 mg freeze dried plant tissues treated with 2,6-DNT (15 mg /L of liquid MS media). The data is the mean from samples (n = 3).

DNT based conjugates	Corrected peak areas from		Uncorrected peak areas from	
	samples with internal standard		samples without internal standard	
	Mean	Standard error	Mean	Standard error

DHAT-O-MG-N-OP (1)	1.7e5	2.2e3	1.71e5	2.7e3
HANT-C-MG (4)	7.04e3	1.7e2	6.9e3	2.1e2
HANT-O-MG (5)	7.27e4	7.7e2	7.3e4	8.3e2
HANT-C-MG-O-M (7)	1.26e4	4.2e2	1.2e4	3.9e2
ANT-C-MG (9)	4.9e3	2.6e2	5.1e3	2.7e2

4.3.1.4 Reliability of measurements:

To analyse any possible interference and increase the reliability of data 20 mg of freeze dried 2,6-DNT (15 mg /L of liquid MS media) was added to 2 ml Eppendorf tubes and extracted in 10% methanol as described earlier. The procedure was repeated in triplicate. In another 2 ml Eppendorf tube, 900 µl of 2,6-DNT (35 mg /L of liquid MS media) treated liquid MS media (in which the *A. thaliana* were grown) was taken and 100 µl of methanol was added. The sample was vortexed for 1 min and centrifuged in Eppendorf centrifuge 5415D (13.2×1000 rpm) for 7 min. The supernatant was decanted and stored in 2 ml HP GC vials. The procedure was repeated in triplicate. Both the tissue samples and extracted media were analysed by LC-MS.

The results (Figure 4.6) from the analysis indicated that the conjugates are not excreted from the plants to the media. This shows that the conjugates are confined and compartmentalised within the plants.

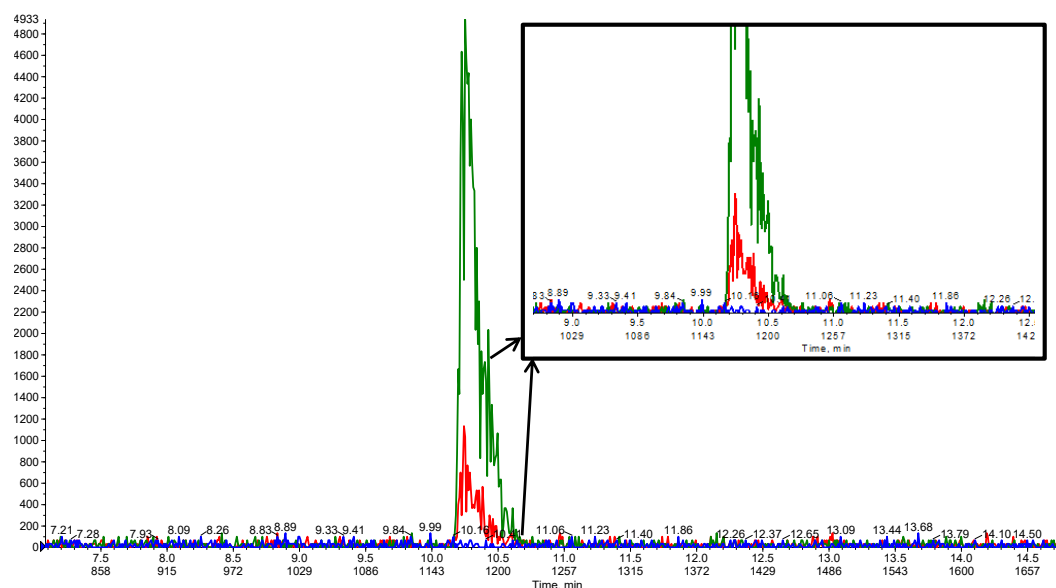


Figure 4.6: MRM scan of media for DHAT-*O*-MG (1) (Table 4.3). The blue colour line is the media extract compared to red (5 mg /L of liquid MS media 2,6-DNT) and green (35 mg /L of liquid MS media 2,6-DNT) treatments. Plant based peaks were not detected in the media.

4.3.2 Quantification and analysis of DNT based conjugates in *A. thaliana*:

The quantification of all DNT based conjugates was done relative to 4-nitrophenyl β -D-glucopyranoside used as an internal standard. This method was adopted as an alternative to isolating each conjugate as this would require extensive purification and characterisation of low abundance compounds which was beyond the scope of this project. 4-Nitrophenyl β -D-glucopyranoside has a similar structure to the glucosylated conjugates and was thought to be a practical solution to the issue of quantification. However, using this compound as an internal standard does have its limitations in so far as it's assumed that the response factors are the same for all conjugates. Thus the quantification of DNT conjugates is relative to this standard.

4.3.2.1 Analysis of conjugates in 2,6-DNT treated *A. thaliana*:

The MRM scan (Figure 4.7) of 2,6-DNT treated *A. thaliana* indicated peaks of glucosylated conjugates. The results indicated that both HANT and ANT were glucosylated. The glucosylation activity was observed to be more with HANTs compared to ANT. The peak with the greatest area was at 11.5 min producing a fragmentation ion of m/z 371[M-H]⁻ that corresponds to a molecule (chemical formula C₁₆H₂₃N₂O₈⁻ anion) predicted to be DHAT with an **O-MG** at one terminal and a propanone on the other (DHAT-O-MG-N-OP (1)). Further fragmentation yielded a major product ion at m/z 151 [M-H]⁻ (Table 4.3); The fragmentation pattern for DHAT-O-MG-N-OP (1) corresponding to DHAT (C₇H₇N₂O₂⁻ anion) with other fragment ions of m/z value of 163 corresponding to glucose (C₆H₁₁O₅⁻ anion) and m/z value of 57 corresponding to propanone (C₃H₅O⁻ anion).

The second most intense peak obtained from mass spectral scan of 2,6-DNT treated *A. thaliana* was obtained at 8.2 min which fragmented to m/z 371[M-H]⁻ as targeted through a product ion scan. This peak further fragmented to diagnostic fragments of m/z of 195 (chemical formula C₉H₇N₂O₃⁻ anion) and neutral losses of m/z 120 predicted to be sequential loss of glucose. This indicated the parent molecule to be DHAT-C-MG-N-OP (2). The third most intense peak was with m/z of 329[M-H]⁻ (chemical formula C₁₃H₁₇N₂O₈⁻) fragmented to m/z of 209 [M-H]⁻ (chemical formula C₉H₉N₂O₄⁻ anion) with neutral loss of m/z 120 predicted to be hydroxyamino nitrotoluene conjugated to a single glucose molecule (HANT-C-MG (4)). The third fourth most intense peak was of ANT-C-MG (9) which fragments into m/z 193 as major peak. The next peaks to follow sequentially was of ANT-N-MG (8) (m/z 313) and then HANT-C-MG-N-OB (3) (m/z 413) (Figure 4.7).

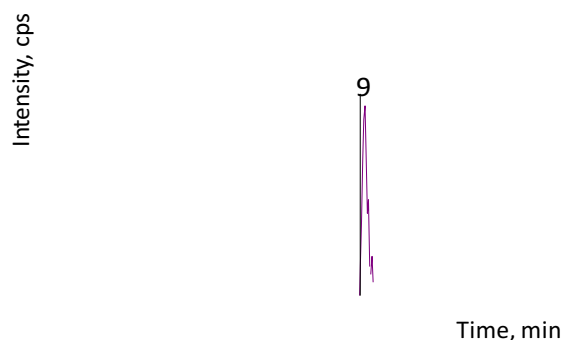
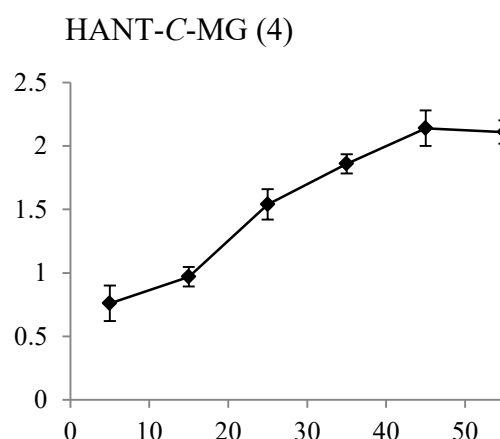
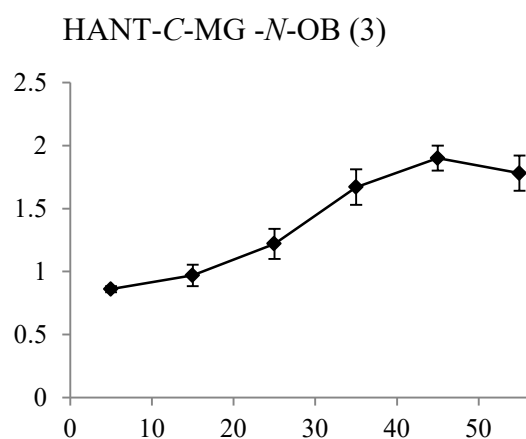
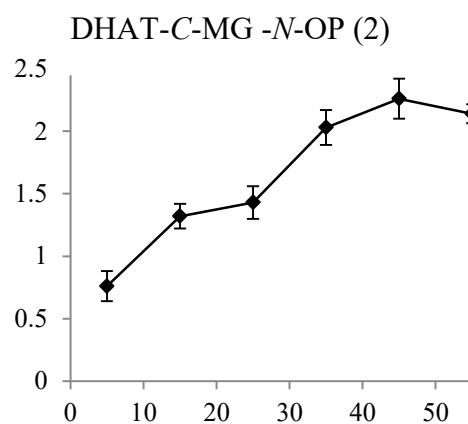
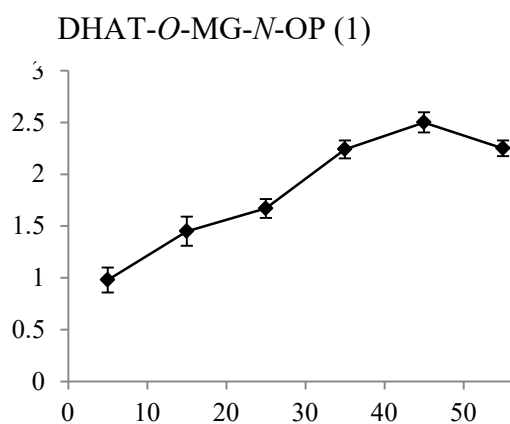


Figure 4.7: MRM scan (one of three technical replicates) of 2,6-DNT related conjugates from *A. thaliana* treated with 5 mg /L of liquid MS media (14 days post cultivation), showing only the most intense peaks. (1) DHAT-*O*-MG-*N*-OP; (2) DHAT-*C*-MG-*N*-OP; (3) HANT-*C*-MG-*N*-OB (4) HANT-*C*-MG; (5) HANT-*O*-MG; (6) HANT-*O*-MG-*O*-M; (7) HANT-*C*-MG-*O*-M; (8) ANT-*N*-MG; (9) ANT-*C*-MG; (10) ANT-*C* & *N*- DG.

When the relative concentrations (Figure 4.8) of different glucosides obtained through the analysis was calculated, it was observed that the highest concentration was for DHAT-*O*-MG-*N*-OP (1) and this fragmented to **DHAT**. The lowest concentration was of HANT-*O*-MG-*O*-M (6). Overall the intensity of glucosylation decreased from *DHAT-O-MG-N-OP* (1) (*highest*) > *DHAT-C-MG-N-OP* (2) > *HANT-C-MG* (4) > *ANT-C-MG* (9) > *ANT-N-MG* (8) > *HANT-C-MG-N-OB* (3) > *HANT-O-MG* (5) > *ANT-C & N- DG* (10) > *HANT-C-MG-O-M* (7) > *HANT-O-MG-O-M* (6) (*lowest*). The data revealed that the relative concentration of DHAT-*O*-MG-*N*-OP (1), being the highest obtained glucoside, ranged from **0.98 (at 2,6-DNT concentration of 5 mg/L of liquid MS media) to 2.5 (at 2,6-DNT concentration of 35 mg/L of liquid MS media) $\mu\text{mol} / \text{g}$ dry tissue weight of *A. thaliana* treated with 2,6-DNT**. This concentration then dropped to **2.25 $\mu\text{mol} / \text{g}$ dry tissue weight of *A. thaliana* at**

2,6-DNT concentration of 55 mg /L of liquid MS media. The relative concentration of HANT-*O*-MG-*O*-M (6), being the lowest available monoglucoside, ranged from **0.56 to 1.21 μmol /g dry tissue weight of *A. thaliana*** at 2,6-DNT concentration of 5 mg /L of liquid MS to 45 mg /L of liquid MS media, this value then dropped to **0.89 μmol /g dry tissue weight of *A. thaliana*** against 55 mg /L of liquid MS media.

Relative concentration of 2,6-DNT conjugates (μmol) /g dry wt of *A. thaliana* tissues



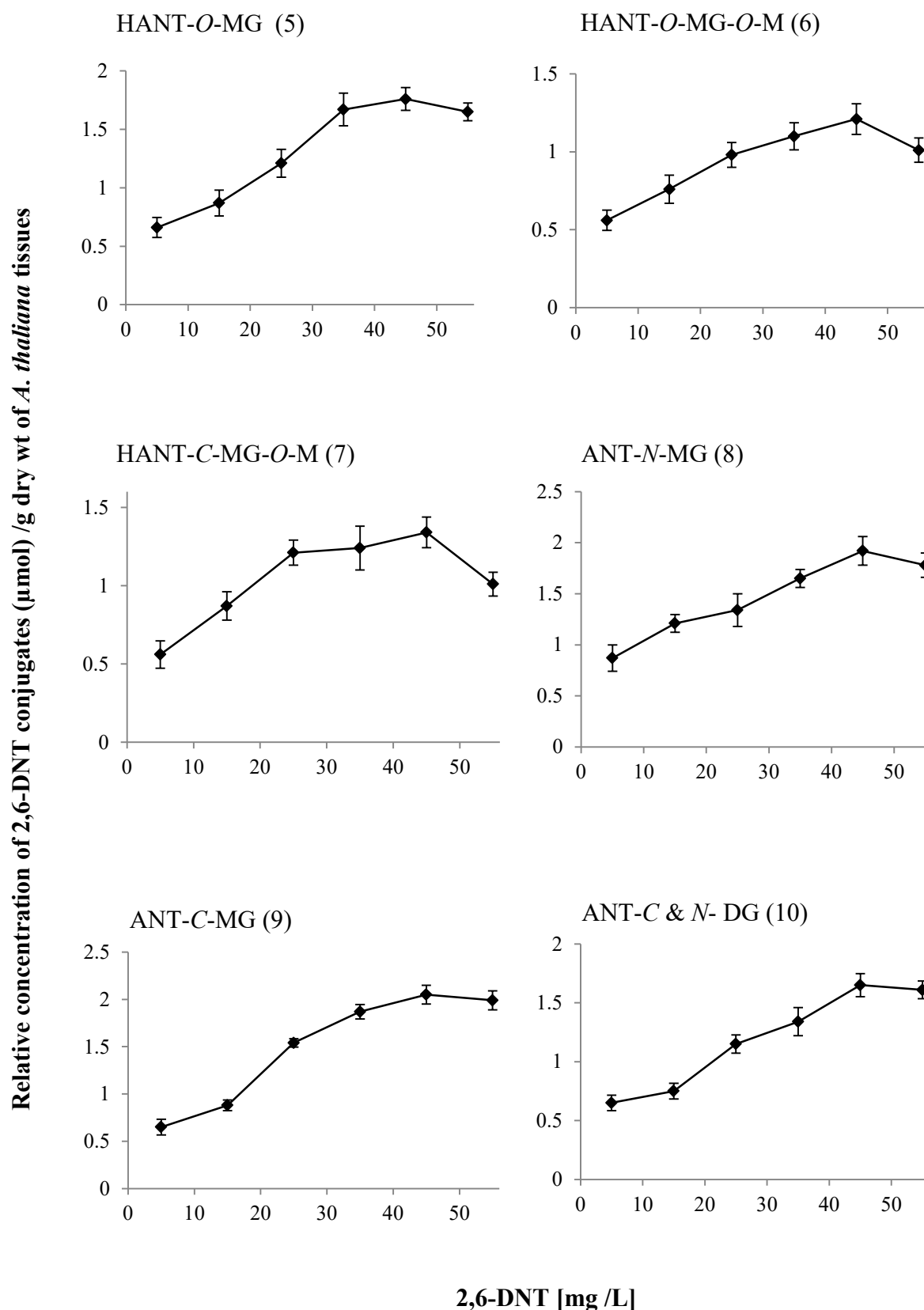


Figure 4.8: Conjugates of reduced 2,6-DNT metabolites recovered from an homogenous mixture of ten 14 day (post cultivation) old *A. thaliana* plant grown in different concentrations of 2,6-DNT in sterile liquid MS media and analysed by LC-MS/MS

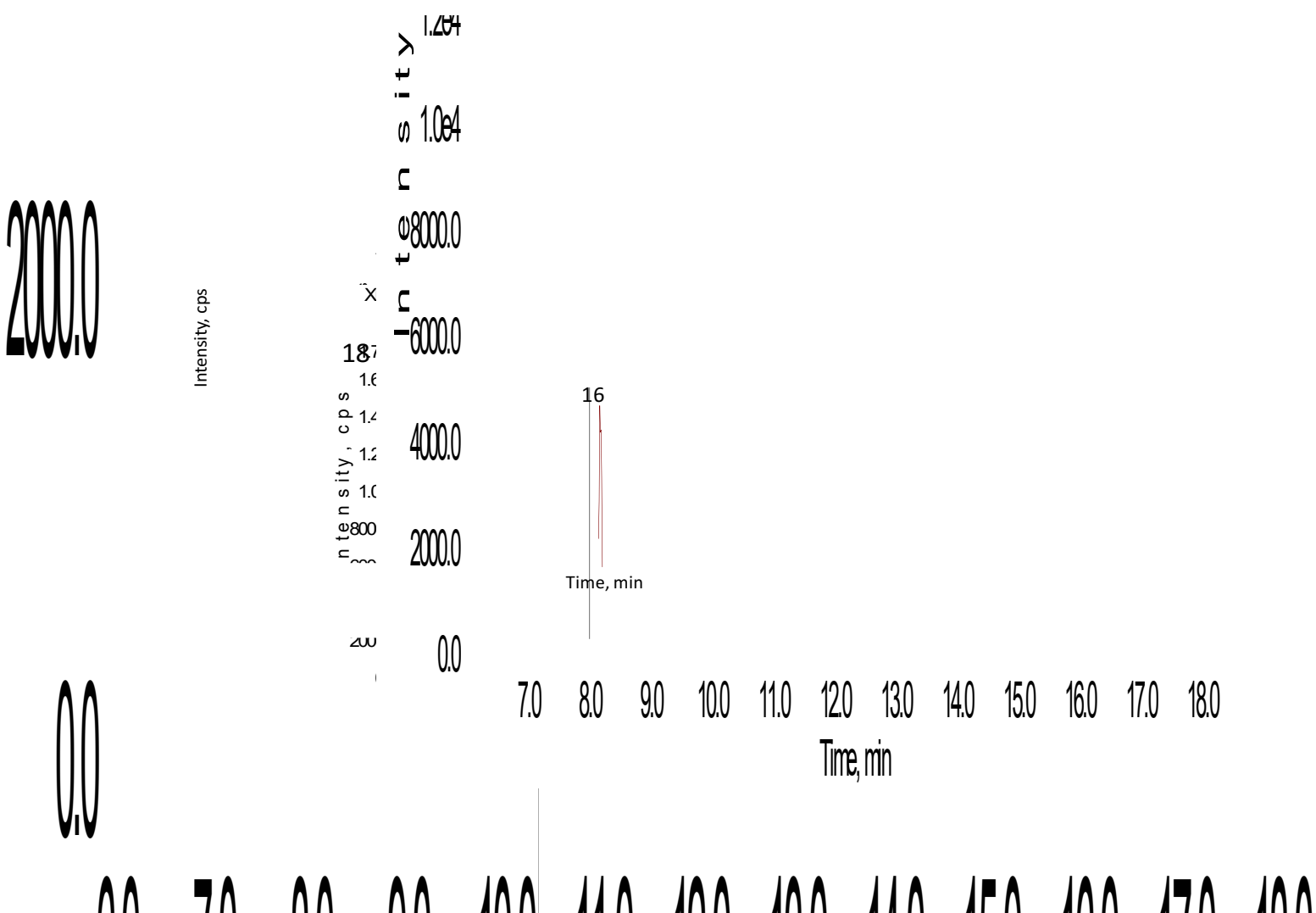
(growth temperature was $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period; constant speed 130 rpm). Data was plotted as mean of four replicates (ten plants from one container were freeze dried and homogenized to give one replicate).

For rest of the isomers the deuterated DNTs were not available, therefore the conjugates were predicted on the basis of deuterated d_3 -2,6DNT data. Further confirmation was done by matching the EPI fragmentation pattern (in the Appendix). It was found that the fragmentation pattern for all the isomers and deuterated d_3 -2,6DNT glucosylated conjugates were exactly same. The retention times were also similar.

4.3.2.2 Analysis of conjugates in 2,3-DNT treated *A. thaliana*:

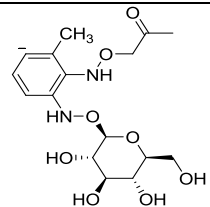
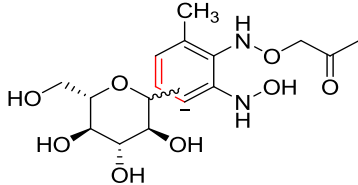
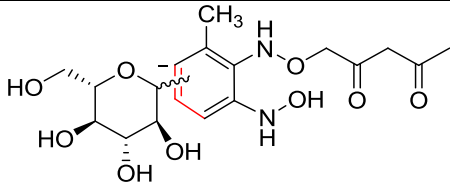
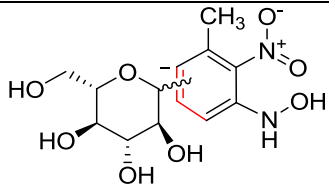
The MRM scan (Figure 4.9) as obtained from the mass spectrometric analysis of 2,3-DNT treated *A. thaliana* is slightly different from that of the 2,6-DNT treated *A. thaliana* tissue analysis. The mass spectra of all conjugates for convenience are listed in the appendix. The highest magnitude peak was of DHAT-C-MG-N-OP (12) with a molecular mass m/z of **371** that further fragmented to a major peak m/z of **195** $[\text{M-H}]^-$ anion (chemical formula $\text{C}_9\text{H}_7\text{N}_2\text{O}_3^-$ anion) and neutral losses. The second most prominent peak obtained from MRM scan and further targeted through product ion scan was of HANT-C-MG-O-M (17) with the nominal molecular mass of m/z **415** $[\text{M-H}]^-$ anion. Further fragmentation yielded the intense product ion of m/z value of **151** $[\text{M-H}]^-$ anion (chemical formula $\text{C}_7\text{H}_7\text{N}_2\text{O}_2^-$ anion) (Table 4.4) The fragmentation pattern for HANT-C-MG-O-M (17) is predicted to be (hydroxyamino toluene) and diagnostic ions with m/z value of **163** (glucose molecule) and other ions with m/z value of **101** (malonyl). The third most intense peak corresponded to a m/z value of **329** $[\text{M-H}]^-$ anion (HANT-O-MG (15)). This precursor ion fragmented to a m/z of **151** $[\text{M-H}]^-$ anion predicting it to be an ANT molecule conjugated to glucose (*N*- monoglucosides). Another prominent peak with m/z value of **313** $[\text{M-H}]^-$ anion fragmented into m/z of **193** $[\text{M-H}]^-$ anion predicted to be ANT molecule and compound refers to *C*-monoglucoside

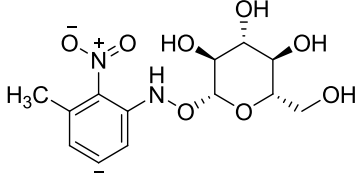
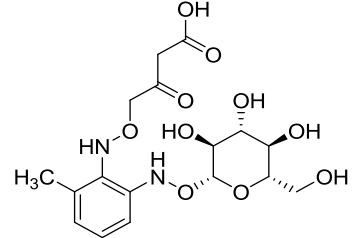
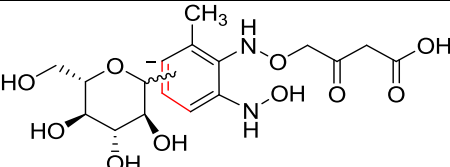
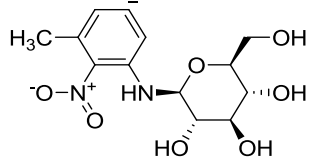
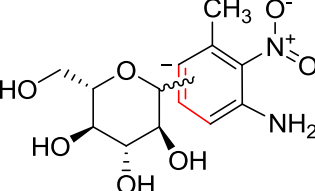
(ANT-C-MG (19). The rest of the peaks fragmented in the similar way as glucosides extracted from 2,6-DNT treated *A. thaliana*.



The peaks were then assigned the names (Table 4.6) on the basis of their fragmentation pattern.

Table 4.6: Glucosyl conjugates derived from 2,3-DNT metabolites with their retention times.

Compound Number	Semi-systematic name	Retention time (min)	Structure [M-H] ⁻	2,3-DNT based precursor /product ions (m/z) [M-H] ⁻
11	Aminohydroxynitro-toluene -O-monoglucoside-N-oxopropanone (DHAT-O-MG-N-OP)	11.5		371/151
12	Aminohydroxynitro-toluene -C-monoglucoside-N-oxopropanone (DHAT-C-MG-N-OP)	8.4		371/195
13	Aminohydroxynitro toluene -C-monoglucoside-N-oxobutanoate (HANT-C-MG-N-OB)	12.6		413/151
14	Aminohydroxy nitrotoluene -C-monoglucoside (HANT-C-MG)	14.06		329/209

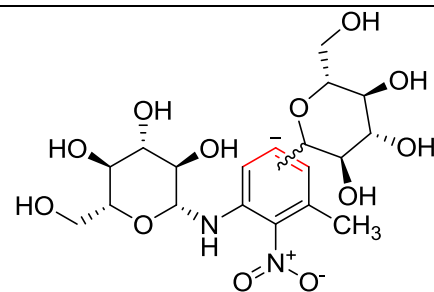
15	Aminohydroxy nitro-toluene – <i>O</i> -monoglucoside (HANT- <i>O</i> -MG)	10.49		329/151
16	Aminohydroxy nitro-toluene – <i>O</i> -monoglucoside- <i>O</i> -malonyl (HANT- <i>O</i> -MG- <i>O</i> -M)	11.2		415/195
17	Aminohydroxy nitrotoluene – <i>C</i> -monoglucoside- <i>O</i> -malonyl (HANT- <i>C</i> -MG- <i>O</i> -M)	10.37		415/151
18	Aminonitro-toluene- <i>N</i> -monoglucoside (ANT- <i>N</i> -MG)	7.18		313/151
19	Aminonitro-toluene- <i>C</i> -monoglucoside (ANT- <i>C</i> -MG)	10.26		313/193

20

Aminonitro-toluene-*C* &
N- diglucoside (ANT-*C* &
N- DG)

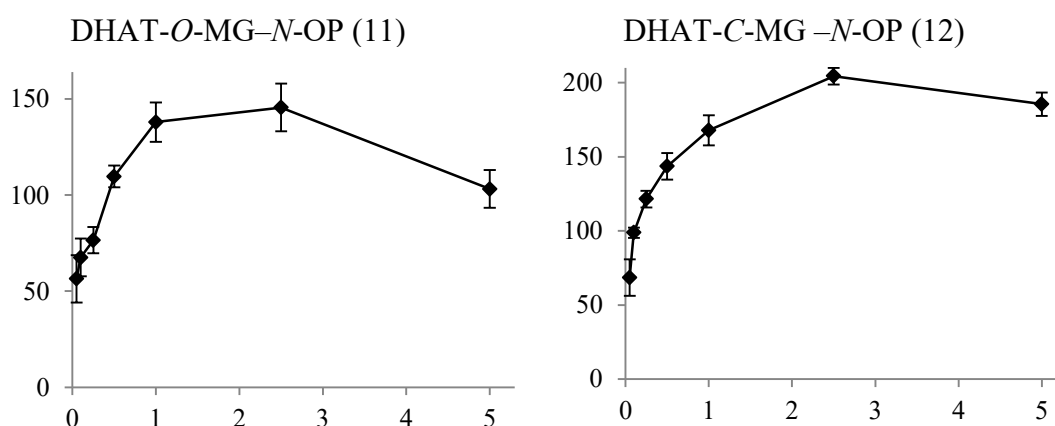
11.25

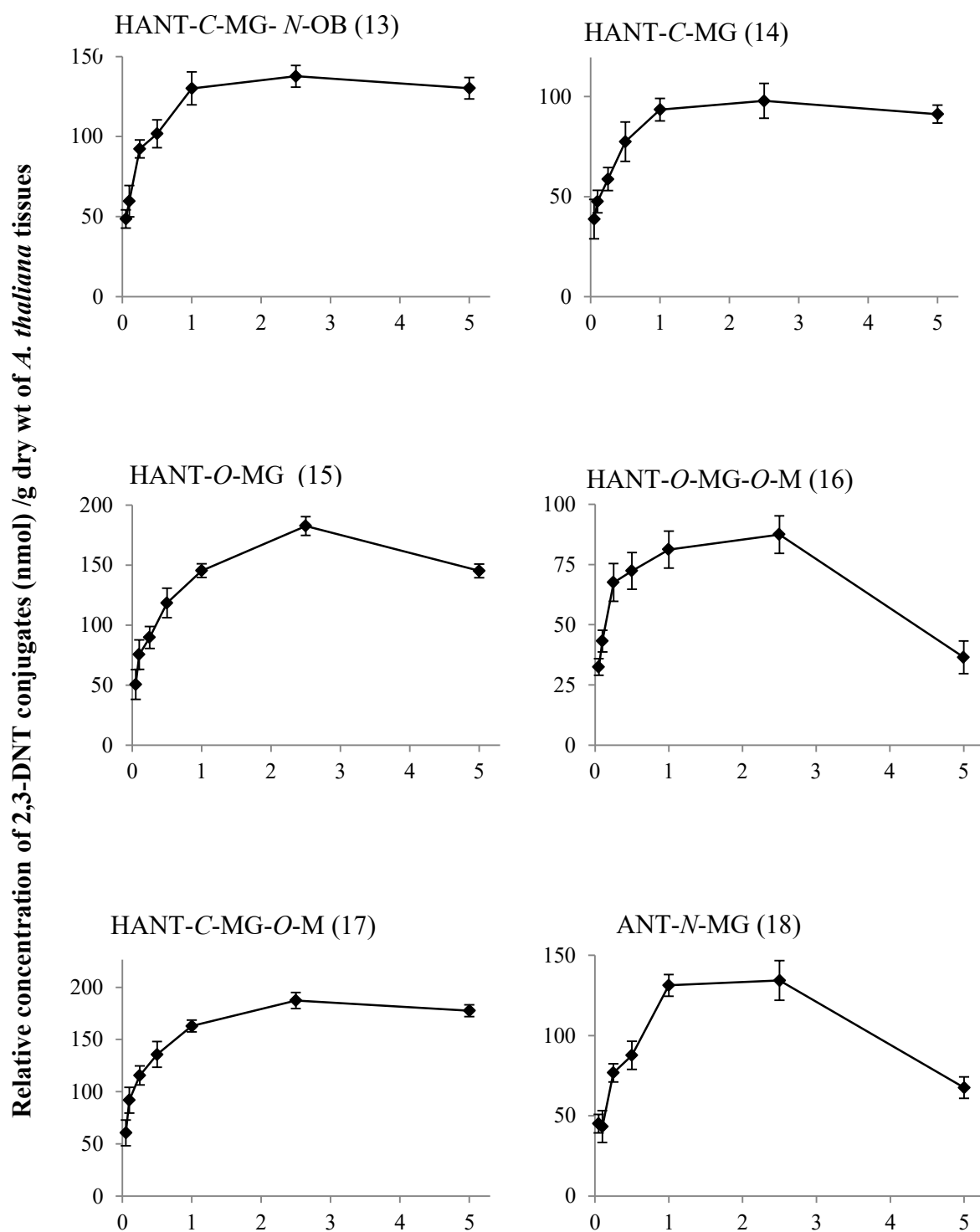
475/149



*the red colour highlighted bonds indicate possible conjugation sites

The highest relative concentration (Figure 4.10) for all conjugates were observed at 2,3-DNT concentration of **2.5 mg /L of liquid MS media** with the exception of **ANT-C & N- DG (20)** and **HANT-O-MG-O-M (16)** having the highest concentrations at **1 mg /L of liquid MS media** (Figure 4.5). The relative concentration of glucosides decreased from *DHAT-C-MG-N-OP (12)* (highest) > *HANT-C-MG-O-M (17)* > *HANT-O-MG (15)* > *ANT-C-MG (19)* > *DHAT-O-MG-N-OP (11)* > *HANT-C-MG-N-OB (13)* > *ANT-N-MG (18)* > *ANT-C & N- DG (20)* > *HANT-O-MG (14)* > *HANT-O-MG-O-M (16)* (lowest). The highest amount of conjugates were observed for hydroxylamino derivatives compared to aminonitrotluenes. The relative concentration of *DHAT-C-MG-N-OP (12)*, being the highest, ranged from **68.45** (at 2,3-DNT concentration of 0.05 mg /L of liquid MS media) to **204.34** (at 2,3-DNT concentration of 2.5 mg/L of liquid MS media) **nmol /g dry tissue weight of *A. thaliana* treated with 2,3-DNT**, which decreased to **185.46 nmol /g dry tissue weight of *A. thaliana***. The relative concentration of *HANT-O-MG-O-M (16)*, being the lowest, ranged from **32.45** (at 2,3-DNT concentration of 0.05 mg /L of liquid MS media) to **87.43** (at 2,3-DNT concentration of 2.5 mg/L of liquid MS media) **nmol /g dry tissue weight of *A. thaliana* treated with 2,3-DNT**, which decreased to **6.45 nmol /g dry tissue weight of *A. thaliana***.





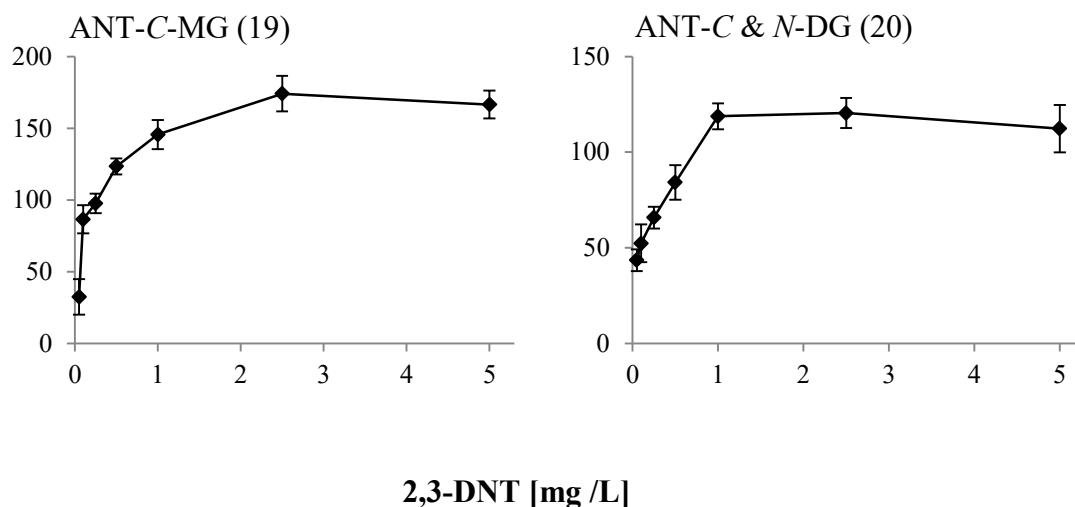


Figure 4.10: Conjugates of reduced 2,3-DNT metabolites recovered from homogenous mixture of ten 14 day (post cultivation) old *A. thaliana* plant grown in different concentrations of 2,3-DNT in sterile liquid MS media and analysed by LC-MS/MS (growth temperature was $20\pm 3^{\circ}\text{C}$ for 16:8 h day:night period; constant speed 130 rpm). Data was plotted as mean of four replicates (ten plants from one container were freeze dried and homogenized to give one replicate).

4.3.2.3 Analysis of conjugates in 2,4-DNT treated *A. thaliana*:

The intense peaks eluted at **10.5 min** (Figure 4.11) had the precursor ion of m/z **329** $[\text{M-H}]^{-}$ anion fragmented to give a main fragment of m/z **151** $[\text{M-H}]^{-}$ (chemical formula $\text{C}_7\text{H}_7\text{N}_2\text{O}_2^{-}$ anion) and m/z **163** $[\text{M-H}]^{-}$ that corresponds to glucose cleaved from HANT molecule. This indicates the presence of *O*-mono-glucoside conjugated to **HANT** (HANT-*O*-MG (25)). At a retention time of **10.36 min** had the main precursor ion m/z **415** $[\text{M-H}]^{-}$ anion (HANT-*C*-MG-*O*-M (27)) with product ion fragmentation gave intense peak with mass m/z of **151** $[\text{M-H}]^{-}$ anion (chemical formula $\text{C}_7\text{H}_7\text{N}_2\text{O}_2^{-}$ anion) (HANT) with the neutral loss of m/z **120**. Another prominent peak was with m/z value of **313** fragmented to m/z value of **151**. This refers to ANT conjugated to monoglucoside at N- terminal (aminonitrotoluene conjugated to glucose) (ANT-*N*-MG (28)). The peaks with molecular mass of m/z of **329** fragmented to m/z

of 209 [M-H]⁻ anion (chemical formula C₉H₁₀N₂O₄⁻ anion) referring to HANT molecule conjugated to glucose (HANT-C-MG (24)) was also observed.

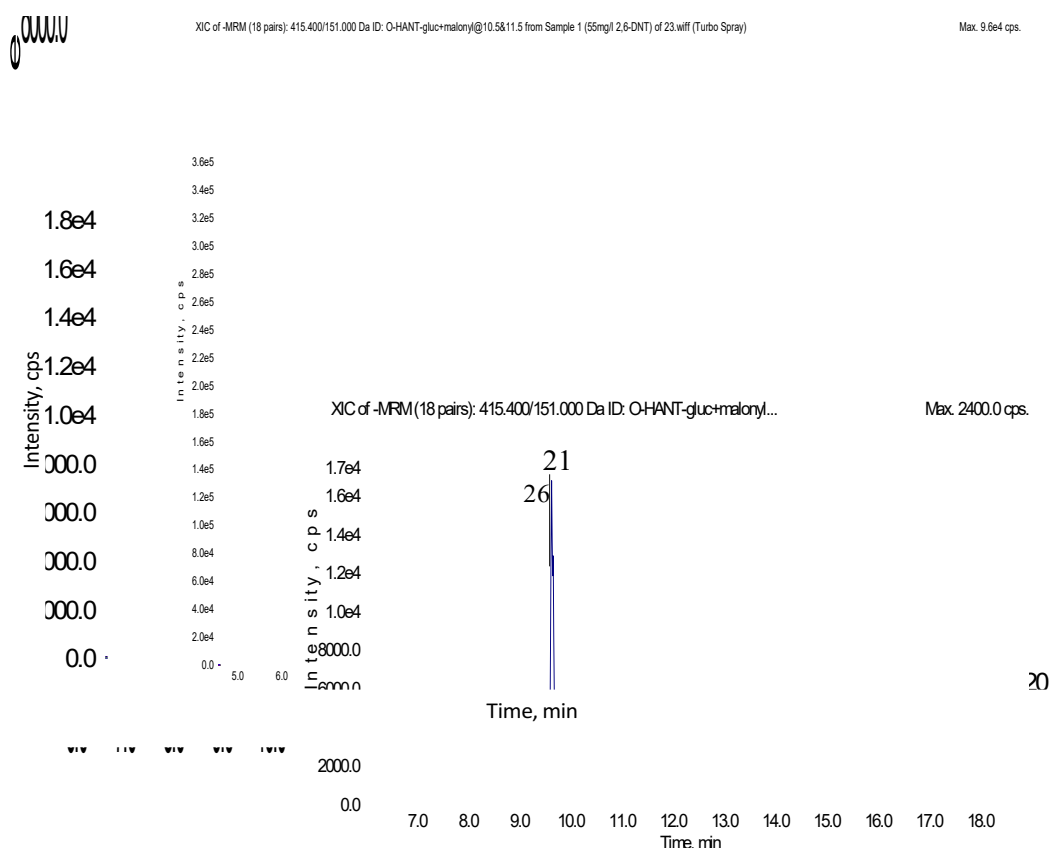
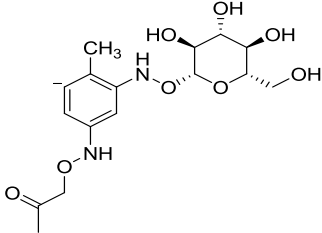
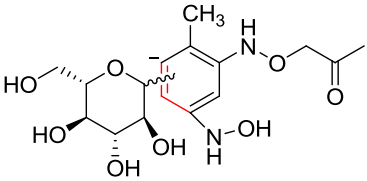
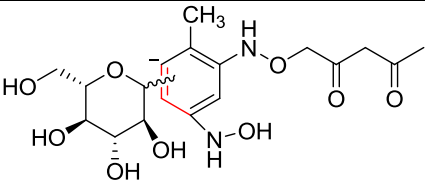
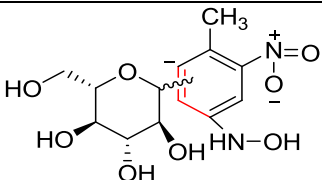
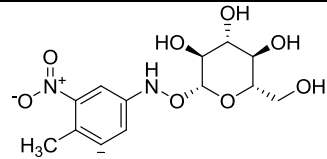
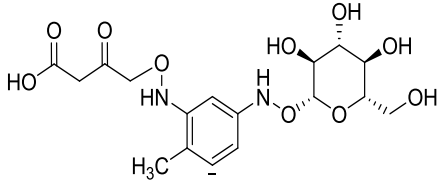
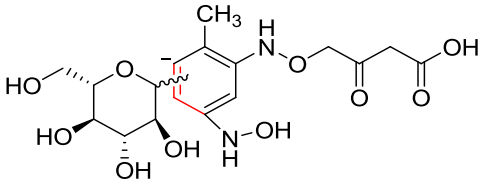
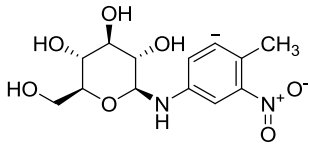
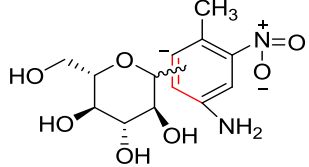


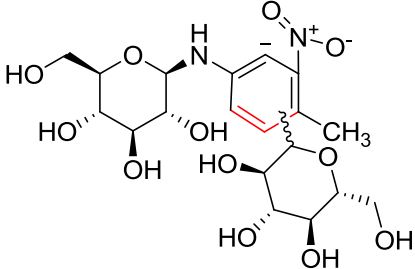
Figure 4.11: MRM scan (one of three technical replicates) of 2,4-DNT related conjugates from *A. thaliana* treated with 0.5 mg /L of liquid MS media (14 days post cultivation) and cultivated under growth temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period. The analysis was carried out on 20 mg of freeze dried tissue. The retention times are 21=11.5, 22= 8.4, 23=12.6, 24=14.22, 25=10.5, 26=11.2, 27=10.37, 28=7.19, 29=10.16, 30= 11.6.

The peaks were the assigned names (Table 4.7) on the basis of their fragmentation pattern.

Table 4.7: Glucosyl conjugates derived from 2,4-DNT metabolites with their retention times.

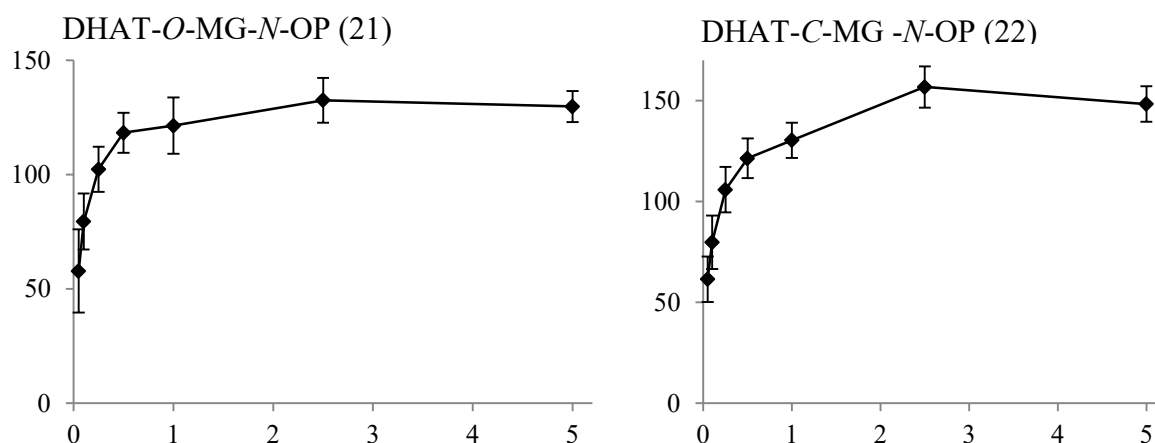
Compound Number	Semi-systematic name*	Retention time (min)	Structure [M-H] ⁻	2,4-DNT based precursor /product ions (<i>m/z</i>) [M-H] ⁻
21	Aminohydroxynitro-toluene –O-monoglucoside- <i>N</i> -oxopropanone (DHAT-O-MG- <i>N</i> -OP)	11.5		371/151
22	Aminohydroxynitro-toluene –C-monoglucoside- <i>N</i> -oxopropanone (DHAT-C-MG- <i>N</i> -OP)	8.4		371/195
23	Aminohydroxynitro toluene –C-monoglucoside- <i>N</i> -oxobutanoate (HANT-C-MG- <i>N</i> -OB)	12.6		413/151
24	Aminohydroxy nitrotoluene –C-monoglucoside (HANT-C-MG)	14.2		329/209

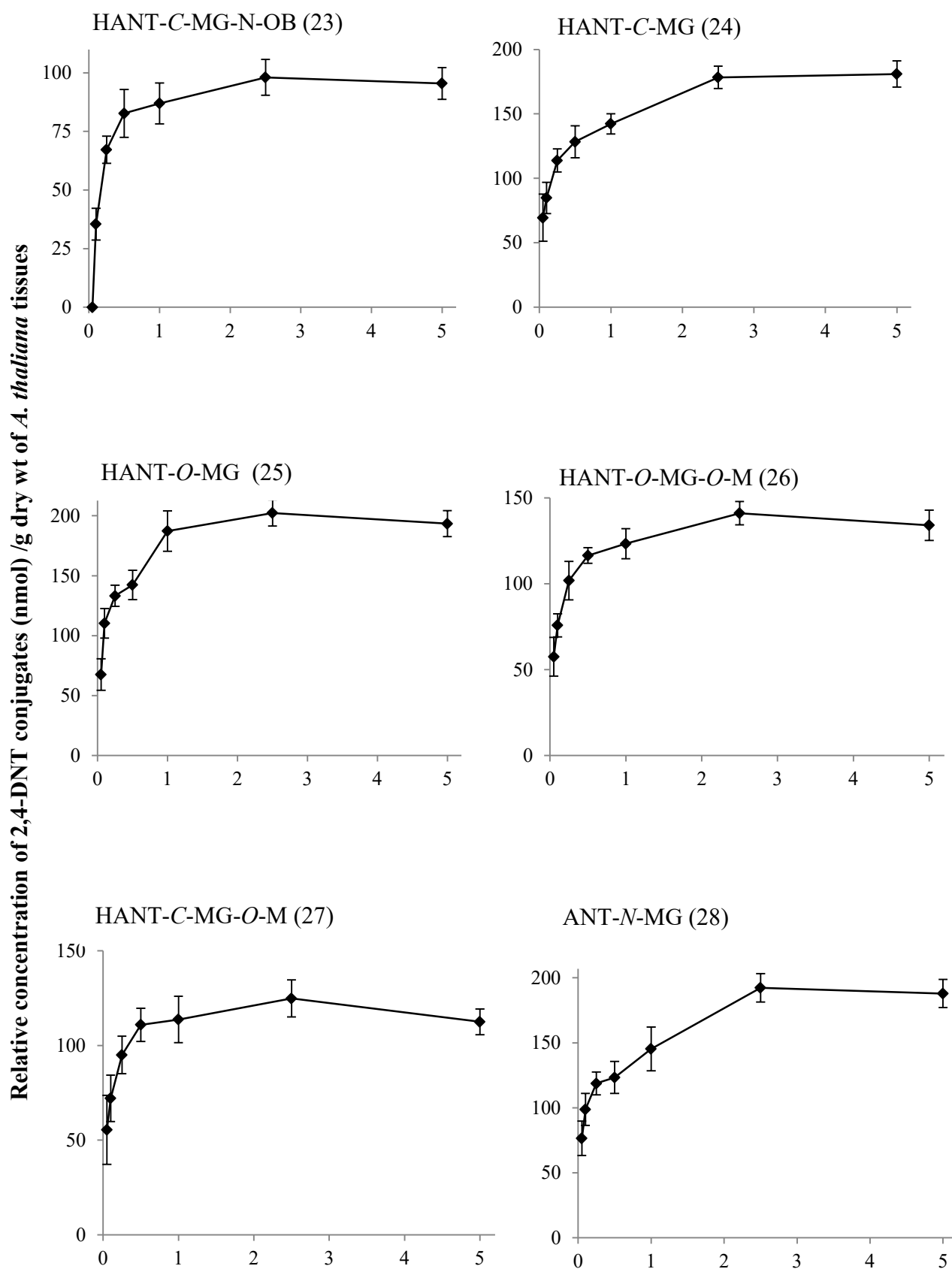
25	Aminohydroxy nitro-toluene –O-monoglucoside (HANT-O-MG)	10.5		329/151
26	Aminohydroxy nitro-toluene –O-monoglucoside-O-malonyl (HANT-O-MG-O-M)	11.19		415/195
27	Aminohydroxy nitrotoluene –C-monoglucoside-O-malonyl (HANT-C-MG-O-M)	10.37		415/151
28	Aminonitro-toluene- N-monoglucoside (ANT-N-MG)	7.19		313/151
29	Aminonitro-toluene- C-monoglucoside (ANT-C-MG)	10.16		313/193

30	Aminonitro-toluene- <i>C</i> & <i>N</i> - diglucoside (ANT- <i>C</i> & <i>N</i> - DG)	11.6		475/149
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*the red colour indicates the possible site for conjugation.

The relative concentrations (Figure 4.12) of all conjugates was observed at 2,4-DNT concentration of **2.5 mg /L of liquid MS media** which decreased at **5 mg /L of liquid MS media** and the least values were obtained at **0.05mg /L of liquid MS media**. The relative glycoside contents decreased from *HANT-O-MG* (25) (*highest*) > *ANT-N-MG* (28) > *HANT-C-MG* (24) > *DHAT-O-MG-N-OP* (22) > *HANT-O-MG-O-M* (26)> *ANT-C-MG* (29) > *DHAT-O-MG-N-OP*(21) > *HANT-C-MG-N-OB* (27) > *ANT-C & N- DG* (30) > *HANT-C-MG-N-OB* (23) (*lowest*). The data showed that the relative concentration of *HANT-O-MG* (25), being the highest, was **67.54 μmol /g dry tissue weight of *A. thaliana* at 2,4-DNT concentration of 0.05 mg /L of liquid MS media** and reached **202.34 nmol /g dry tissue weight of *A. thaliana* at 2,4-DNT concentration of 2.5 mg /L of liquid MS media** treated with 2,3-DNT. These then decreased to **198.42 nmol /g dry tissue weight of *A. thaliana* at 5 mg /L of liquid MS media**. For the lowest obtained glycoside (*HANT-C-MG-N-OB* (23)), no conjugates recovered from 2,4-DNT concentration of 0.05 mg /L of Phytigel media and a relative concentration of **35.46 nmol /g dry tissue weight of *A. thaliana*** was obtained at 2,4-DNT concentration of 0.1 mg /L of liquid MS media after attaining a maximum concentration of **98.07 nmol /g dry tissue weight of *A. thaliana* at 2,4-DNT concentration of 2.5 mg /L of liquid MS media**.





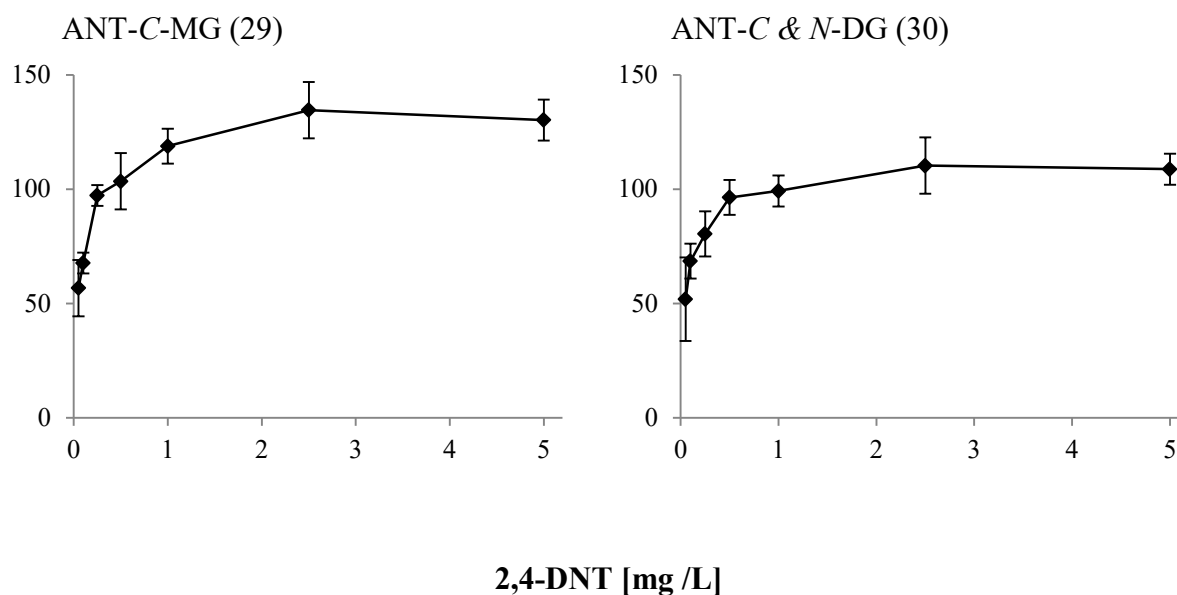


Figure 4.12: Conjugates of reduced 2,4-DNT metabolites recovered from homogenous mixture of ten 14 day (post cultivation) old *A. thaliana* plant grown in different concentrations of 2,4-DNT in sterile liquid MS media and analysed by LC-MS/MS (growth temperature was $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period; constant speed 130 rpm). Data was plotted as mean of four replicates (ten plants from one container were freeze dried and homogenized to give one replicate).

4.3.2.4 Analysis of conjugates in 3,4-DNT treated *A. thaliana*:

The mass spectrometric (Figure 4.13) analysis of 3,4-DNT treated *A. thaliana* showed the intense peak of glycosylated compound HANT-C-MG (34) with the nominal molecular mass of m/z 329 $[\text{M-H}]^{-}$. Further fragmentation yielded a highest ion of m/z value of 209 $[\text{M-H}]^{-}$ anion referring to 3-methyl-4-nitro-1-hydroxypyrrolo-2,3-benzene (chemical formula $\text{C}_9\text{H}_{10}\text{N}_2\text{O}_4^{-}$ anion) (Fragmentation pattern for HANT-C-MG (4)). Another prominent peak scanned through MRM and then further analysed by EPI scan was of ANT-C & N- DG (40) which gave a fragment m/z value of 475 $[\text{M-H}]^{-}$ further fragmented to m/z value of 149 $[\text{M-H}]^{-}$ (chemical formula $\text{C}_7\text{H}_5\text{N}_2\text{O}_2^{-}$) which refers to 3-methyl-4-nitro-1H-pyrrolo-2,3-benzene rearranged after fragmentation of *N*-monoglucoside (aminonitrotoluene conjugated to

glucose). Third most intense peak was of ANT-*N*-MG (38) with molecular mass of m/z 313 $[M-H]^-$ anioin fragmented to 151 $[M-H]^-$ anion.

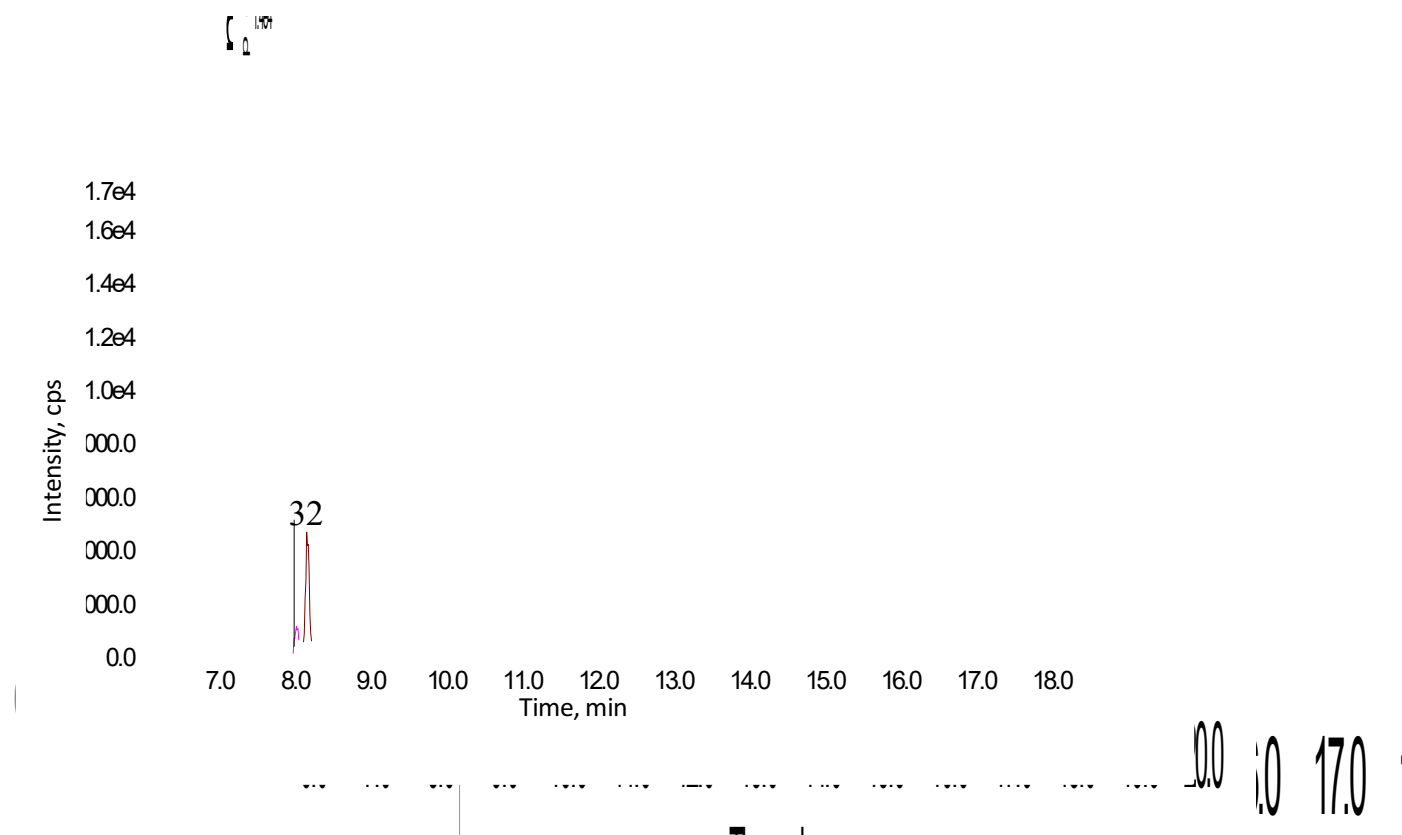
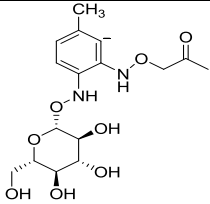
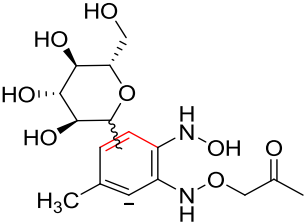
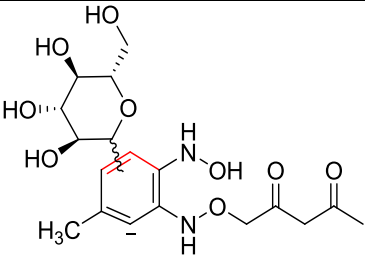
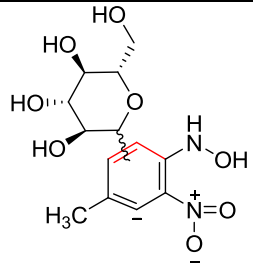
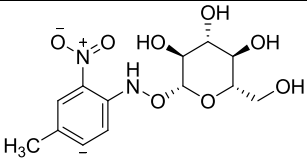
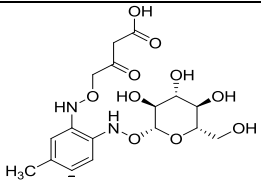
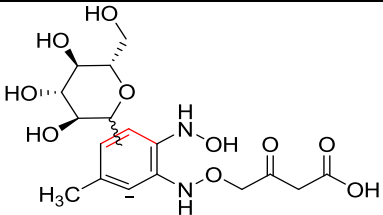
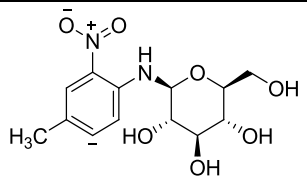


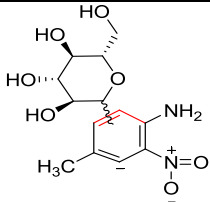
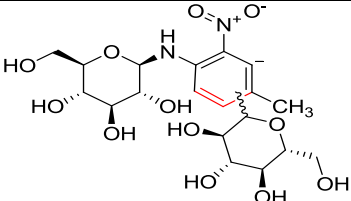
Figure 4.13: MRM scan (one of three technical replicates) of 3,4-DNT related conjugates from *A. thaliana* treated with 0.5 mg /L of liquid MS media (14 days post cultivation) and cultivated under growth temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period. The analysis was carried out on 20 mg of freeze dried tissue. The retention times are 31=11.5, 32= 8.2, 33=12.55, 34=14.11, 35=10.45, 36=11.15, 37=10.37, 38=7.19, 39=10.2, 40= 11.4.

The peaks were the assigned names (Table 4.8) after matching and contrasting their fragmentation patterns.

Table 4.8: Glucosyl conjugates derived from 3,4-DNT metabolites with their retention times.

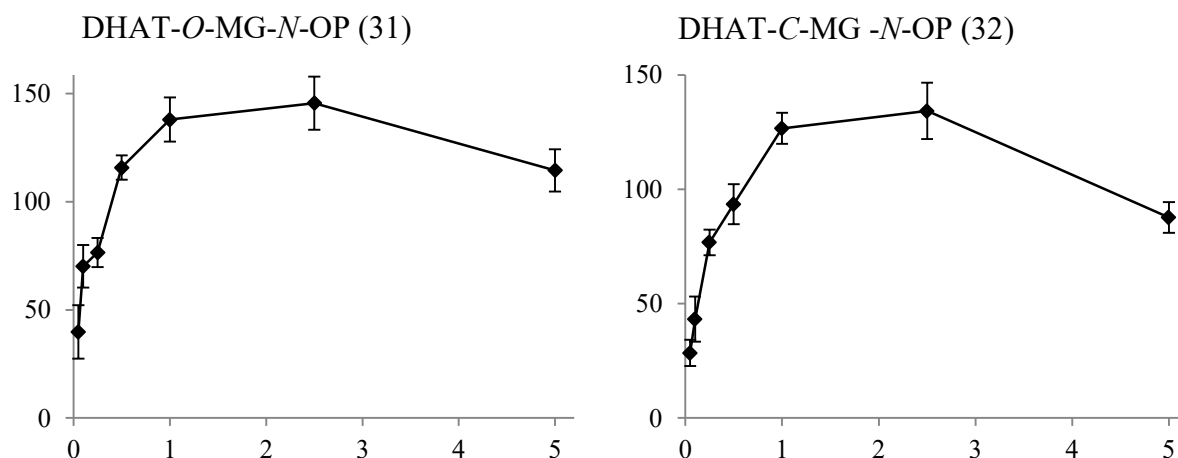
Compound Number	Semi-systematic name*	Retention time (min)	Structure [M-H] ⁻	3,4-DNT based precursor /product ions (<i>m/z</i>) [M-H] ⁻
31	Aminohydroxynitro-toluene –O-monoglucoside- <i>N</i> -oxopropanone (DHAT- <i>O</i> -MG- <i>N</i> -OP)	11.5		371/151
32	Aminohydroxynitro-toluene –C-monoglucoside- <i>N</i> -oxopropanone (DHAT- <i>C</i> -MG- <i>N</i> -OP)	8.2		371/195
33	Aminohydroxynitro toluene –C-monoglucoside- <i>N</i> -oxobutanoate (HANT- <i>C</i> -MG- <i>N</i> -OB)	12.55		413/151

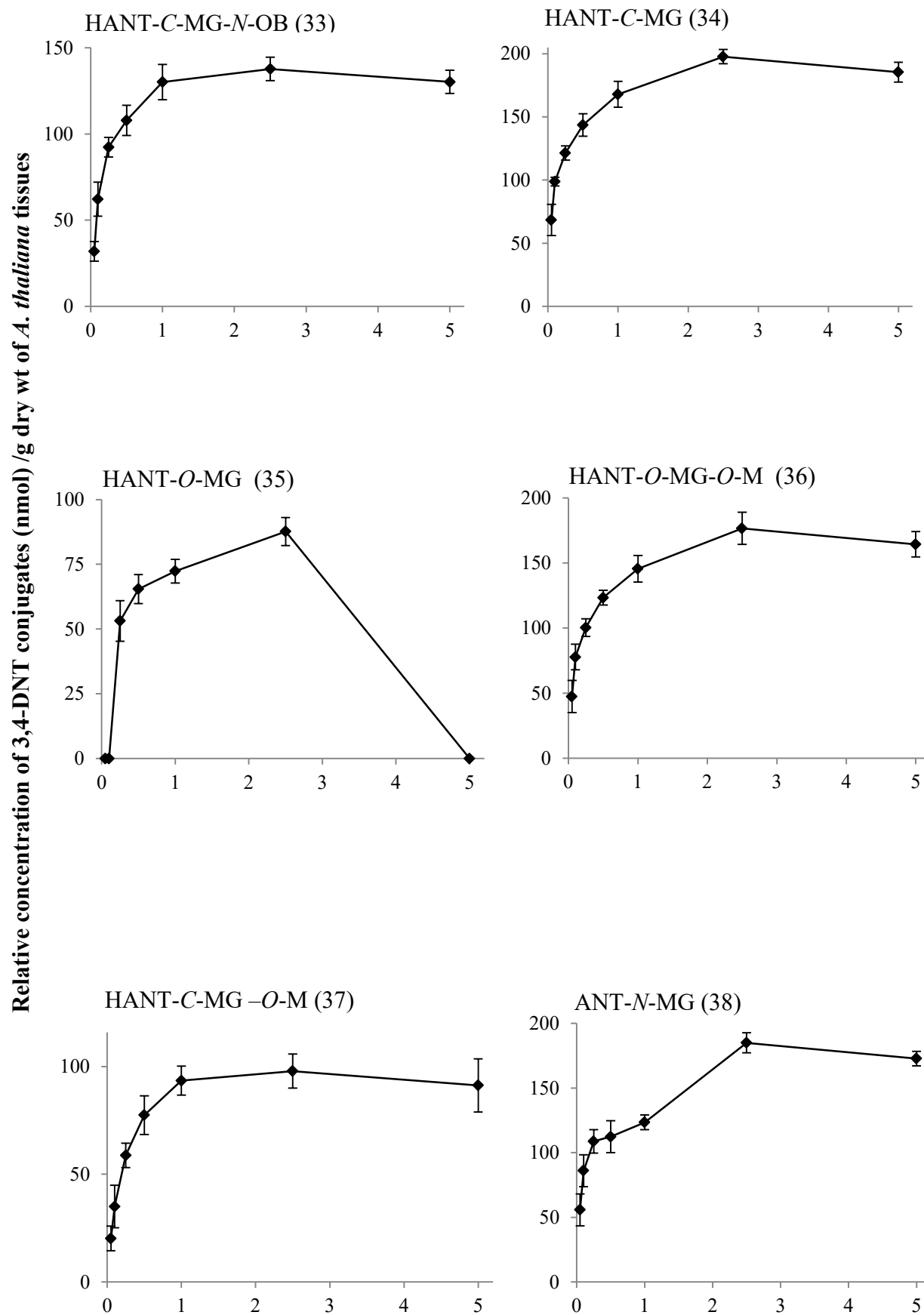
34	Aminohydroxy nitrotoluene –C-monoglucoside (HANT-C-MG)	14.11		329/209
35	Aminohydroxy nitro-toluene –O-monoglucoside (HANT-O-MG)	10.45		329/151
36	Aminohydroxy nitro-toluene –O-monoglucoside-O-malonyl (HANT-O-MG-O-M)	11.16		415/195
37	Aminohydroxy nitrotoluene –C-monoglucoside-O-malonyl (HANT-C-MG-O-M)	10.37		415/151
38	Aminonitro-toluene- N-monoglucoside (ANT-N-MG)	7.2		313/151

39	Aminonitro-toluene- C- monoglucoside (ANT-C- MG)	10.2		313/193
40	Aminonitro-toluene-C & N- diglucoside (ANT-C & N- DG)	11.4		475/149

*the red colour indicates tge possible conjugation sites.

The highest relative concentration (Figure 4.14) of all conjugates was observed at 3,4-DNT concentration of **2.5 mg /L of liquid MS media** which decreased at **5 mg /L of liquid MS media** and the least values were obtained at 0.05mg /L of liquid MS media. The relative glucoside contents decreased from *HANT-C-MG (34)(highest)* > *ANT-C & N- DG (40)* > *ANT-N-MG (38)* > *HANT-O-MG-O-M (36)* > *DHAT-O-MG-N-OP (31)* > *HANT-C-MG-N-OB (33)* > *DHAT-O-MG-N-OP (32)* > *ANT-C-MG (39)* > *HANT-O-MG-N-OB (37)* > *HANT-O-MG (35) (lowest)*. The data showed that the relative concentration of *HANT-O-MG (34)*, being the highest, was **68.45 nmol /g dry tissue weight of *A. thaliana* at 3,4-DNT concentration of 0.05 mg /L of liquid MS media** and reached **197.68 nmol /g dry tissue weight of *A. thaliana* at 3,4-DNT concentration of 2.5 mg /L of liquid MS media**. These then decreased to **185.46 nmol /g dry tissue weight of *A. thaliana* at 5 mg /L of liquid MS media**. For the lowest obtained glucoside (*HANT-O-MG (35)*), no conjugates were obtained at **3,4-DNT concentration of 0.05 and 0.1 mg /L of liquid MS media**. A relative concentration of **87.65 nmol /g dry tissue weight of *A. thaliana*** was obtained at 3,4-DNT concentration of 2.5 mg /L of liquid MS media.





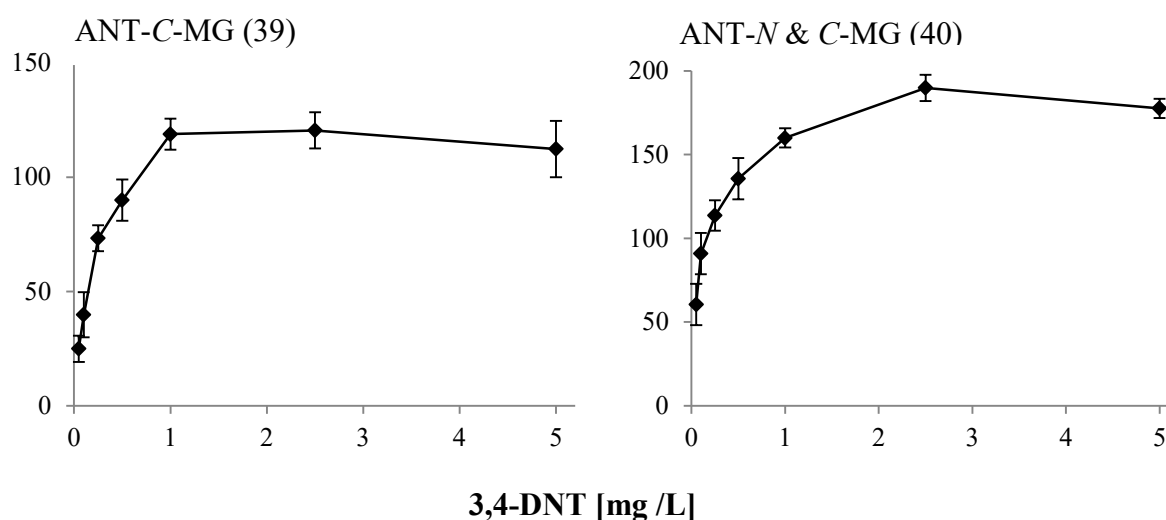


Figure 4.14: Conjugates of reduced 3,4-DNT metabolites recovered from homogenous mixture of ten 14 day (post cultivation) old *A. thaliana* plant grown in different concentrations of 3,4-DNT in sterile liquid MS media and analysed by LC-MS/MS (growth temperature was $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period; constant speed 130 rpm). Data was plotted as mean of four replicates (ten plants from one container were freeze dried and homogenized to give one replicate).

4.4 Discussion:

Previous studies and results from the previous chapter (Chapter 3) have shown the disappearance of DNT based reduced metabolites. It was assumed that the metabolites were further processed (either conjugated or sequestered) and therefore progressively disappeared and became undetectable. To validate the conjugation of reduced DNT metabolites, the present study was conducted. The plants were grown in sterile liquid MS media for this particular experiment to avoid any external interaction, avoid stressing the plant while sample collection and other enzyme activity to influence glucoside profile and to get maximum possible conjugates through maximum exposure of plant to DNTs. The results have shown that *A. thaliana* can conjugate DNT metabolites with glucose most likely derived from UDP-glucosides via an enzymatic reaction with a glucosyl transferase. Ten of the conjugates were

shown in 2,6-DNT treated *A. thaliana* tissues and confirmed by deuterated d₃-2,6DNT treated *A. thaliana* analysis. Deuterated d₃-2,6DNT was fed under exactly similar conditions to non deuterated compounds. The analysis of extracts and comparison of their masses confirmed that the conjugates were DNT based and solely generated by *A. thaliana* based enzymes. The quantity of conjugates was not enough to be isolated and would require at least a milligram of pure compound for NMR analysis, therefore LC-MS was carried out to estimate the possible position of conjugates and side chains. The possible positions for C-glucosylation for the available carbons on the benzene ring (highlighted (red) in the structures (Table 4.3-4.6)) as it was not possible to confirm the glucylation site, since LC-MS cannot determine the spatial orientation of functional groups, only NMR or X-ray analysis can do this.

The intense fragments produced in Q1 were further fragmented in Q3 quadrupoles of Linear Ion Trap Quadrupole LC/MS/MS Mass Spectrometer. The intense fragments of Q3 were combined to predict the possible conjugates and possible structures were confirmed through available literature (PubChem archived in 2012; SciFi Scholar, 2009). Previous studies have shown the glucosylation reaction of reduced metabolites of nitroaromatic compounds in *A. thaliana* with the glucose donor molecule (UDP- glucosides) through the activity of glucosyltransferases (Brazier-Hicks et al., 2005 and 2007; Gandia-Herrero et al., 2008; Louveau et al., 2011; Ahn et al., 2012).

From the initial structure prediction it was found that most of the conjugates were with hydroxylamino derivatives (HA- derivatives) compared to amino derivatives (A- derivatives). In case of HA-derivatives, the hydroxyl groups provide better effective conjugation sites (Zhang et al., 2011). High concentrations of C and O- glucosides were obtained from the present study (Table 4.3). This conjugation is in agreement with previous studies of *A. thaliana* treated with trinitrotoluene (TNT) and dichloroaniline (Brazier-Hicks et al., 2007; Gandia-Herrero et al., 2008; Dosnon-Olette et al., 2011). The glucosides observed were in

agreement with the glucosides obtained by Gandia-Herrero et al., 2008 through glucosylation of reduced TNT metabolites observed through LC-MS/MS (Figure 4.15).

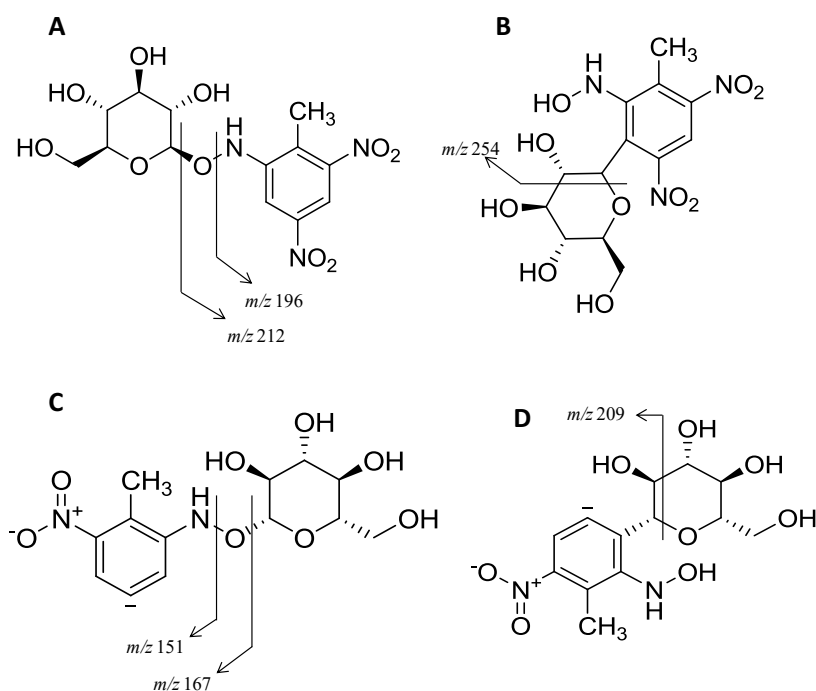


Figure 4.15: (A) *O*- and (B) *C*- glucosides for hydroxylaminodinitrotoluene (HADNT) (reduced product of TNT) as observed by Gandia-Herrero et al., 2008 and in contrast (C) *O*- and (D) *C*- glucosides for hydroxylaminotoluenes (HANT) (reduced product of 2,6-DNT) observed in the present study. An *m/z* difference of 45 can be observed in both the metabolites due to one nitro group less in DNTs compared to TNT.

Most of the *O*-glucosylation was observed with HA-derivatives of DNTs (Table 4.3). This may be due to the deprotonation requirement of acceptor nucleophile where HANTs are readily deprotonated. The ability of His-19 of UGTs (UGT72B1) to orientate and change nucleophilic attack contributes towards *N*-glucosyl conjugation, instead of proton abstraction from the aniline acceptor (Brazier-Hicks et al., 2007; Diamon et al., 2010). The *N*-glucosylation of dichloroaniline has been observed in plants, which is due to the high activity of abundant UGT72B1, which is an important component to regulate the metabolism of anilines and other nitrogen based xenobiotics in crops (Loutre et al., 2003).

The formation of *C-N* glucosidic bond (as observed for ANT-*N*-MG (9)) is very important in generating non-hydrolyzable glycosides (Mittler et al., 2007). The observation of *C*-glucosyl conjugates (*C-C* glucosidic bond, as observed in DHAT-*C*-MG-*N*-OP (2), HANT-*C*-MG (4), HANT-*C*-MG-*O*-M (7), ANT-*C*-MG (9) and ANT-*C* & *N*- DG (12)) (Table 4.3) in the present study showed the possibility of non-labile glucosidic bonds (Brazier-Hicks et al., 2007). Previously *C*-glucosidic bonds have been shown by Gandia-Herrero et al., 2008 through TNT conjugation assisted by UGT73C1 in *A. thaliana*.

For a good remediation system, the metabolites should not get distributed into the media. The assay for media was conducted in the present study and it was found that glucosidic conjugates were not excreted into the media, in fact they remained confined to the plants (Figure 4.5).

According to the results of present study, it was observed that the conjugation was highly substrate specific. When glucosides, as extracted from *A. thaliana* treated with different DNT isomers, were compared it was found that in each treatment the glucosides had different intensities (Table 4.9).

Table 4.9: Four observed glucosides (highest relative concentrations) extracted from *A. thaliana* treated with DNT.

DNT	Glucosyl conjugates with precursor /product ions (<i>m/z</i>)			
isomers	1	2	3	4
2,6-DNT	DHAT- <i>O</i> -MG –N-OP (1) 371/151	DHAT- <i>C</i> -MG–N-OP (2) 371/195	HANT- <i>C</i> -MG (4) 329/209	ANT- <i>C</i> -MG (9) 313/193
2,3-DNT	DHAT- <i>C</i> -MG –N-OP (12) 371/191	HANT- <i>C</i> -MG- <i>O</i> -M (17) 415/151	HANT- <i>O</i> -MG (15) 329/151	ANT- <i>N</i> -MG (18) 313/151
2,4-DNT	HANT- <i>O</i> -MG (25) 329/151	ANT- <i>N</i> -MG (28) 313/151	DHAT- <i>C</i> -MG- <i>N</i> -OP (22)	HANT- <i>C</i> -MG (24) 329/209

			371/195	
3,4-DNT	HANT-C-MG (34)	ANT-C & N-	ANT-O-MG (38)	HANT-O-MG-
	329/209	DG (40)	313/151	O-M (36)
		475/149		415/191

One of the reasons for this variation in glucoside concentration with DNT isomers is that the activity of potential UGTs involved in the catalysis of the reaction seems to have distinct substrate specificity and preference of the conjugation site. This inter-individual variability in UGTs activity has been shown to vary with the position of the side chain. The glycosidic side chain is sometimes acquired frequently by a molecule to attain stability (Wang et al., 2011). This result in high intensity conjugation of a particular metabolite compared to the other. Another reason could be the available glycosylation sites. When UGTs come across a specific acceptor molecule, the side chains determine potential glycosylation site. The structure of the acceptor molecule determines available sites to covalently bind to glucose (Halim et al., 2012). This variation in conjugation due to the substrate specificity has been reported in previous studies (Brazier-Hicks et al., 2007; Abad-Garcia et al., 2009; Tryfona et al., 2010).

When relative concentrations of different conjugates, as obtained through different isomer treatment, were evaluated it was found that high concentration of conjugates were observed with hydroxyl derivatives of DNT compared to ANTs (Figure 4.8, 4.10, 4.12). This is again in agreement with a TNT based study using *A. thaliana* where hydroxyl derivatives had higher intensity than amino derivatives of TNT in *A. thaliana* (Gandia-Herrero et al., 2008). Another interesting conjugation with hydroxyl compounds was of malonyl side chain donated by malonic acid. The malonyl conjugates have been observed in the earlier studies in Lemnaceae callus cultures treated with 3-Trifluoromethylphenol (Reinhold et al., 2011) and in marine microalga *Tetraselmis* spp. treated with para-chlorophenol (p-CP) where

malonylated gluco-conjugate (6-*O*-malonyl conjugates) were isolated (Petroutsos et al., 2007).

The present study suggested that for all the glucosyl conjugates, the relative concentrations increased gradually with the rate of DNT in the media. They then reached at a maximum concentration against the second highest considered DNT concentration in the media (Figure 4.7, 4.9, 4.11 and 4.12). The value then suddenly dropped. This might be due to conjugation of these glucosides with the lignin (phase III reaction) or otherwise the highest considered concentration for all DNT isomers was extremely toxic (Chapter 2; section 2.3) that function of plant based enzymatic machinery was compromised under these conditions. Since DNTs are toxic for plants at higher concentrations, therefore they might have impaired the cellular function to metabolise and conjugate DNT isomers at highest considered concentration in the media. Previous studies have shown reduced xenobiotic metabolism in plants after a specific concentration of xenobiotics in media (Zu and Jing, 2012; Oliveira et al., 2010).

The possible disappearance of reduced metabolites reported in Chapter 3 may be due to their conversion to conjugates and was therefore proved in the present study. For example Figure 4.16 shows that ANT_s decreased in quantity after a certain concentration and glucosyl conjugates started to increase.

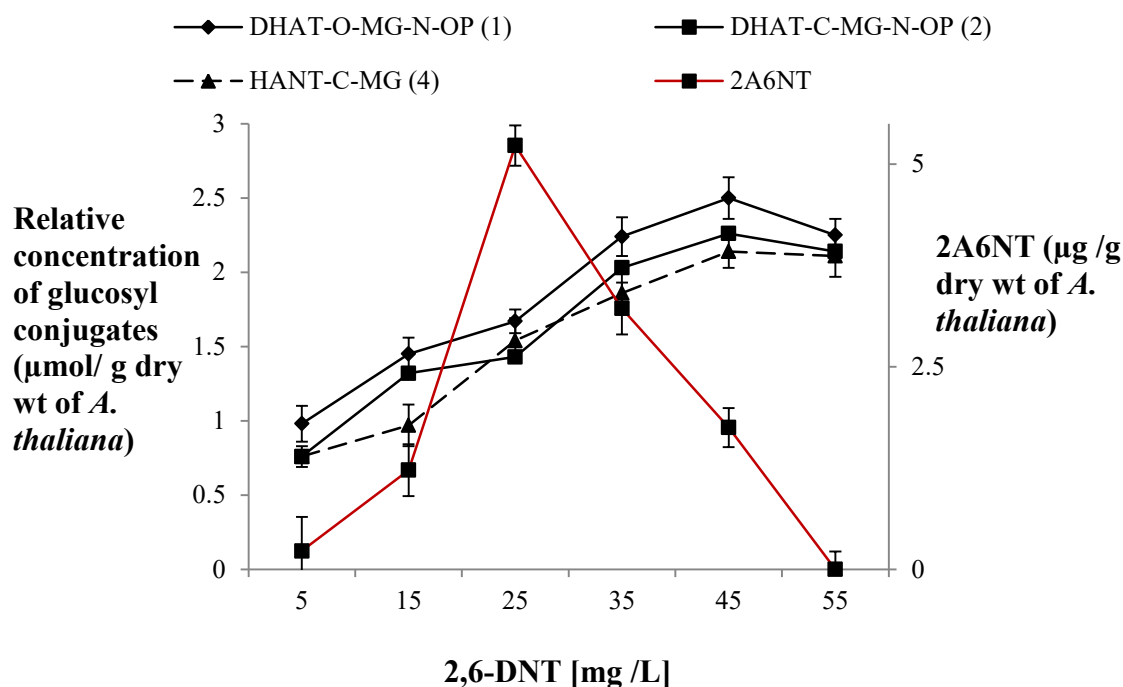


Figure 4.16: Comparison of 2-amino-6-nitrofluene (2A6NT) (Chapter 3) to three highest glucosyl conjugates analysed from 2,6-DNT treated *A. thaliana*. Both the plants were treated in sterile media.

The relative concentration of different DNT based conjugates was also very low compared to their concentration in the media. It was considered that the conjugates might have been sequestered into the cell wall of the plant cells limiting their availability through analysis. Another reason could be their conjugation with glutathione. Although nitroamines have been shown to have limited conjugation with glutathione and prefer conjugation with glucose moiety (UDP- glycosides through glucosyltransferases) and malonic acid (malonate through malonyltransferases) (Brazier-Hicks et al., 2007; Diamon et al., 2010). To observe the conjugation of DNT metabolites with glutathione a preliminary study was conducted through *in situ* labelling of 2,6-DNT treated *A. thaliana* with monochlorobimane (MCB). MCB acts as xenobiotic and combines with glutathione (GSH) to form glutathione *S*-bimane (GSB) and therefore a competition for GSH to bind with MCB and xenobiotic appears. The dye fluoresces under a multiphoton microscope only when GSB is formed, so reduction in

intensity of fluorescence due to competition in MCB and xenobiotics and control can be detected to show GSH conjugation (Melissa et al., 2005).

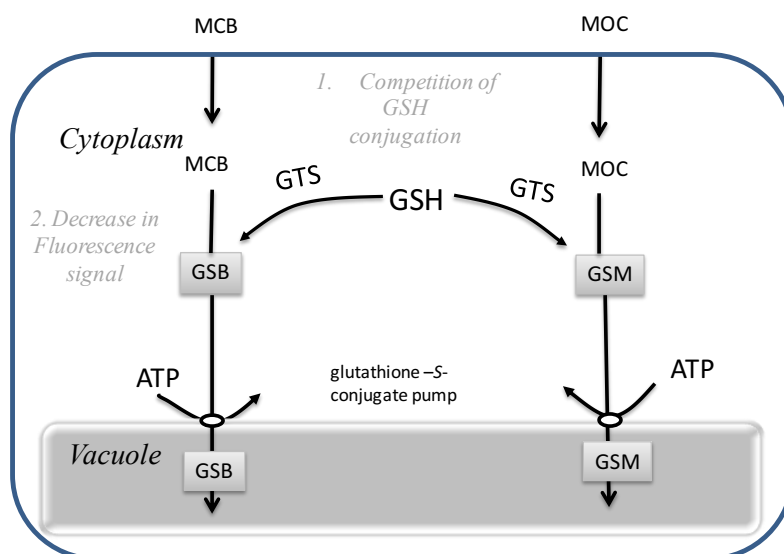


Figure 4.17: Formation of GSB offers a competition for GSH binding to xenobiotics (Melissa et al., 2005).

An intact root of 8 day old *A. thaliana* seedling treated with 5 mg /L of Phytigel media (for 6 days) and intact root of untreated 8 day old *A. thaliana* seedling was placed on a glass slide and transferred to a drop of MCB (100 mM stock prepared in DMSO and diluted to 5 mM through distilled water) with NaN_3 (depletes ATP concentration and avoid GSB vacuolar sequestration). The coverslip was placed gently to avoid root crushing and sealed (with Vaseline, lanolin, paraffin at 1:1:1 ratio) to avoid evaporation. The fluorescence was measured soon after the treatment with fluorescence of GSB was recorded at $\lambda_{\text{em}}=770\text{nm}$. The results showed high intensity colour in controls compared the treated *A. thaliana*, which shows a competition in GSH availability due to MCB and 2,6-DNT. The experiment was conducted for three replicates.

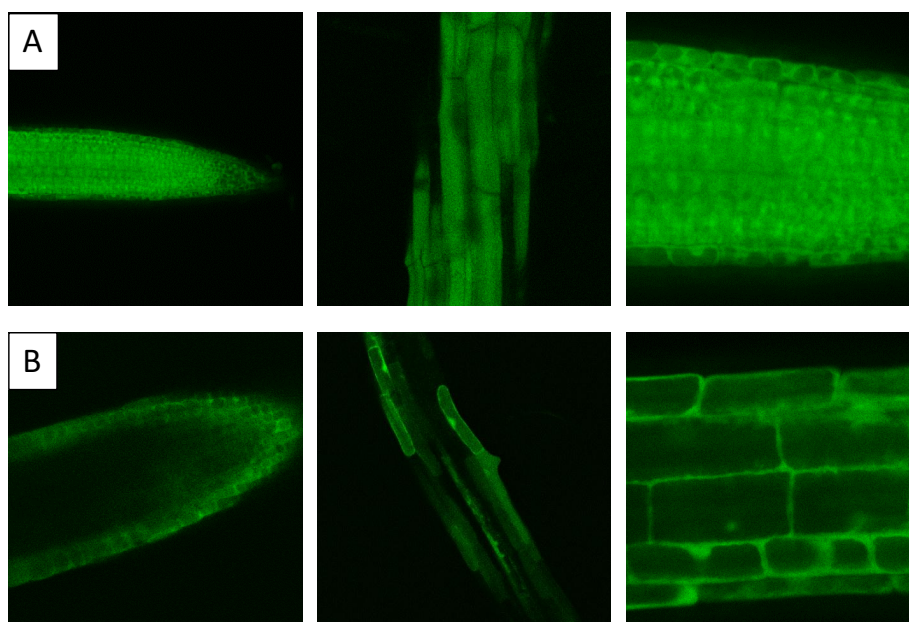


Figure 4.18 : (A) Images for control (untreated roots) which shows high concentration of GSB due to MCB (5) 5 mg 2,6-DNT /L of Phytigel media treated *A. thaliana* roots showing less intensity of fluorescence due to competition in GSH binding: A preliminary experiment.

Following conjugation the metabolites remain in the cytosol and are then transported to either the vacuole or are incorporated into the lignin wall (Figure 4.19). Previous studies utilizing TNT as substrate have shown that the conjugation reaction occurs in the cytosol of the cell as no membrane spanning signals (vital if reduced TNT metabolites are conjugated in some cell organelle and transported out in the cytosol) have been identified. TNT derived intermediates have been found to be incorporated in the cytoplasm and cell wall equally which shows that they bind to the cell wall (Gandia-Herrero et al., 2008). In case of chlorinated phenols and aniline the conjugates have been shown to be sequestered in the vacuole. In tobacco cell suspension cultures, due to the lack of lignin (a sink to bind and retain conjugates within plant), the mono and diglucosides of 2- and 4-HADNT found distributed in all cell culture (Vila et al., 2005).

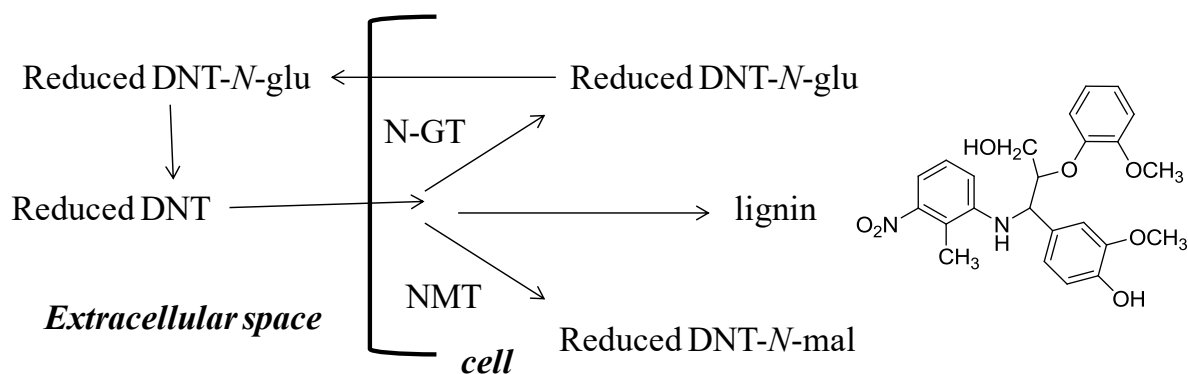


Figure 4.19: The reduced DNTs (ANTs or HANTs) conjugate through activity of *N*-glucosyltransferases (N-GT) to *N*-glucoside (reduced DNT *N*-glucoside) and through activity of *N*-malonyltransferases (NMT) to *N*-malonate (reduced DNT *N*-mal).

Chapter 5

The effects of DNT contaminated *Arabidopsis thaliana* on the growth, fecundity and survival of a specialist (*Brevicoryne brassicae*) and generalist (*Myzus persicae*) aphid

5.1 Introduction:

According to the results of previous chapter (Chapter 5), it has been found that ANT_s and HANT_s (reduced products of DNT_s) are conjugated to glucosides in *A. thaliana*. If phytoremediation is considered as a practical approach for DNT_s then herbivore feeders could potentially accumulate these compounds, since they are unable to discriminate in the food source. This can result in accumulation and propagation of DNT based contaminants in the ecosystem through predation of contaminated prey. Dichlorodiphenyltrichloroethane DDT, a pesticide, is one example and has been studied for its potential accumulation in the ecosystem as it poses a problem for the health of higher animals (Cole et al., 1976; Matozzo et al., 2012; Kannan et al., 2012; Ondarza et al., 2012).

The extensive use of DDT_s as an insecticide has led to its accumulation in the body fat of higher birds through predation of the contaminated prey and finally affected their reproduction (Espin et al., 2012). p,p'-dichlorodiphenyl dichloroethylene (DDE). It has been shown to be transported across the remote areas in the arctic ranges after it has accumulated in Antarctic Krill (*Euphausia superba*) when their larvae are exposed to contaminated aquatic plants. The larval development was severely affected and accumulation of DDE was observed among the survivors (Poulsen et al., 2011).

The photosynthate, nutrients and hazardous compounds absorbed by the plants from the soil travel in the vascular tissues and are acquired by the predatory animal, birds and insects. Several studies (Daugherty et al., 2004; Retuerto et al., 2004) have demonstrated the role of plants in the transport of xenobiotics from soil into the ecosystem since the photosynthates are the main food source for phloem-feeding herbivores. DDT, carbaryl, thiamethoxam, imidacloprid or pyrethroids have been shown to travel in the vascular tissues and are then acquired by herbivores (Matsukura et al., 2012; Seagraves et al., 2012).

Amongst the herbivores, phloem-feeding arthropods are the most important and widely studied for their involvement in the propagation of plant based contaminants due to their wide geographical distribution (Blanch et al., 2012; Scollon et al., 2012). Insecticides such as DDT, carbaryl, imidacloprid or pyrethroids have been shown to travel from plant source to the aphid feeding insects and birds (Scollon et al., 2012; Espin et al., 2012). Thiomethoxam has also been shown to accumulate in the aphid survivors and transported to a higher predator, causing toxic effects on this population (Seagraves et al., 2012). Imidacloprid accumulation by potato psyllid (*Bactericera cockerelli*) has been shown after its transport through the phloem to the aphid body via the stylet (Butler et al., 2012). Neem and tobacco based synthetic insecticides have been shown to affect the population of aphid predators that indicate their transport through the food chain (Shabozoi et al., 2011). Organophosphate (OP) and carbamate detection in the pellets of western burrowing owls after predation on contaminated aphids has led to the discontinuation of the use of insecticides in United States by 2002 to stop the decline in the owl population (Engelman et al., 2011). The above examples show the movement of xenobiotics through the different trophic levels.

Generally there are two modes of propagation of contaminants- direct and indirect propagation. The direct method involves transport through feeding of a contaminated aphid and indirect is through feeding contaminated honey dew produced by these aphids (Marzaro

et al., 2011). Phloem feeding insects generate honey dew which is the food of several ant species. Ants preference for honey dew that contain borates and toxic compounds has been extensively studied (Klotz et al., 2000; Warner et al., 2005). These toxicants can then impact on ant eaters (mammals belonging to the sub-order Vermilingua and anteater reptiles) once they become the prey on the contaminated ants. Usually predators consume large amounts of prey at a time resulting in bio magnification of the contaminants as the trophic level increases.

Furthermore, the aphids (source of propagation of contaminants) are either specialists (feeding on selective closely related plant species) or generalist (consuming a wide variety of plant species) on the basis of their feeding pattern. Specialists feed on a narrower range of plant species can be hyperaccumulators if the range of the plant consumed is contaminated with xenobiotics (Francis et al., 2004; Pineda et al., 2012). Studies have shown the fecundity and survival of specialists to be affected more by plant based contaminants compared to generalists.

This study was therefore designed to focus on phloem feeding insects -oligophagus (*Brevicoryne brassicae*) and polyphagus (*Myzus persicae*) aphids. To analyse the propagation of DNTs it is necessary to observe their survival, relative growth rate and fecundity in relation to the contaminated plants on which they are feeding. If aphids can survive on contaminated plants then the propagation of DNT metabolites is likely throughout the trophic levels in the ecosystem.

5.1.1 Hypotheses:

The hypotheses on which the present study is based are as follows:

- Both *M. persicae* and *B. brassicae* have different tolerance levels for different isomers of DNTs.
- The increases in different concentrations of DNTs affect the weight of aphids of both species feeding on them.
- A gradual death occurs in aphids, as the contaminant accumulation reaches upto a certain extent.
- The reproduction of aphids is severely inhibited with the contamination of the food source.
- Aphids being phloem feeders can accumulate DNT based metabolites in their bodies.

5.1.2 Aims:

Based on the hypotheses as described above, the aims of this study are therefore to;

- To analyse the survival and LC₅₀ (lethal concentration 50%; calculated through indirect way) of *M. persicae* and *B. brassicae* feeding on DNT treated Phytigel grown *A. thaliana*.
- To conduct the mean relative growth rate (MRGR) to analyse the rate in weight loss over a period of exposure.
- To carry out the time course study for weight loss in aphids over a time of exposure.
- To analyse the fecundity of different aphids on treated *A. thaliana*.
- To observe aminotoluenes (ANTs) and DNTs accumulated in both *M. persicae* and *B. brassicae* feeding on contaminated *A. thaliana*.
- To analyse possible DNT based glycosyl conjugates in *M. persicae* and *B. brassicae* through LC-MS/MS analysis.

Due to time restraints it was only possible to use the Phytigel agar based model systems.

5.2. Material and methods:

5.2.1 Chemicals

As described in the section 2.2.1 of Chapter 2.

2,4-DNT (purity $\geq 98\%$), 2,3-DNT (purity $\geq 99\%$), 2,6-DNT (purity $\geq 99\%$) and 3,4-DNT (purity $\geq 99\%$) were purchased from Sigma Aldrich (UK). Acetonitrile (purity $\geq 97.8\%$, HPLC grade) and methanol (purity $\geq 97\%$) were reagent grade and obtained from VWR, UK. Murashige and Skoog salts (MS), Phytigel (P8169), Agrose (A9539), 2-(*N*-morpholino) ethanesulfonic acid (MES) and sucrose (99.8%) were purchased from Sigma Aldrich (UK).

5.2.2 Insects and plant material:

5.2.2.1 Cultivation of plants

5.2.2.1.1 DNT stock solutions for the plant growth media

The concentrations of DNT isomers used for the treatment of *A. thaliana* grown in the Phytigel media is as described in the Table 3.2 (Chapter 3).

5.2.2.2 Cultivation of plants:

Seeds were surface sterilized with 70% ethanol and 25% sodium hypochlorite containing 1% triton following the procedure as described in Chapter 3 (section 3.2). They were then allowed to grow in Phytigel containing petri plates placed vertically in growth room under $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ and temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h of a day:night period.

After four days the seedlings with uniform root length and shoot size were transferred to the tissue culture boxes containing 60 ml of half strength MS, 1% sucrose and 0.75% phytigel adjusted to pH 5.7 with 1M KOH amended with the specific concentration of DNT isomer (solution prepared in 100% ethanol). Sterile aluminium foil was carefully layered on the top

of the Phytigel media to avoid contamination with aphids. A sterile needle was used to make a fine hole in the foil and plant roots were gently passed through them such that roots should not break or damage. The foil with seedlings was then fixed on the top of the agar such that roots were immersed in the agar and shoots were upright on the foil.

The plants were allowed to grow for 12 days, under the constant light conditions of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Hanstech Quantitherm lightmeter; attained by Starcoat T5 high output Hungry fluorescent bulbs) and temperature of $20 \pm 3^{\circ}\text{C}$ for 16:8 h of a day:night period, prior to the infestation of the specific aphid species.

5.2.2.3. *Insects:*

An anholocyclic crucifer generalist aphid *M. persicae* (polyphagus) and crucifer specialist aphid *B. brassicae* (oligophagus) were obtained from Dr Glen Powell and associates (Imperial College London) from a maintained culture on the cabbage plants. The *M. persicae* of clone US1L and *B. brassicae* of clone from South-East UK were used for this study.

To get a uniform age group of insects, isolated individuals were maintained on the *A. thaliana*. Five adult insects of uniform size and morphology were selected for both species of insects and allowed to grow and reproduce on the 12 day old *A. thaliana* for 24 hours at 18°C with 16:8 h day:night photoperiod. These plants were covered individually with perforated plastic bags (10 cm diameter and 50 cm long sieved plastic bags open at one end). Six nymphs were collected and reared on another 12 day old *A. thaliana* plants (grown in the black $6 \times 6 \times 6$ cm pots containing compost; 4:1 organic compost – Levington's F2 with sand and perlite) for 8 days at 18°C with 16:8 h day:night photoperiod with the individual plants covered with perforated plastic bags. This procedure was followed to get the adults of uniform age group. The nymphs of these first instar adults so obtained were then used to infest the treated *A. thaliana* plants for the present study.

5.2.3 Survival:

To determine the survival of *M. persicae* and *B. brassicae* on DNT treated plants grown on sterile Phytigel medium, seven 2 day old starved nymphs (kept in petri plates for 30 min) were placed on a basal leaves of *A. thaliana* within the tissue culture box having a layer of aluminium foil on the top of the sterile Phytigel media. This layer of foil kept the media sterile after the infestation of the plants with the aphids. The aphids were weighed before being placed on the plants and weighed again after the termination of the experiment.

The aphid survival rate was determined by counting the number of live aphids over 4 days subsequent to infestation. LC_{50} was calculated through non-linear regression analysis by GraphPad Prism 5 at 95% Confidence Interval (95% CI). The equation for the calculation of LC_{50} (GraphPad Prism 5) is as follows:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((EC50 - X) * \text{HillSlope})})$$

LC_{50} is an x-axis value corresponding to halfway of Y-axis values.

HillSlope is the steepness of curve. A value of 1 for *HillSlope* is standard whereas greater than 1 is steeper and lesser than 1 is shallower slope.

Top and Bottom are values of the plateaus in the units of the Y axis.

The weights of the aphids were determined on a microbalance (Mettler/Toledo MX5) before being released onto each plant and after the termination of the experiment.

The mean relative growth rates (MRGR) were determined by using the formula

$$\text{MRGR} = \frac{[\log_e W_{\text{final}} - \log_e W_{\text{orig}}]}{[T]}$$

Where T= total time (in days) between start and termination of experiment, which is 4 days for the current experiment.

Units for MRGR are g /g.d = mass increase (g) per aboveground mass (g) per day (d).

The experiment was done for 15 replicates per concentration.

5.2.4. Growth:

To determine the growth of *M. persicae* and *B. brassicae* on DNT treated *A. thaliana*, a 7 day old starved (kept in petri dish for 30 min) aphid was placed on the leaf of 9 day old plants. After 24 hr adults and nymphs were removed leaving one first instar nymph. At 48 hr periods (2 days; procedure repeated for 6 days before the onset of reproduction) aphids were removed and weighed on a microbalance. Ten replicates per treatment were analysed. The data was analysed against the controls (plants grown in Phytigel media without DNT and with 0.25% ethanol).

5.2.5 Fecundity:

The procedure for fecundity of *M. persicae* and *B. brassicae* born on *A. thaliana* was followed as described by Daniels et al., 2009. For this purpose 12 day old treated plants were placed in individual plastic cages (10 cm diameter and 50 cm long sieved plastic bags open at

one end). Each plant was infested with a 7 day old aphid. After 24 h, all the nymphs were removed leaving one first instar nymph on each plant. The aphid was kept on the plant for 8 days until the onset of reproduction. After the reproduction (allowed to reproduce just once), the number of nymphs produced per aphid was counted and data was analysed. The data was analysed for 15 replicates per treatment.

5.2.6 GC-MS Analysis:

To determine the DNT and its metabolites in *M. persicae* and *B. brassicae* feeding on DNT treated plants grown on sterile Phytigel medium, six 2 day old starved nymphs (kept in petri plates for 30 min) were placed on a basal leaves of *A. thaliana* within the tissue culture box having a layer of aluminium foil on the top of the sterile Phytigel media amended with DNT. The aphids were collected after 5 days in 1 ml Eppendorf tubes and flash frozen in liquid nitrogen and then freeze dried (HETO Dry Winner) for 48 h. Aphids collected from 10 replicates were combined to give a homogeneous mixture.

Sample (10 mg) was taken from the homogenous mixture of freeze dried aphids and placed into 2 ml Eppendorf tubes. A stainless steel ball (5/32") and acetonitrile (1ml) was added to the tubes and they were processed using a QIAGEN Retsch Tissue Lyser at a frequency of 30.0 Hz/sec for 2 min. The remaining procedure of extraction was carried out as described in Chapter 3 (section 3.2.3.2.1). Four replicates were processed following the above procedure for each DNT concentration.

5.2.7 LC-MS/MS Analysis:

Similar procedure of sample collection was followed as described in the above section (section 5.2.5).

Sample (10 mg) was taken from the homogenous mixture of freeze dried aphids on the treated *A. thaliana* plants and placed into 2 ml Eppendorf tubes. A stainless steel ball (5/32") and 10% methanol (2 ml) was added to the tubes and they were processed using a QIAGEN Retsch Tissue Lyser at a frequency of 30.0 Hz/sec for 2 min. The rest of the procedure of extraction was carried out as described in Chapter 4 (section 4.2.3.1). Three replicates were processed following the above procedure for each DNT concentration.

Calculations:

The calculations concentrations of metabolites (from GC-MS analysis) and conjugates (LC-MS analysis) was carried out as described in the sections 3.2.3.2.4 and 4.2.4 respectively.

5.2.8 Statistical Analysis:

Data analysis including calculation of average values, calculation of percentage rates and standard deviations were completed using Microsoft Excel 2007. Non linear regression analysis through GraphPad prism 5 was used to find out (lethal concentration 50%) LC₅₀ for both *M. persicae* and *B. brassicae* from the survival data obtained against different concentrations of different isomers of DNTs.

5.3 Results:

5.3.1 *M. persicae* and *B. brassicae* on 2,6-DNT treated *A. thaliana*

5.3.1.1 Survival

M. persicae and *B. brassicae* aphids did not survive on plants treated with 2,6-DNT concentrations of 55 mg /L in Phytigel media (Figure 5.1). *M. persicae* aphid survival was reduced by 38.5 ± 2.11 % compared to the control at the lowest considered 2,6-DNT concentration (5 mg /L of Phytigel media) in the media. This reduction continued and reached $93.4 \pm 1.33\%$ to that of the control at a 2,6-DNT concentration of 45 mg /L of Phytigel media. The reduction in survival of *B. brassicae* increased from $37.1 \pm 2.11\%$ at 5 mg /L to $98 \pm 2.03\%$ of the control at 45 mg /L. The LC_{50} value as obtained through GraphPad Prism 5 (95% CI) was 11.4 mg /L of Phytigel media for *B. brassicae* with $r^2=0.978$ and 13.9 mg /L of Phytigel media for *M. persicae* with $r^2=0.988$.

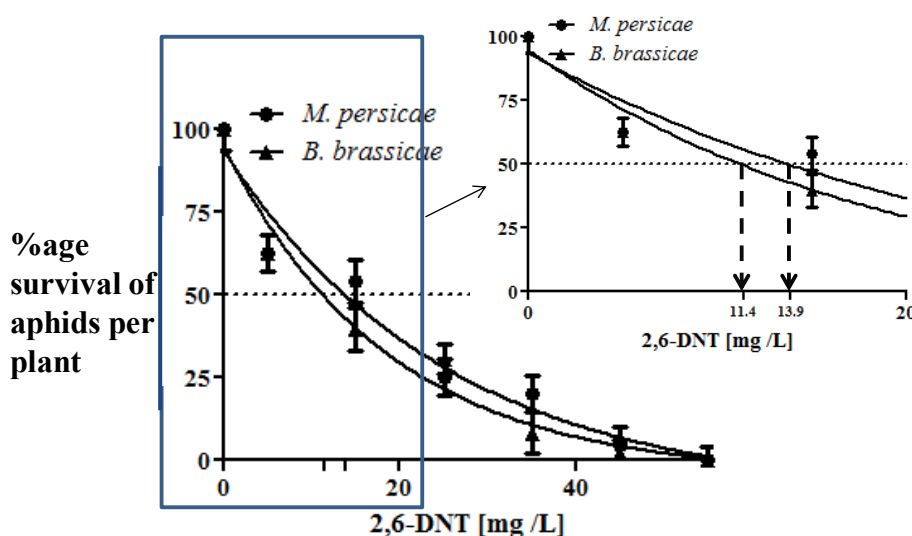


Figure 5.1: LC_{50} determination of *M. persicae* and *B. brassicae* on 2,6-DNT treated *A. thaliana* by counting number of aphids survived (%) after 4 days of exposure to 14 day old (post cultivation) plants treated with different concentrations of 2,6-DNT (in Phytigel media) compared to control (aphids on plants with unamended Phytigel media) as analysed through a dose-response curve ($r^2=0.988$ for *M. persicae* $r^2=0.978$ for *B. brassicae*) and fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$; GraphPad Prism 5). The aphids were reared at a temperature of $18^{\circ}C$ for 16:8 h day:night period. Data points are the mean of aphid survival from 15 replicates (7 aphids per treatment). The LC_{50} for *M. persicae* and *B.*

brassicae at which aphids only survived by 50% of the control was 13.9 and 11.4 mg /L of Phytigel media respectively. (data in Table 1 of Appendix IV)

5.3.1.2 MRGR

A comparison of the mean relative growth rate of *M. persicae* and *B. brassicae* showed a decrease in the aphid weight with increasing 2,6-DNT concentration in the media (Figure 5.2). The reduction in weight of *B. brassicae* was observed to be more frequent than *M. persicae*. A negative value (weight loss) was observed for *M. persicae* beyond 25 mg /L of Phytigel media and the negative value was observed for *B. brassicae* beyond 5 mg /L of Phytigel media

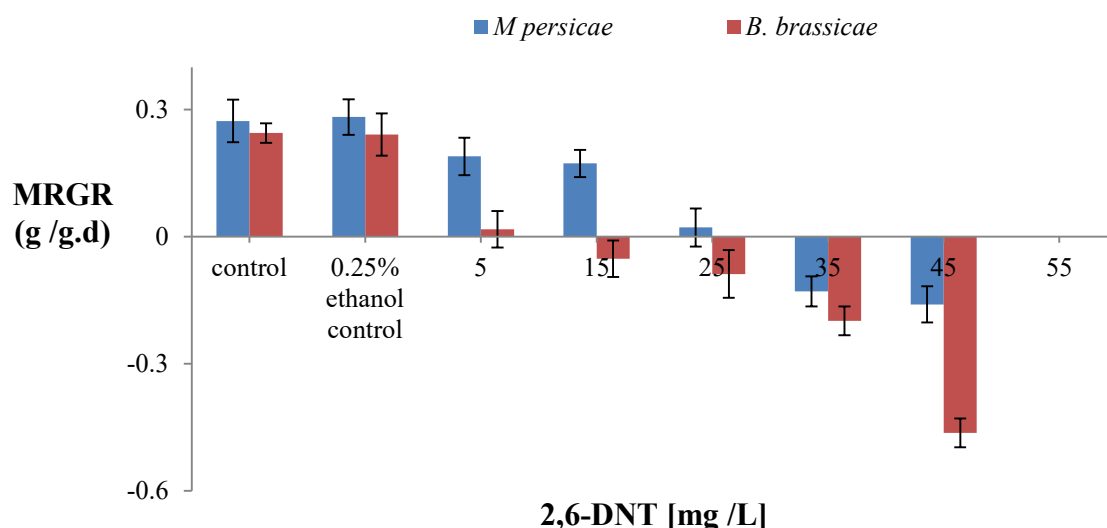


Figure 5.2: Mean Relative Growth Rate (MRGR) of *M. persicae* and *B. brassicae* on *A. thaliana* treated with 2,6-DNT in Phytigel media. The data is the mean of weight from ten aphids (on 10 replicates-12 day old plants) at 18°C with 16:8 h day:night photoperiod. The negative values show the aphid weight loss. Units stand for mass increase (g) per aboveground mass (g) per day (d). (data in Table 2 of Appendix IV).

5.3.1.3 Time course study for reduction in weight

The results from time course experiment indicated that both *B. brassicae* lost weight more frequently (Figure 5.3 B) compared to *M. persicae* (Figure 5.3 A) over a period of time when exposed to different concentrations of 2,6-DNT in the media. *M. persicae* (Figure 5.3 A) grown at 2,6-DNT treated plants below 35 mg /L of Phytigel media increased in weight on 4th day but then no further weight gain was observed until 6th day of exposure. Complete mortality for *B. brassicae* and *M. persicae* was obtained on 55 mg /L of 2,6-DNT on 6th day of exposure.

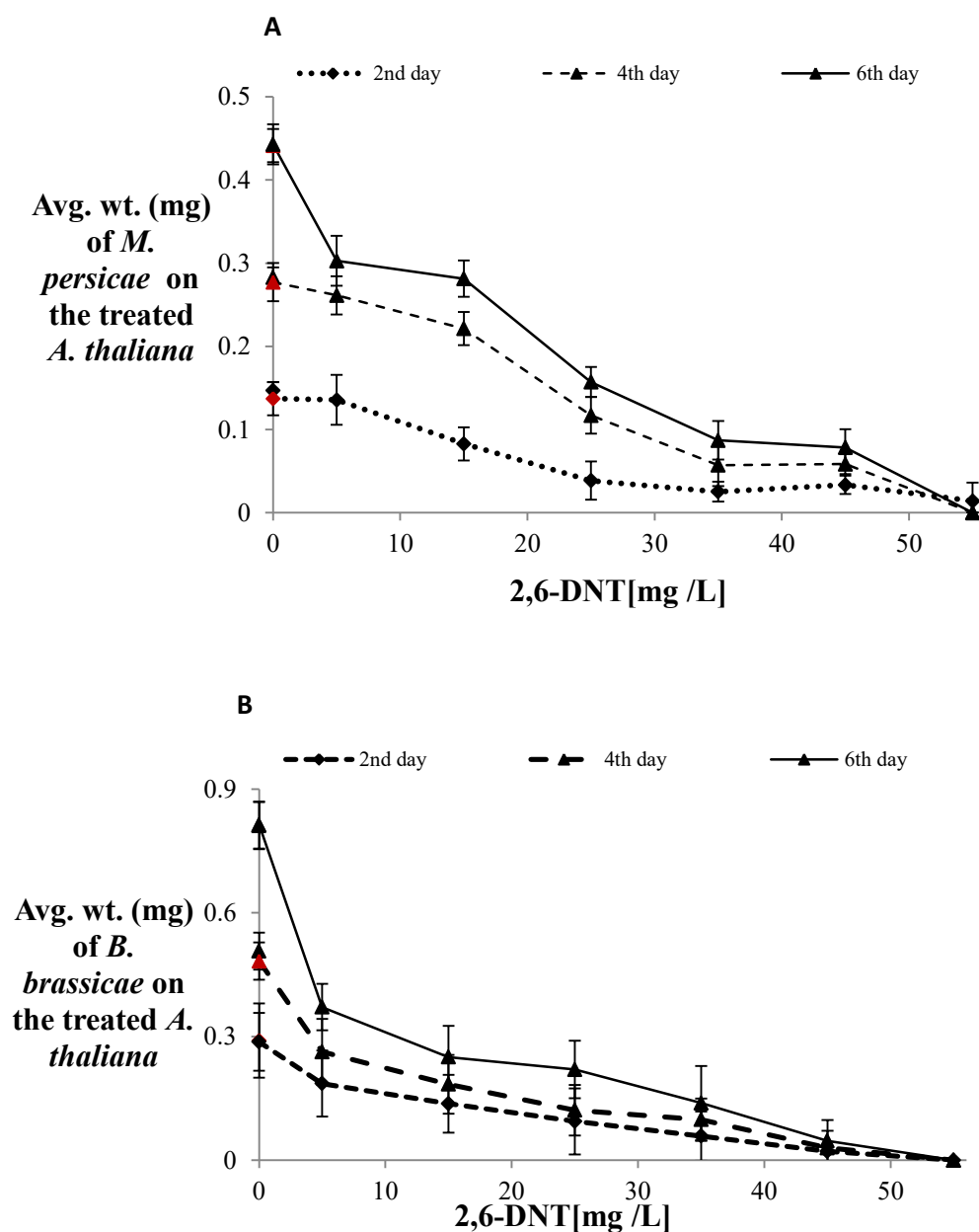


Figure 5.3: Weight (mean) of (A) *M. persicae* and (B) *B. brassicae* on the Phytagel media grown *A. thaliana* against different concentrations of 2,6-DNT. The first instar nymphs were analysed after 4 days of infestation on 2,6-DNT treated *A. thaliana* (14 days old plants after 2 days of post germination). The survival data is mean and weight is average of six aphids on 10 replicates (plants) at 18⁰C with 16:8 h day:night photoperiod. The red data point represents mock control (0.25% ethanol control). (data in Table 4 of Appendix IV).

5.3.1.4 Fecundity:

For Phytagel grown 2,6-DNT treated *A. thaliana*, *M. persicae* showed a reduction in fecundity by **26.43±2.87%** in relation to the control at a 2,6-DNT concentration of 5 mg /L of Phytagel media and this reached **79.44±3.12%** at a 2,6-DNT concentration of 45 mg /L of Phytagel media. For *B. brassicae* the reduction in fecundity started from **21.61±2.11%** compared to control against 2,6-DNT concentration of 5 mg /L of Phytagel media and reached a value of **85.13±2.78%** at 2,6-DNT concentration of 45 mg /L of Phytagel media. No aphids were recovered from 2,6-DNT concentration of 55 mg /L of Phytagel media, therefore fecundity data could not be obtained at these concentrations (Figure 5.4).

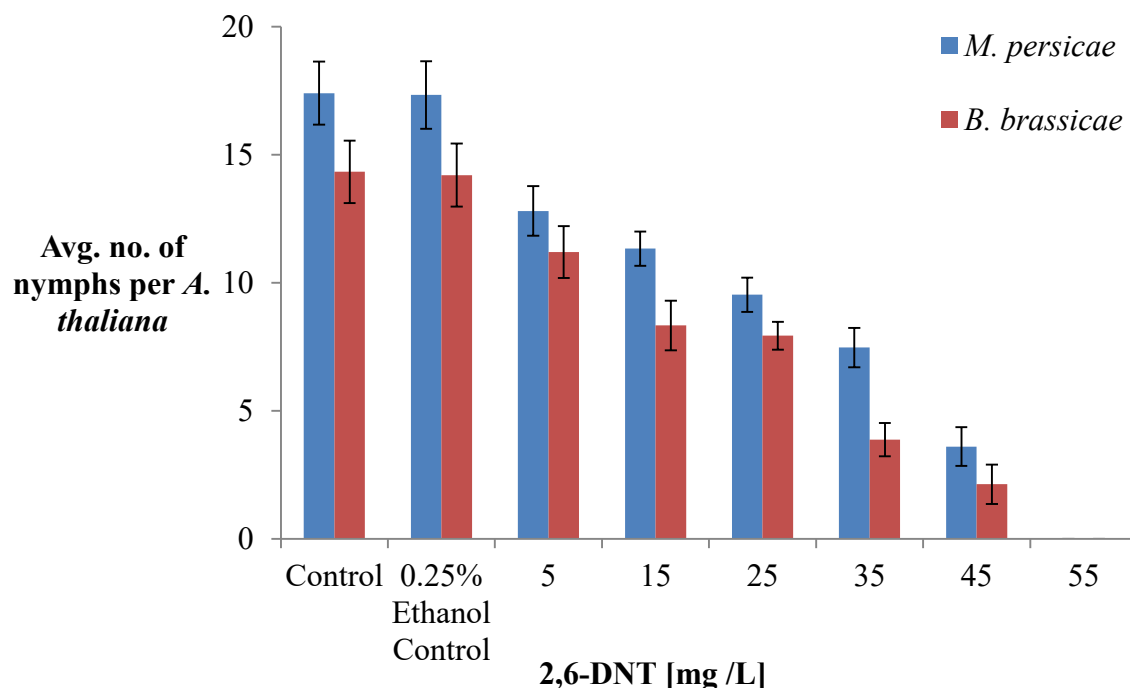


Figure 5.4: Fecundity of *M. persicae* and *B. brassicae* on Phytagel grown 2,6-DNT treated *A. thaliana*. The data is mean of no. of nymphs (produced by a single aphid) from 15 replicates (plants) at 18°C with 16:8 h day:night photoperiod. (data in Table 3 of Appendix IV).

5.3.2 *M. persicae* and *B. brassicae* on 2,4-DNT treated *A. thaliana*:

5.3.2.1 Survival:

For *M. persicae* aphids reared on 2,4-DNT treated *A. thaliana* grown in Phytagel media (Figure 5.5) an inhibition of $43.75 \pm 2.18\%$ of control in survival was observed at 0.05 mg /L of Phytagel media which reached $90.57 \pm 2.41\%$ of the control at 2,4-DNT concentration of 2.5 mg /L of Phytagel media.

The *B. brassicae* aphid survival was reduced by $39.6 \pm 2.76\%$ to that of the control at a 2,4-DNT concentration of 0.05 mg /L of Phytagel media. This reduction then increased and reached $89.59 \pm 2.18\%$ at a 2,4-DNT concentration of 1 mg /L of Phytagel media. No *B. brassicae* were recovered at 2,4-DNT concentrations of 5 mg /L of Phytagel media

The LC₅₀ value as obtained through GraphPad Prism 5 (95% CI) was **0.343 mg /L of Phytigel media** for *B. brassicae* with $r^2=0.96$ and **0.24 mg /L of Phytigel media** for *M. persicae* with $r^2=0.95$.

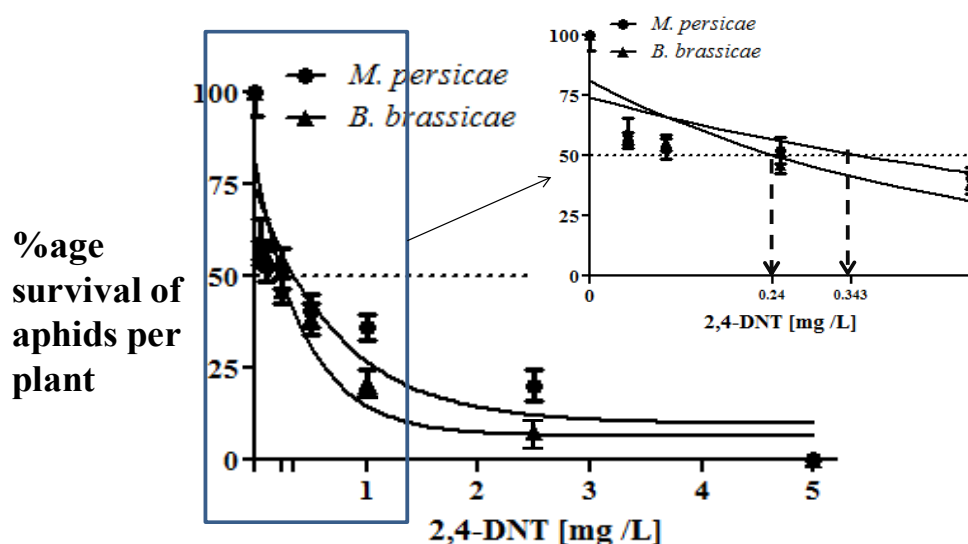


Figure 5.5: LC₅₀ determination of *M. persicae* and *B. brassicae* on 2,4-DNT treated *A. thaliana* by counting number of aphids survived (%) after 4 days of exposure to 14 day old (post cultivation) plants treated with different concentrations of 2,4-DNT (in Phytigel media) compared to control (aphids on plants with unamended Phytigel media) as analysed through a dose-response curve ($r^2=0.96$ for *M. persicae* $r^2=0.95$ for *B. brassicae*) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$; GraphPad Prism 5). The aphids were reared at a temperature of 18°C for 16:8 h day:night period. Data points are the mean of aphid survival from 15 replicates (7 aphids per treatment). The LC₅₀ for *M. persicae* and *B. brassicae* at which aphids only survived by 50% of the control was 0.343 and 0.24 mg /L of Phytigel media respectively. (data in Table 5 of Appendix IV).

5.3.2.2 MRGR

Figure 5.6 shows that no *B. brassicae* and *M. persicae* aphids survived beyond 2,4-DNT concentration of 2.5 mg /L of Phytigel media. In the case of *M. persicae*, a significant decrease in weight was observed above a 2,4-DNT concentration of 0.25 mg /L of Phytigel

media compared to *B. brassicae* but as the concentration increased *M. persicae* tolerance was more comparable (Figure 5.6) to *B. brassicae*.

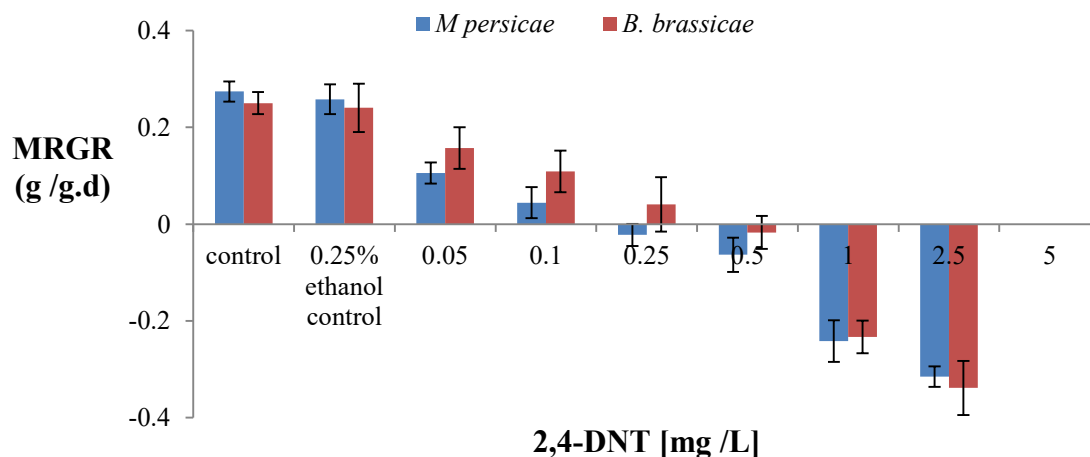


Figure 5.6: Mean Relative Growth Rate (MRGR) of *M. persicae* and *B. brassicae* on *A. thaliana* treated with 2,4-DNT in Phytigel media. The data is the mean of weight from ten aphids (on 10 replicates-12 day old plants) at 18°C with 16:8 h day:night photoperiod. The values in negative show the aphid weight loss. Units stand for mass increase (g) per aboveground mass (g) per day (d).

5.3.2.3 Time course study for reduction in weight

The weight loss in *M. persicae* is more frequent compared to the weight loss in *B. brassicae* (Figure 5.7). The results suggest that the frequency of weight gain in both *M. persicae* and *B. brassicae* was reduced with the increase in 2,4-DNT concentration in the media. In case of *B. brassicae* exposed to 2,4-DNT concentration of 2.5 mg /L of Phytigel media a very slight weight gain was observed on 4th day of exposure compared to 2nd and 6th day of exposure (Figure 5.7 B).

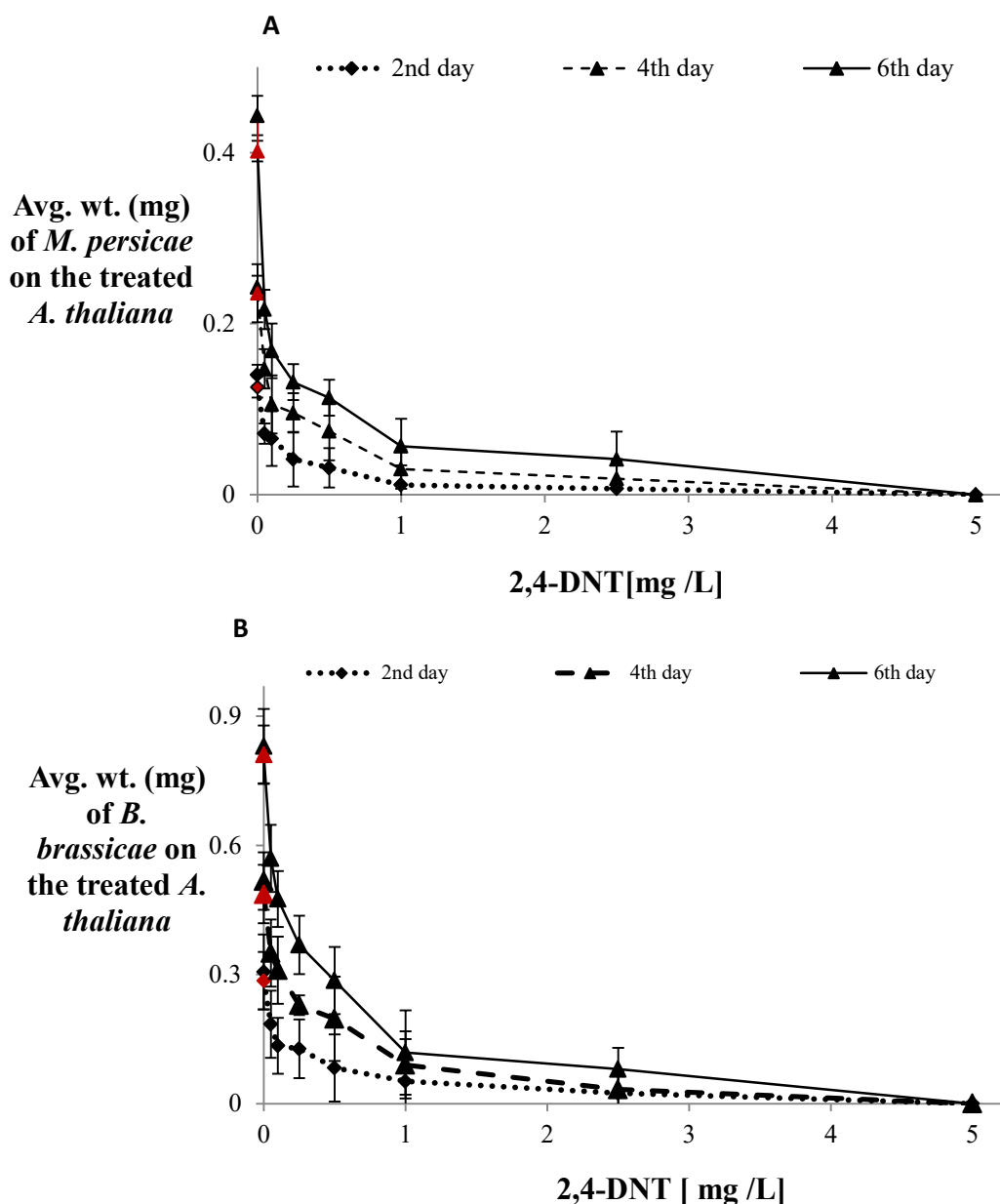


Figure 5.7: Weight (mean) of (A) *M. persicae* and (B) *B. brassicae* on Phytigel media grown *A. thaliana* against different concentrations of 2,4-DNT. The first instar nymphs were analysed after 4 days of infestation on 2,4-DNT treated *A. thaliana* (14 days old plants after 2 days of post germination). The survival data is mean and weight is average of six aphids on 10 replicates (plants) at 18⁰C with 16:8 h day:night photoperiod. The red data point represents mock control (0.25% ethanol control).

5.3.2.4 Fecundity:

M. persicae showed a reduction in fecundity of $16.54 \pm 2.13\%$ compared to the control at 2,4-DNT concentration of 0.05 mg /L of Phytigel media and this value increased and reached a

value of $76.73 \pm 2.31\%$ at 2,4-DNT concentration of 1 mg /L of Phytigel media (Figure 5.8). For *B. brassicae* the reduction in fecundity started from $9.65 \pm 2.11\%$ compared to the control at a 2,4-DNT concentration of 0.05 mg /L of Phytigel media and reached a value of $69.79 \pm 3.19\%$ at 2,4-DNT concentration of 1 mg /L of Phytigel media.

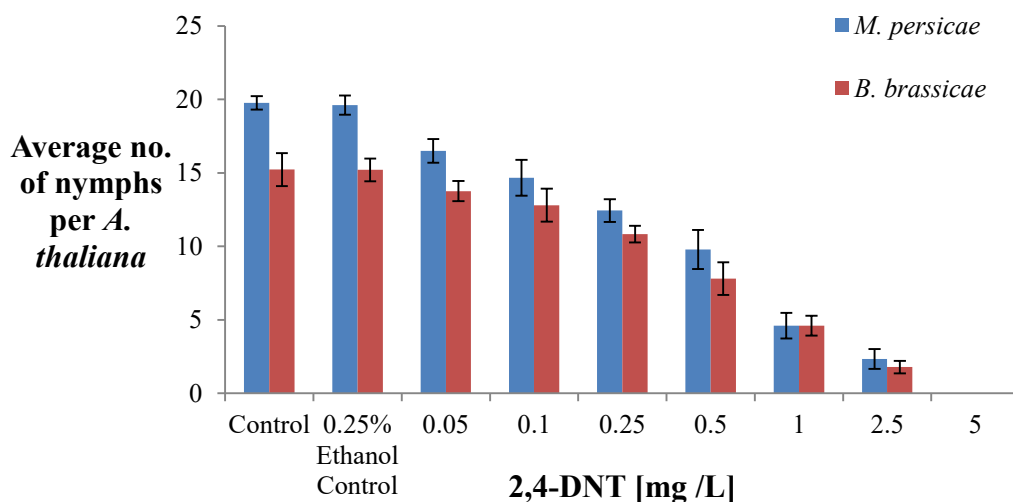


Figure 5.8: Fecundity of *M. persicae* and *B. brassicae* on Phytigel grown 2,4-DNT treated *A. thaliana*. The data is mean of no. of nymphs (produced by a single aphid) from 15 replicates (plants) at 18°C with 16:8 h day:night photoperiod.

5.3.3 *M. persicae* and *B. brassicae* on 2,3-DNT treated *A. thaliana*:

5.3.3.1 Survival:

No aphids survived on the plants treated with and beyond 2.5 mg /L of Phytigel media. The survival of *M. persicae* was reduced by $29.03 \pm 2.33\%$ compared to the control with the lowest considered concentration (0.05 mg /L) of 2,6-DNT and reaching a value of $95.71 \pm 1.98\%$ at 1 mg /L of Phytigel media (Figure 5.9).

The survival of *B. brassicae* was reduced by $59.13 \pm 1.93\%$ at the lowest considered concentration (0.05 mg /L of Phytigel media) of 2,3-DNT reaching a value of $96.28 \pm 2.21\%$

at a 2,3-DNT concentration of 1 mg /L of Phytigel media. No aphids were observed at and above a 2,3-DNT concentration of 2.5 mg /L of Phytigel media.

The LC_{50} value as obtained through GraphPad Prism 5 (95% CI) was **0.08 mg /L of Phytigel media** for *B. brassicae* with $r^2=0.975$ and **0.14 mg /L of Phytigel media** for *M. persicae* with $r^2=0.976$.

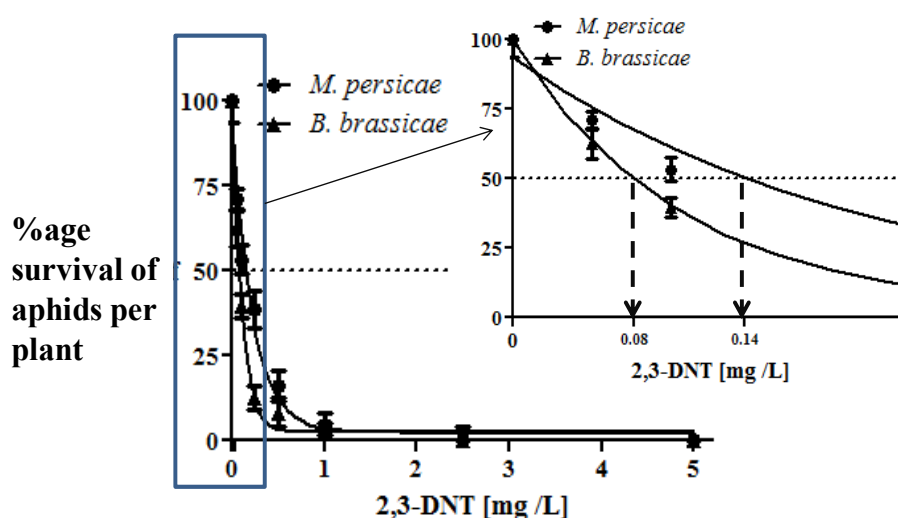


Figure 5.9: LC_{50} determination of *M. persicae* and *B. brassicae* on 2,3-DNT treated *A. thaliana* by counting number of aphids survived (%) after 4 days of exposure to 14 day old (post cultivation) plants treated with different concentrations of 2,3-DNT (in Phytigel media) compared to control (aphids on plants with unamended Phytigel media) as analysed through a dose-response curve ($r^2=0.976$ for *M. persicae* $r^2=0.975$ for *B. brassicae*) fitted for non-linear regression ($Y=Bottom + (Top-Bottom)/(1+10^{((EC50-X)*HillSlope)})$); GraphPad Prism 5). The aphids were reared at a temperature of 18°C for 16:8 h day:night period. Data points are the mean of aphid survival from 15 replicates (7 aphids per treatment). The LC_{50} for *M. persicae* and *B. brassicae* at which aphids only survived by 50% of the control was 0.14 and 0.08 mg /L of Phytigel media respectively. (data in Table 9 of Appendix IV).

5.3.3.2 MRGR:

A sudden reduction in the weight of *B. brassicae* aphids was observed when allowed to grow on 2,3-DNT treated *A. thaliana* grown in the Phytigel media (Figure 5.10). The negative

value (reduction in weight) was observed for *B. brassicae* at and beyond 2,3-DNT concentration of 0.5 mg /L of Phytigel media. For *M. persicae* this negative value was achieved at 1 mg /L of Phytigel media with no aphids recovered beyond this concentration (Figure 5.10).

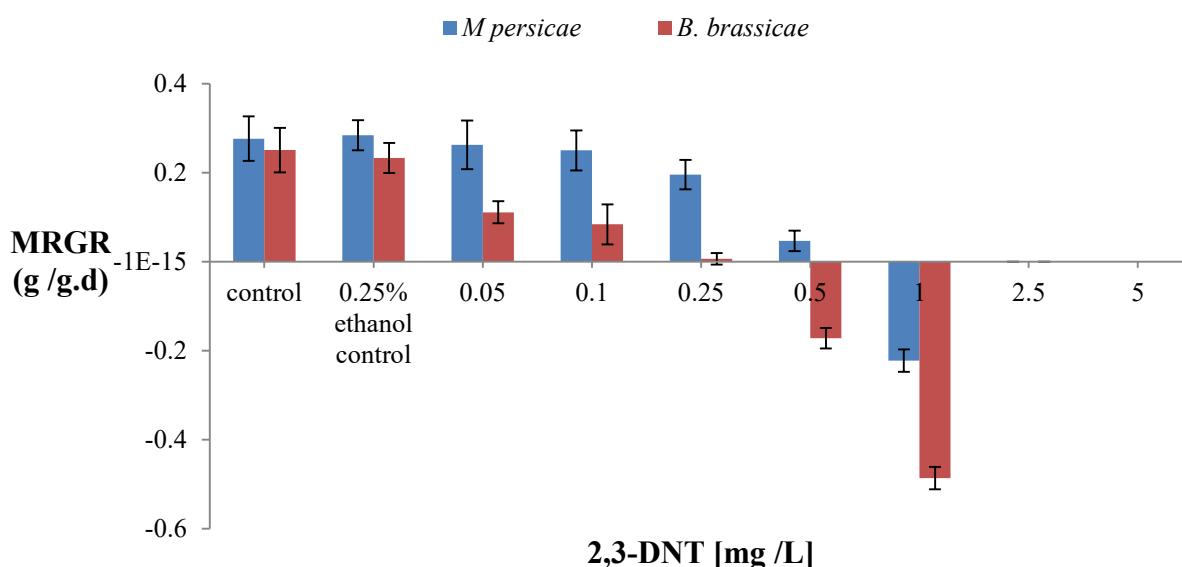


Figure 5.10: Mean Relative Growth Rate (MRGR) of *M. persicae* and *B. brassicae* on *A. thaliana* treated with 2,3-DNT in Phytigel media. The data is the mean of weight from ten aphids (on 10 replicates-12 day old plants) at 18⁰C with 16:8 h day:night photoperiod. The values in negative show the aphid weight loss. (data in Table 10 of Appendix IV).

5.3.3.3 Time course study for reduction in weight

According to the results of time course study (Figure 5.11), the weight loss in *B. brassicae* was more frequent compared to *M. persicae* when allowed to grow against different concentrations of DNT in the media. For *M. persicae*, reared on *A. thaliana* cultivated on 2,3-DNT concentration of 2.5 mg /L of Phytigel media, weight gain was observed only on 2nd day of exposure after which no aphid survived at this concentration on 4th and 6th day of exposure (Figure 5.11 A).

For both *B. brassicae* and *M. persicae* very slow weight gain was observed beyond 2,3-DNT concentration of 0.5 mg /L of Phytigel media compared to the other treatments.

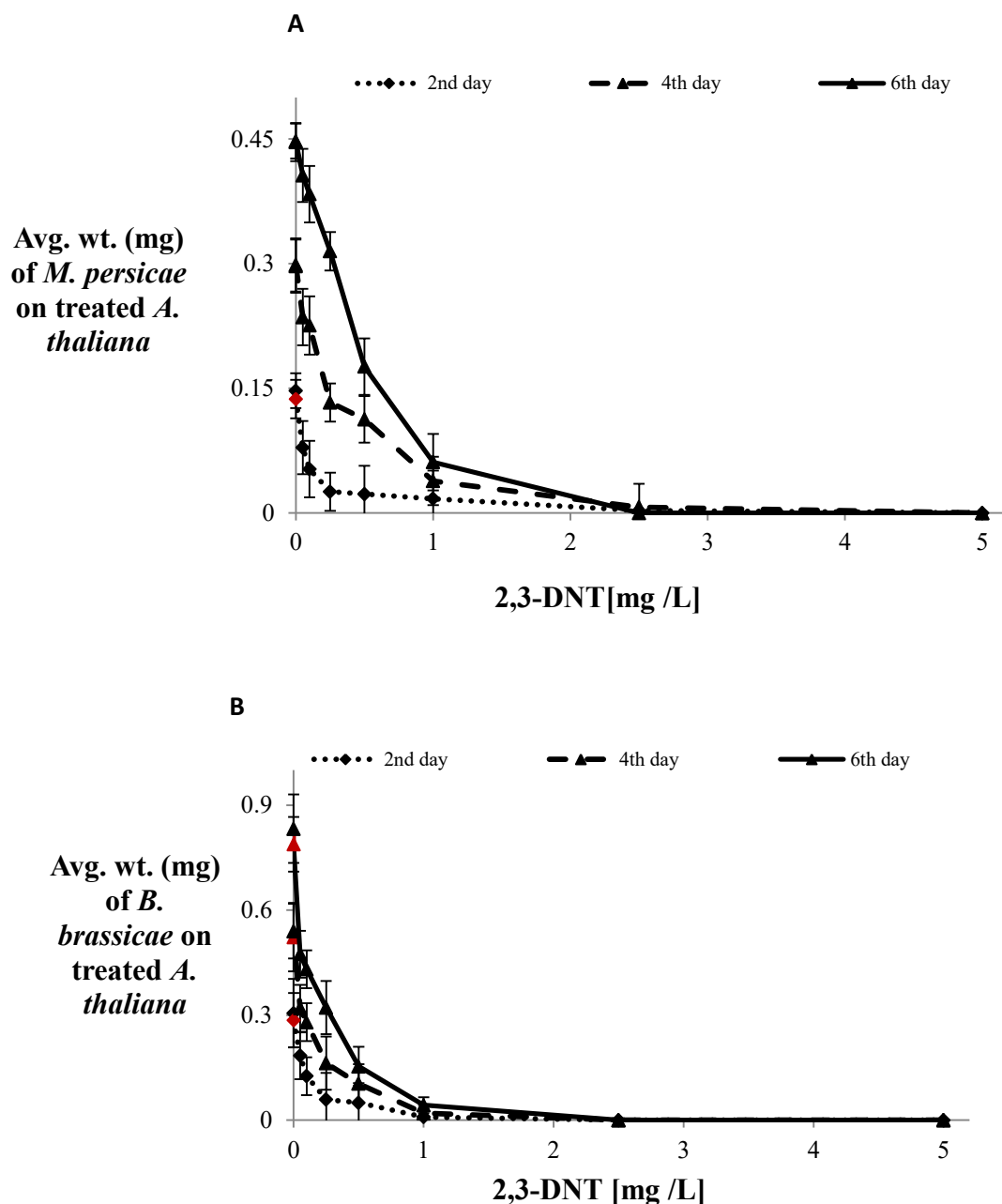


Figure 5.11: Weight (mean) of (A) *M. persicae* and (B) *B. brassicae* on Phytigel media grown *A. thaliana* against different concentrations of 2,3-DNT. The first instar nymphs were analysed after 4 days of infestation on 2,3-DNT treated *A. thaliana* (14 days old plants after 2 days of post germination). The weight is represented as the mean of six aphids on 10 replicates (plants) at 18⁰C with 16:8 h day:night photoperiod. The red data

point represents mock control (0.25% ethanol control). (data in Table 12 of Appendix IV).

5.3.3.4 Fecundity

For 2,3-DNT treated *A. thaliana*, *M. persicae* showed a reduction in fecundity by $12.49 \pm 1.99\%$ compared to control at 2,3-DNT concentration of 0.05 mg /L of Phytigel media reaching a value of $53.41 \pm 2.31\%$ at 2.5 mg /L of Phytigel media (Figure 5.12). For *B. brassicae* the reduction in fecundity started from $10.70 \pm 2.76\%$ compared to control against 2,3-DNT concentration of 0.05 mg /L of Phytigel media and reached a value of $67.43 \pm 2.16\%$ at 2,3-DNT concentration of 1 mg /L of Phytigel media.

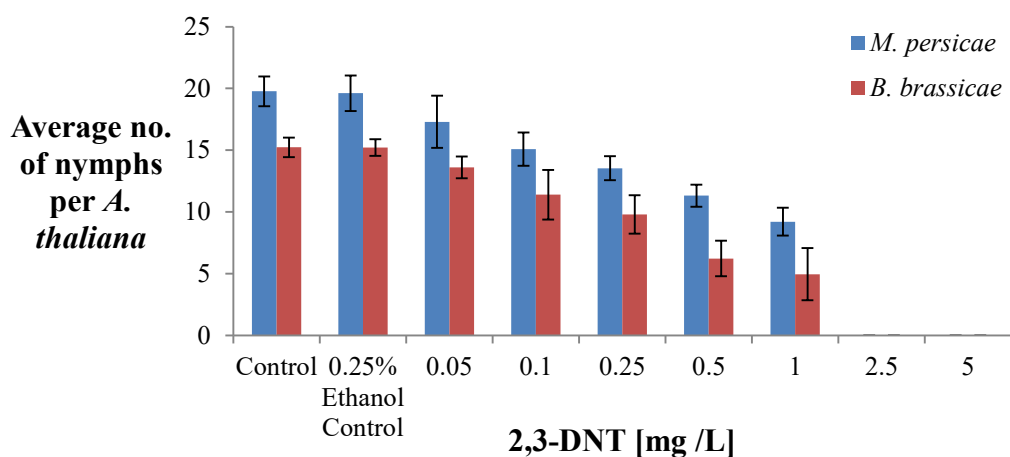


Figure 5.12: Fecundity of *M. persicae* and *B. brassicae* on Phytigel grown 2,3-DNT treated *A. thaliana*. The data is mean of no. of nymphs (produced by a single aphid) from 15 replicates (plants) at 18°C with 16:8 h day:night photoperiod.

5.3.4 *M. persicae* and *B. brassicae* on 3,4-DNT treated *A. thaliana*:

5.3.4.1 Survival:

The survival of *M. persicae* was inhibited by $33.87 \pm 2.05\%$ to that of the control against the lowest considered concentration (0.05 mg /L of Phytigel media) of 3,4-DNT and reached a value of $91.43 \pm 1.98\%$ by 2.5 mg /L of Phytigel media (Figure 5.13). No *M. persicae* aphid survived after this concentration in the media.

The survival of *B. brassicae* was inhibited by $42.55 \pm 2.06\%$ to that of control by the lowest considered concentration (0.05 mg /L of Phytigel media) of 3,4-DNT and reached a value of $94.66 \pm 2.33\%$ at 1 mg /L of Phytigel media. No aphids were observed at and beyond 3,4-DNT concentration of 2.5 mg /L of Phytigel media.

The LC₅₀ value as obtained through GraphPad Prism 5 (95% CI) was **0.1 mg /L of Phytigel media** for *B. brassicae* with $r^2=0.96$ and **0.138 mg /L of Phytigel media** for *M. persicae* with $r^2=0.92$.

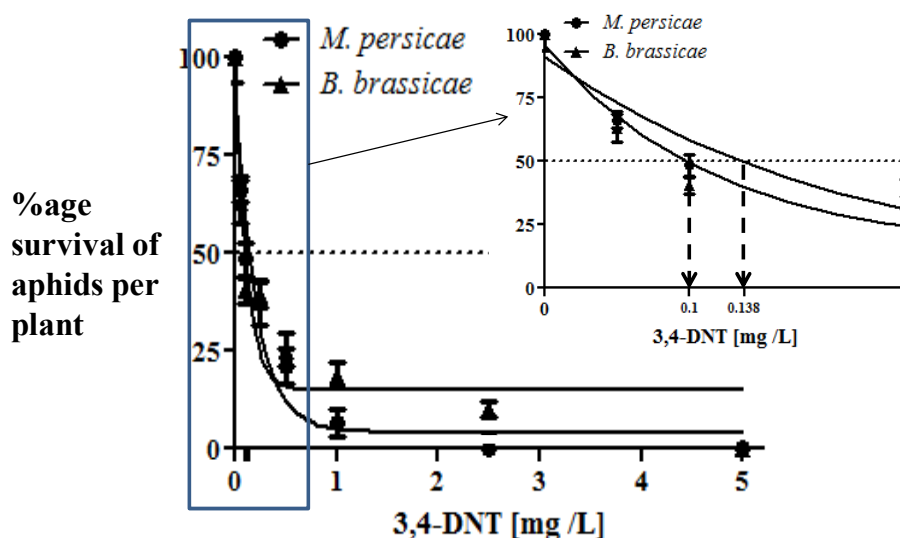


Figure 5.13: LC₅₀ determination of *M. persicae* and *B. brassicae* on 3,4-DNT treated *A. thaliana* by counting number of aphids survived (%) after 4 days of exposure to 14 day old (post cultivation) plants treated with different concentrations of 3,4-DNT (in Phytigel media) compared to control (aphids on plants with unamended Phytigel media) as analysed through a dose-response curve ($r^2=0.92$ for *M. persicae* $r^2=0.96$ for *B.*

brassicae) fitted for non-linear regression ($Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{EC}_{50} - X) * \text{HillSlope}))}$); GraphPad Prism 5). The aphids were reared at a temperature of 18°C for 16:8 h day:night period. Data points are the mean of aphid survival from 15 replicates (7 aphids per treatment). The LC₅₀ for *M. persicae* and *B. brassicae* at which aphids only survived by 50% of the control was 0.138 and 0.1 mg /L of Phytigel media respectively. (data in Table 13 of Appendix IV).

5.3.4.2 MRGR

The mean relative growth rate of *M. persicae* was more compared to *B. brassicae* throughout the different concentrations of 3,4-DNT in the media (Figure 5.14). A significant weight loss (negative value) for *B. brassicae* was observed at 3,4-DNT concentration of 1 mg /L of Phytigel media where *M. persicae* was observed to tolerate this concentration. The weight loss of *M. persicae* was observed at 3,4-DNT concentration of 2.5 mg /L of Phytigel media beyond which no aphids survived.

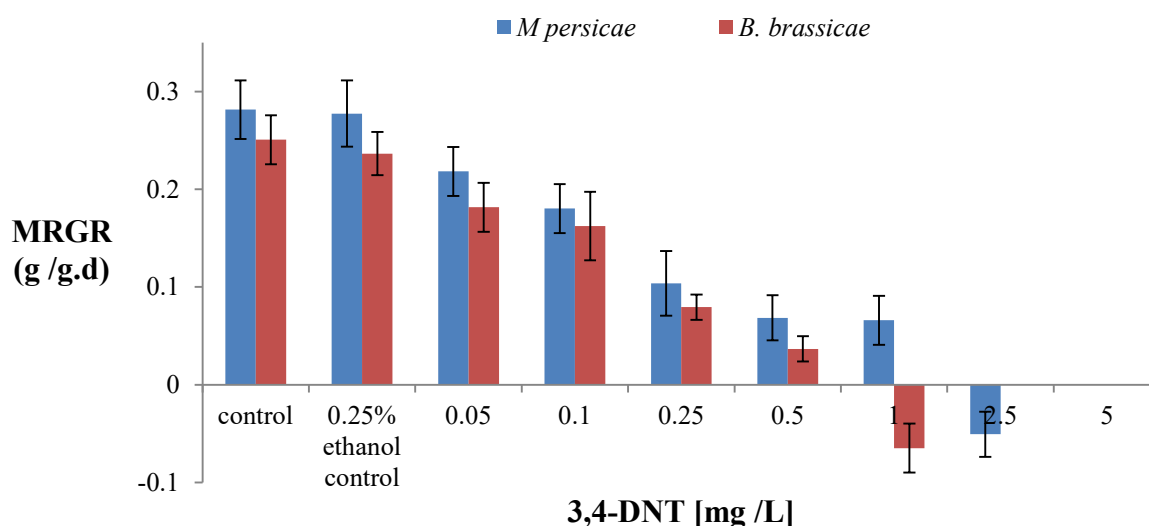


Figure 5.14: Mean Relative Growth Rate (MRGR) of *M. persicae* and *B. brassicae* on *A. thaliana* treated with 3,4-DNT in Phytigel media. The data is the mean of weight from ten aphids (on 10 replicates-12 day old plants) at 18°C with 16:8 h day:night photoperiod. The values in negative show the aphid weight loss. Units stand for mass increase (g) per aboveground mass (g) per day (d). (data in Table 14 of Appendix IV).

5.3.4.3 Time course study for the reduction in weight

For time course study of weight gain in both *M. persicae* and *B. brassicae* against different concentrations of 3,4-DNT in the media it was observed that the weight loss was more frequent in *B. brassicae* than *M. persicae*. For *M. persicae* the weight gain was observed to decrease gradually with the increase in 3,4-DNT concentration in the media with the increase in time of exposure (Figure 5.15 A). For *B. brassicae* although the weight loss was gradual at and below 3,4-DNT concentration of 1 mg /L of Phytigel media, no aphid could survive at 3,4-DNT concentration of 2.5 mg /L of Phytigel media (Figure 5.15 B).

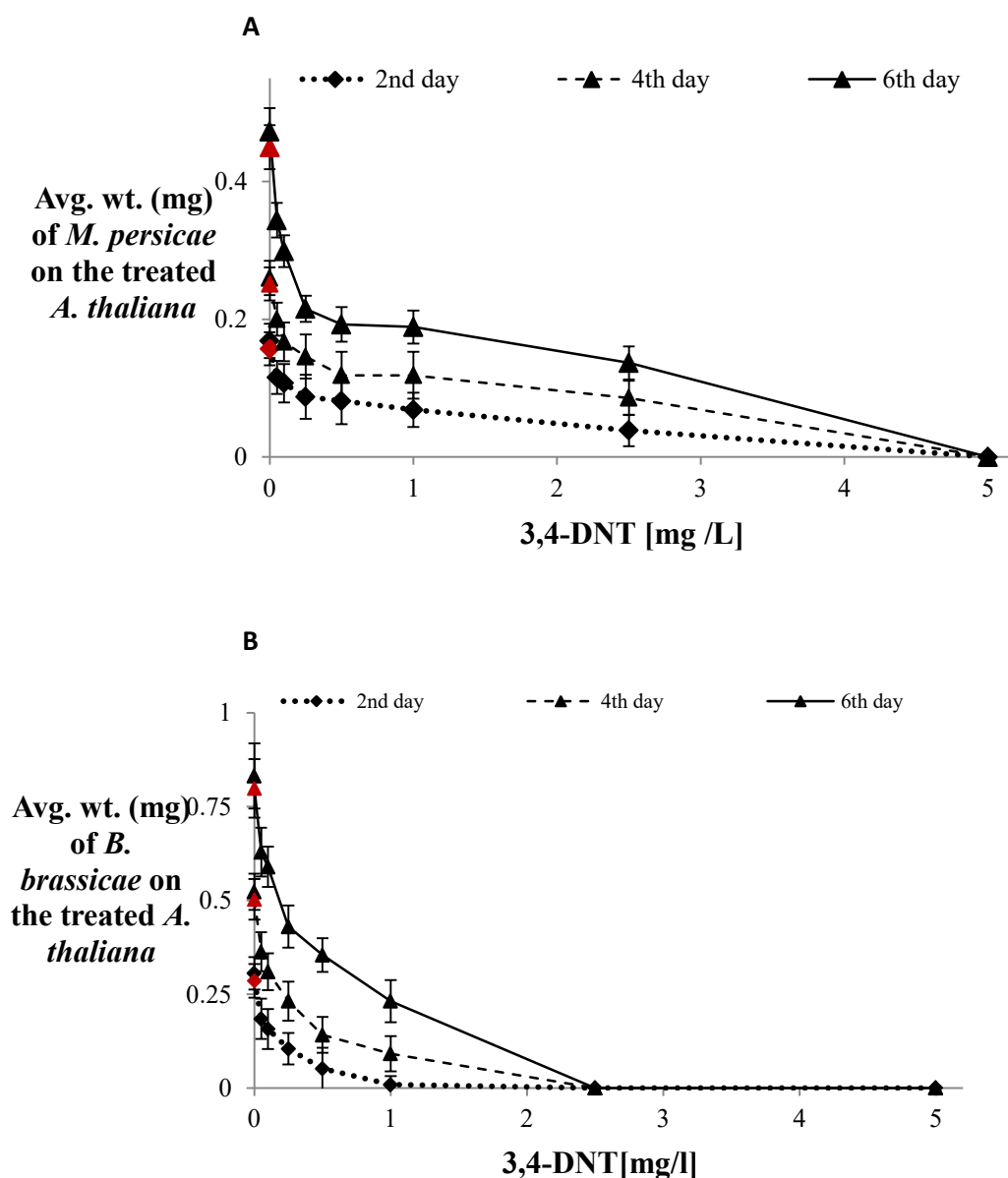


Figure 5.15: Weight (mean) of (A) *M. persicae* and (B) *B. brassicae* on Phytigel media grown *A. thaliana* against different concentrations of 3,4-DNT. The first instar nymphs were analysed after 4 days of infestation on 3,4-DNT treated *A. thaliana* (14 days old plants after 2 days of post germination). The data is the average weight of six aphids on 10 replicates (plants) at 18°C with 16:8 h day:night photoperiod. The red data point represents mock control (0.25% ethanol control).

5.3.4.4 Fecundity:

The fecundity of *M. persicae* was reduced by $10.67 \pm 2.54\%$ compared to control at 3,4-DNT concentration of 0.05 mg /L of Phytigel media and reached a value of $73.69 \pm 3.12\%$ at 3,4-DNT concentration of 1 mg /L of Phytigel media (Figure 5.16). In case of *B. brassicae* the reduction in fecundity started from $16.41 \pm 2.44\%$ compared to the control at a 3,4-DNT concentration of 0.05 mg /L of Phytigel media and reached a value of $80.43 \pm 3.11\%$ at 3,4-DNT concentration of 1 mg /L of Phytigel media. No aphids were recovered from and above 1 mg /L of Phytigel media.

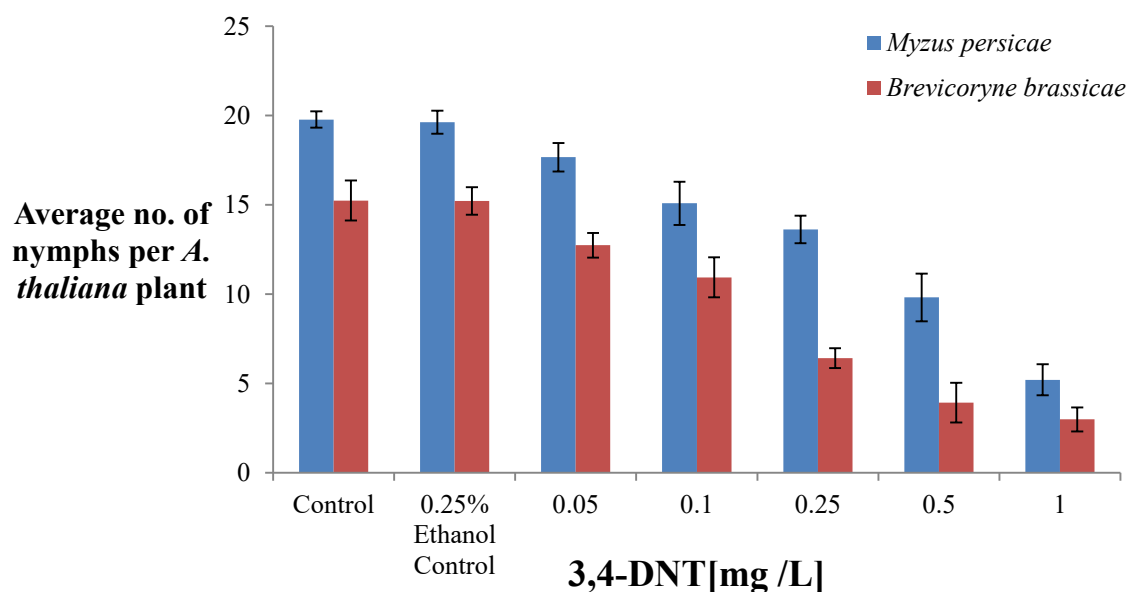


Figure 5.16: Fecundity of *M. persicae* and *B. brassicae* on Phytagel grown 3,4-DNT treated *A. thaliana*. The data is mean of no. of nymphs (produced by a single aphid) from 15 replicates (plants) at 18⁰C with 16:8 h day:night photoperiod.

5.3.5 DNT and its metabolites in *M. persicae* and *B. brassicae*:

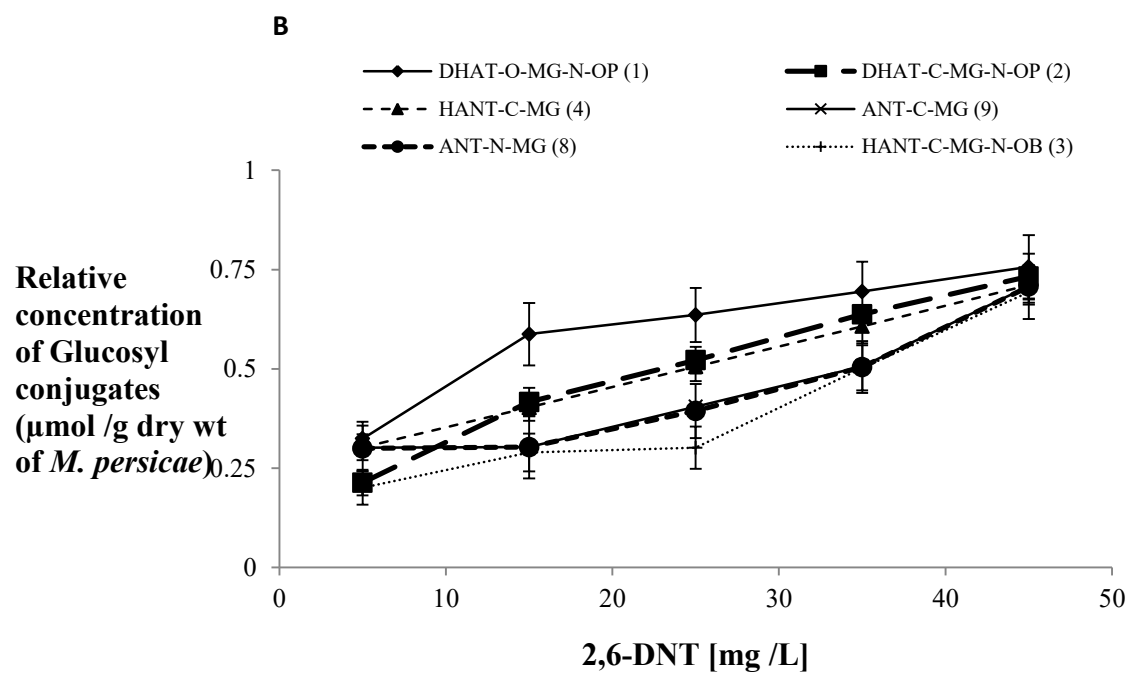
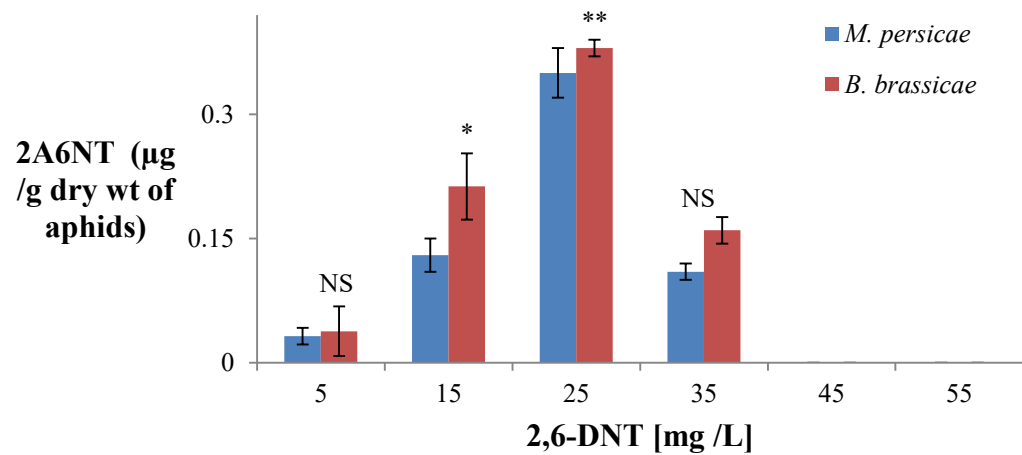
5.3.5.1 Analysis of aphids feeding on 2,6-DNT treated *A. thaliana*:

GC-MS analysis showed that both *M. persicae* and *B. brassicae* absorbed ANTs however no DNTs were detected at any stage of analysis (Figure 5.17 A). The highest ANT concentration was recovered from the aphids fed on plants treated with a 2,6-DNT concentration of 25 mg /L of Phytagel media. No ANTs were recovered from the aphids reared on the plants corresponding to the 2,6-DNT concentration of 45 and 55 mg /L of Phytagel media.

LC-MS/MS analysis showed glucosyl conjugates in both *M. persicae* and *B. brassicae*. Six conjugates (Figure 1 of Appendix IV) were recovered from aphids of both species feeding on the 2,6-DNT treated *A. thaliana*. For both the aphids the highest relative concentration of conjugates was observed at a 2,6-DNT concentration of 45 mg /L of Phytagel media. Overall higher relative concentrations of glucosyl conjugates were recovered from *B. brassicae* compared to *M. persicae*. The total concentration of all glucosyl conjugates ranged from **0.22 to 0.75 μ mol /g dry weight of *M. persicae* and 0.29 to 0.95 μ mol /g dry weight of *B. brassicae***. No conjugates were recovered from the highest considered concentration of 2,6-DNT (55 mg /L of Phytagel media).

The highest relative concentration of conjugates was recovered from aphids feeding on plants grown on a concentration of **2,6-DNT of 45 mg /L** of Phytagel media.

A



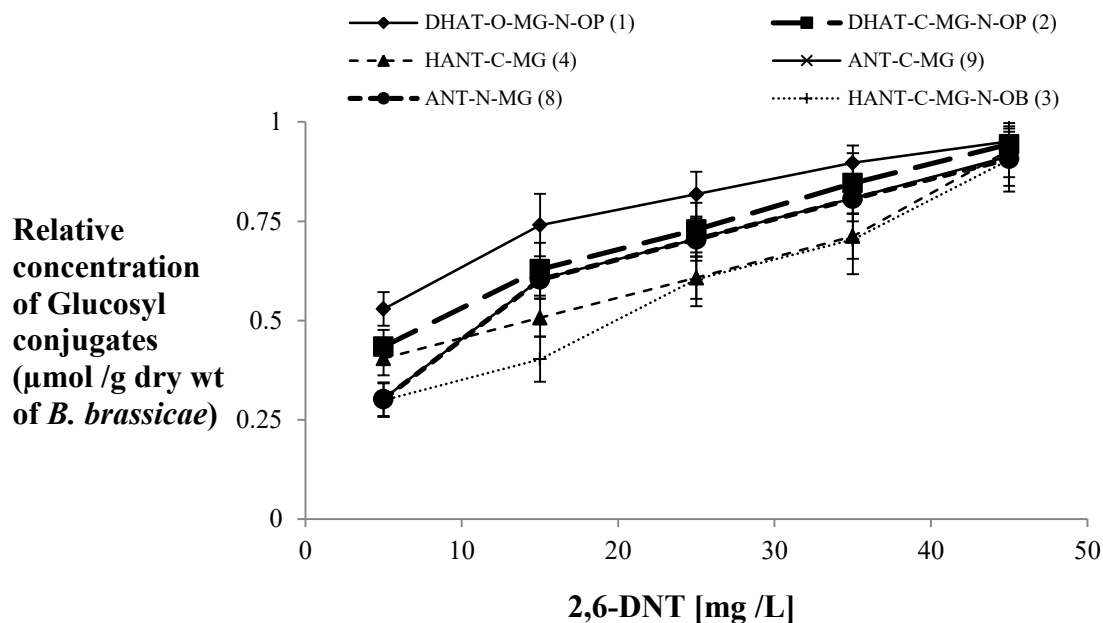


Figure 5.17: (A) 2A6NT concentrations recovered from 7 days old aphids (5 days after infestation of 2 day old nymphs) of *M. persicae* and *B. brassicae* on 14 day old *A. thaliana* plants grown in different concentrations of 2,6-DNT in sterile Phytigel media (growth temperature was 18⁰C for 16:8 h day:night period) and analysed by GC-MS. Data was plotted as a mean of three replicates (aphids from one plant were combined to give one replicate). The aphids on control and 0.25% mock control treated plants were analysed with exactly the same procedures as DNT treated plants and the values of all results are shown in Table 17, 18 and 19 of Appendix IV (NS= not significant, * = significant ($p < 0.05$) and ** = highly significant ($p < 0.005$)). Relative concentrations of glucosyl conjugates of DNT in (B) *M. persicae* (C) *B. brassicae* recovered and analysed by LC-MS/MS from the samples as described in GC-MS experiment. Data points are the mean of three replicates. Dihydroxyaminotoluene-*O*-monoglycoside-*N*-oxopropanone (DHAT-*O*-MG-*N*-OP), Dihydroxyaminotoluene-*C*-monoglycoside-*N*-oxopropanone (DHAT-*C*-MG-*N*-OP), Hydroxyaminonitrotoluene-*C*-monoglycoside (HANT-*C*-MG), Aminonitrotoluene-*C*-monoglycoside (ANT-*C*-MG), Aminonitrotoluene-*N*-monoglycoside (ANT-*N*-MG), hydroxyaminonitrotoluene-*C*-monoglycoside-*N*-oxobutanoate (HANT-*C*-MG-*N*-OB).

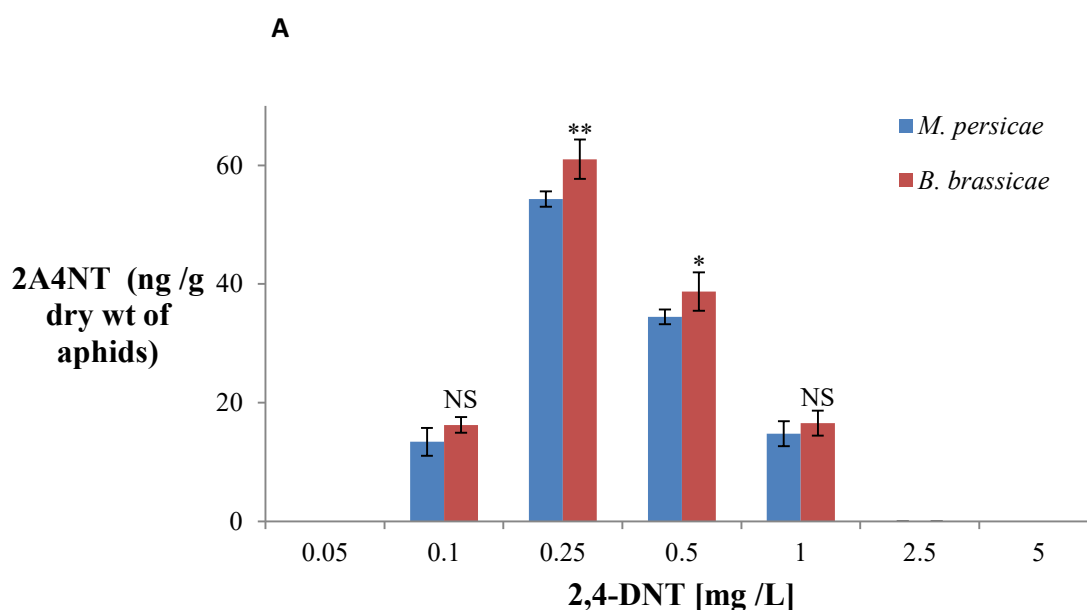
5.3.5.2 Metabolites in aphids feeding on 2,4-DNT treated *A. thaliana*

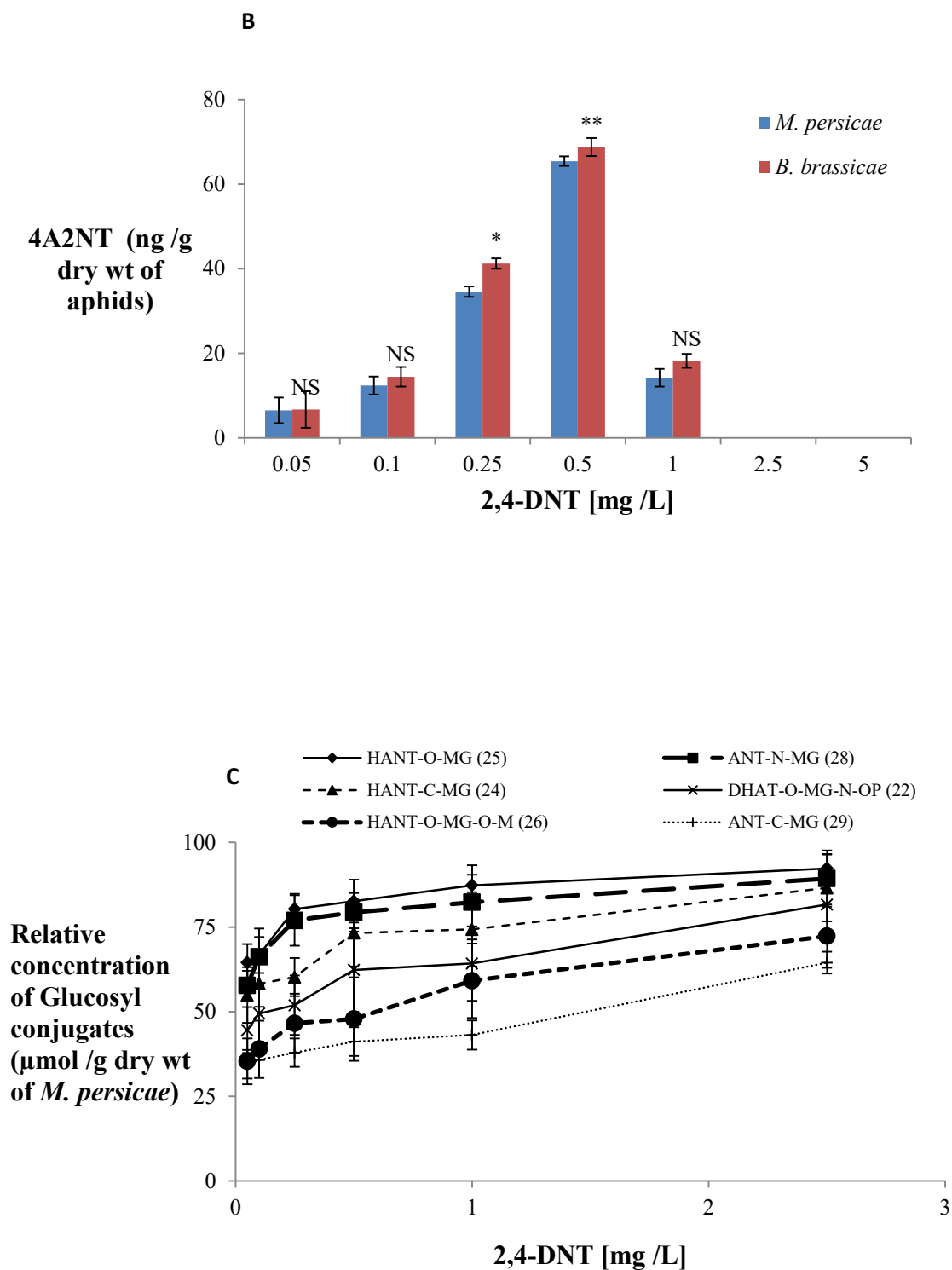
The results from the GC-MS analysis showed that both *M. persicae* and *B. brassicae* had absorbed 2A4NT and 4A2NT from 2,4-DNT treated *A. thaliana*, however no DNT was detected (Figure 5.18). 2A4NT was not present in aphids feeding on plants treated with 0.05mg /L of Phytigel media. The highest 2A4NT concentration was obtained from the aphids feeding on plants treated

with a 2,4-DNT concentration of **0.25 mg /L of Phytigel media** and highest 4A2NT concentration was obtained from the aphids at 0.5 mg /L of Phytigel media. No ANT_s were recovered from the aphids reared on the plants at 2,4-DNT concentrations of 1mg /L of Phytigel media and above.

Glucosyl conjugates were also obtained from both *M. persicae* and *B. brassicae* species. Six conjugates (Figure 1 of Appendix IV) were recovered from the aphids of both species feeding on the 2,4-DNT treated *A. thaliana*. For both the aphids the highest relative concentration of conjugates was observed corresponding to 2,4-DNT concentration of 2.5 mg /L of Phytigel media and no conjugates were obtained from concentrations higher than that. Overall *B. brassicae* had a greater total concentration of all the conjugates compared to *M. persicae*. The concentration of all glucosyl conjugates ranged from **34.45 to 92.33 nmol /g dry weight of *M. persicae*** and **35.67 to 95.34 nmol /g dry weight of *B. brassicae*** which shows only a marginal difference.

DNT_s were not detected in aphids on plants corresponding to a 2,4-DNT concentration of 2.5 mg /L of Phytigel media; however, the highest relative concentration of conjugates was recovered from the aphids feeding on plants treated at this concentration.





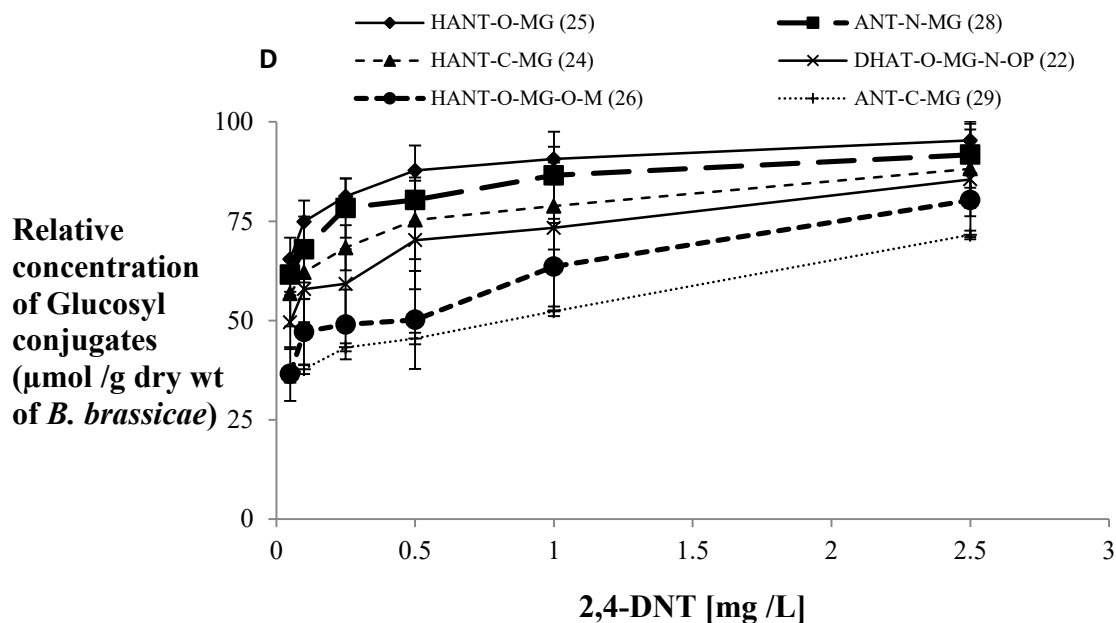


Figure 5.18: (A) 2A4NT and (B) 4A2NT concentrations recovered from 7 days old aphids (5 days after infestation of 2 day old nymphs) of *M. persicae* and *B. brassicae* on 14 day old *A. thaliana* plants grown in different concentrations of 2,4-DNT in sterile Phytigel media (growth temperature was 18⁰C for 16:8 h day:night period) and analysed by GC-MS. Data was plotted as a mean of three replicates (aphids from one plant were combined to give one replicate). The control and 0.25% mock control treated plants were analysed with exactly the same procedures as DNT treated plants and the values of all results are shown in Table 20, 21 and 22 of Appendix IV (NS= not significant, * = significant ($p < 0.05$) and ** = highly significant ($p < 0.005$)). Relative concentrations of glucosyl conjugates of DNT in (C) *M. persicae* (D) *B. brassicae* recovered from the samples as described for aminonitrotoluenes (ANTs) and analysed by LC-MS/MS. Data points are the mean of three technical replicates. Dihydroxyaminotoluene-*O*-monoglycoside-*N*-oxopropanone (DHAT-*O*-MG-*N*-OP), Hydroxyaminonitrotoluene-*O*-monoglycoside (HANT-*O*-MG), Hydroxyaminonitrotoluene-*O*-monoglycoside-*O*-malonyl (HANT-*C*-MG-*O*-M), Aminonitrotoluene-*C*-monoglycoside (ANT-*C*-MG), Aminonitrotoluene-*N*-monoglycoside (ANT-*N*-MG), Hydroxyaminonitrotoluene-*C*-monoglycoside (HANT-*C*-MG).

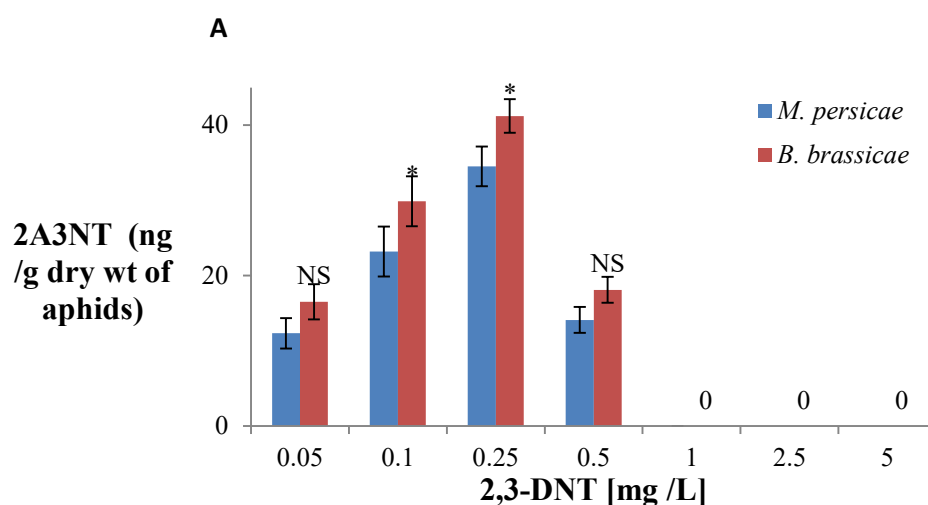
5.3.4.3 Metabolites in aphids feeding on 2,3-DNT treated *A. thaliana*:

The results showed that highest 2A3NT from both *M. persicae* and *B. brassicae* was observed from the aphids at plants having 2,3-DNT concentration of 0.25 mg /L of Phytigel media (Figure

5.19). No ANTs were recovered from the aphids reared on the plants at a 2,3-DNT concentration of 1mg /L of Phytigel media and above.

Glucosyl conjugates were also obtained from both *M. persicae* and *B. brassicae* species. For both the aphids the highest relative concentration of conjugates was observed corresponding to 2,3-DNT concentration of 2.5 mg /L of Phytigel media and no conjugates were obtained at concentrations above that. The concentration of all glucosyl conjugates ranged from **33.01 to 95.33 nmol /g dry weight of *M. persicae*** and **45.63 to 96.34 nmol /g dry weight of *B. brassicae***.

DNTs were not detected in aphids feeding on plants corresponding to 2,3-DNT concentration of 2.5 mg /L of Phytigel media; however, the highest relative concentration of conjugates in the aphid was at this concentration (5.19).



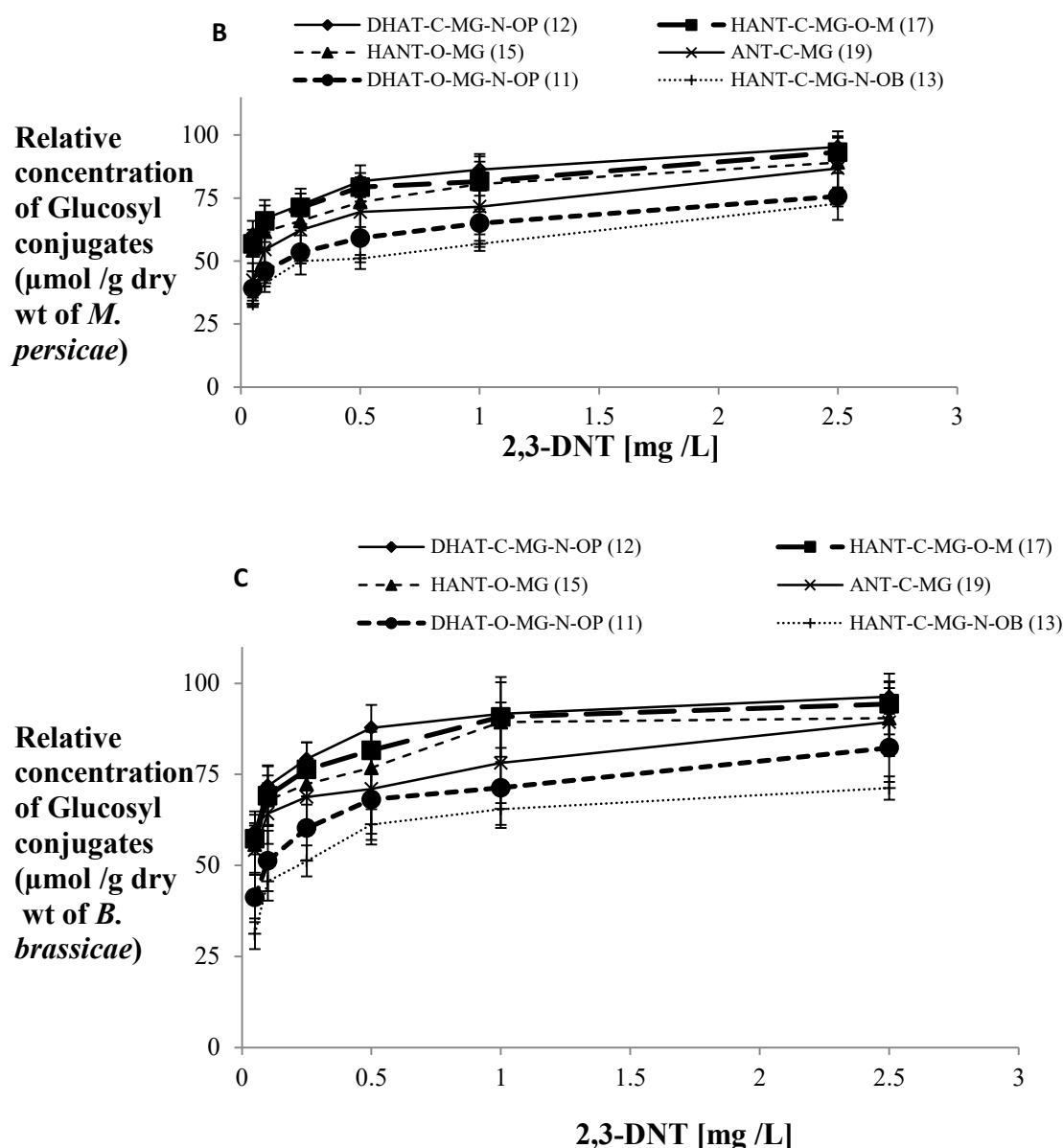


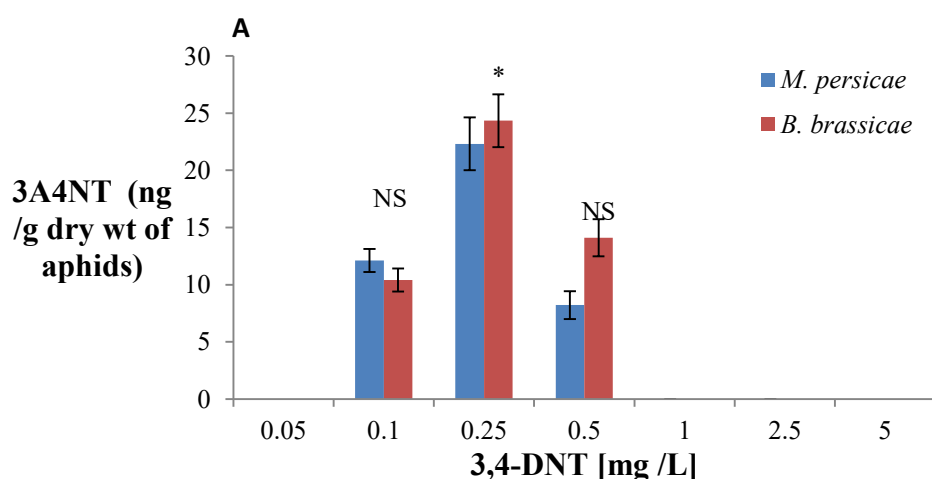
Figure 5.19: (A) 2A3NT concentrations recovered from 7 days old aphids (5 days after infestation of 2 day old nymphs) of *M. persicae* and *B. brassicae* on 14 day old *A. thaliana* plants grown in different concentrations of 2,3-DNT in sterile Phytigel media (growth temperature was 18⁰C for 16:8 h day:night period) and analysed by GC-MS. Data was plotted as a mean of three replicates (aphids from one plant were combined to give one replicate). The control and 0.25% mock control treated plants were analysed with exactly the same procedures as DNT treated plants and the values of all results are shown in Table 24, 25 and 26 of Appendix IV (NS= not significant, * = significant ($p < 0.05$) and **= highly significant ($p < 0.005$)). Relative concentrations of glucosyl conjugates of DNT in (B) *M. persicae* (C) *B. brassicae* recovered from the samples as described for aminonitrotoluenes (ANTs) and analysed by LC-MS/MS. Data points are the mean of three replicates. Dihydroxyaminotoluene-*C*-monoglycoside-*N*-oxopropanone (DHAT-*C*-MG-*N*-OP), Dihydroxyaminotoluene-*O*-monoglycoside-*N*-oxopropanone (DHAT-*O*-MG-*N*-OP), Hydroxyaminonitrotoluene-*O*-monoglycoside

(HANT-*O*-MG), Aminonitrotoluene-*C*-monoglycoside (ANT-*C*-MG), hydroxyaminonitrotoluene-*C*-monoglycoside-*N*-oxobutanone (HANT-*C*-MG-*N*-OB).

5.3.4.4 Metabolites in aphids feeding on 3,4-DNT treated *A. thaliana*

The highest 3A4NT concentration was obtained from the aphids at plants at a concentration of 3,4-DNT of 0.25 mg /L of Phytigel media. ANTs were not detected from the aphids fed on plants at a 3,4-DNT concentration of 1 mg /L of Phytigel media and above (Figure 5.20).

Six glucosyl conjugates recovered from the aphids of both aphid species feeding on the 3,4-DNT treated *A. thaliana* had highest relative concentration corresponding to 3,4-DNT concentration of 2.5 mg /L of Phytigel media and no conjugates were obtained from concentration higher than that. Overall *B. brassicae* had a greater concentration of total conjugates compared to *M. persicae*. The concentration of total conjugates ranged from 34.32 to 91.78 nmol /g dry weight of *M. persicae* and 35.12 to 92.34 μ mol /g dry weight of *B. brassicae*, which does not show high difference.



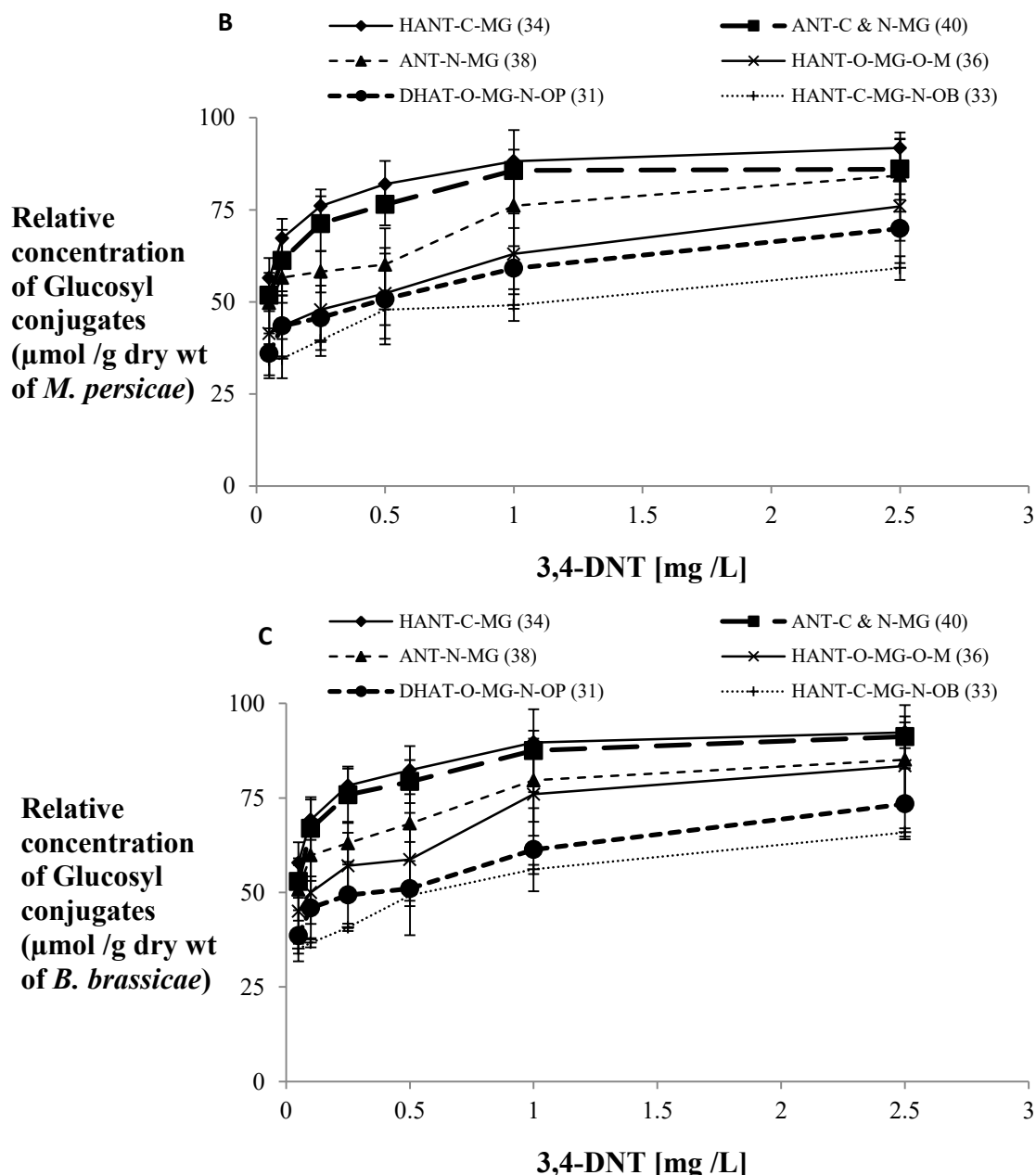


Figure 5.20: (A) 3A4NT concentrations recovered from 7 days old aphids (5 days after infestation of 2 day old nymphs) of *M. persicae* and *B. brassicae* on 14 day old *A. thaliana* plants grown in different concentrations of 3,4-DNT in sterile Phytigel media (growth temperature was 18°C for 16:8 h day:night period) and analysed by GC-MS. Data was plotted as a mean of three replicates (aphids from one plant were combined to give one replicate). The control and 0.25% mock control treated plants were analysed with exactly the same procedures as DNT treated plants and the values of all results are shown in Table 27, 28 and 29 of Appendix IV (NS= not significant, * = significant ($p < 0.05$) and ** = highly significant ($p < 0.005$)). Relative concentrations of glucosyl conjugates of DNT in (B) *M. persicae* (C) *B. brassicae* recovered from the samples as described for aminonitrotoluenes (ANTs) and analysed by LC-MS/MS. Data points are the mean of three technical replicates. Aminonitrotoluene-*N*-monoglycoside (ANT-*N*-

MG), Hydroxyaminonitrotoluene-*C*-monoglycoside (HANT-*C*-MG), Dihydroxyaminonitrotoluene-*O*-monoglycoside-*N*-oxopropanone (DHAT-*O*-MG-*N*-OP), Aminonitrotoluene-*C* and *N*-diglycoside (ANT-*C* & *N*-DG), Hydroxyaminonitrotoluene-*C*-monoglycoside-*N*-oxobutanoate (HANT-*C*-MG-*N*-OB), Hydroxyaminonitrotoluene-*O*-monoglycoside-*O*-malonyl (HANT-*O*-MG-*O*-M).

5.4 Discussion:

The findings of the previous Chapters (Chapter 3 and 4) have shown the uptake of DNT and its transformation and storage of metabolites in plants. If DNT metabolites are stored in the plants then it is likely that they are available to the herbivore feeders. This increases the chance of hyperaccumulation of DNT based metabolites in the ecosystem via the predation of contaminated food. The present study considered a model system of aphid (arthropods being abundant) and plant interaction to show the distribution of DNT based metabolites at two trophic levels. The survival (LC₅₀), growth and fecundity of specialist (*B. brassicae*) and generalist (*M. persicae*) morphs on *A. thaliana* were analysed to observe their response to the contaminated food source. For surviving aphids (especially ones below LC₅₀ value), the metabolites (both amines and glucosylated conjugates) were observed in total body tissue which is a strong indicator that these metabolites can pass to the next trophic level e.g. ladybirds (*Adalia punctata*) (Francis et al., 2000).

The results showed that all fitness parameters for both the aphids were affected by the DNT isomers present in the plant growth media. At the highest concentration in the plant growth media, aphids were not able to survive. Both ANT_s and glucosylated ANT_s were also observed in the aphid tissue. This is in agreement with the findings of previous studies where plant based contaminants have been shown to be absorbed in feeding aphids (Pineda et al., 2012; Fonseca et al., 2011; Laurent and Rathahao, 2003).

The survival of both *M. persicae* and *B. brassicae* was analysed when they were allowed to grow on DNT treated *A. thaliana* to show if aphids could tolerate the treatment. In the case of tolerance it is highly likely that contaminants may get transported to different trophic levels throughout the ecosystem.

When the survival of aphids in relation to the contaminated plants was compared, it was found that overall *M. persicae* was more resistant to the treatment compared to *B. brassicae* (*M. persicae* survived at 3,4-DNT concentration of 2.5 mg /L however *B. brassicae* did not survive at and beyond this concentration). One of the reasons for this difference in survival could be due to the greater body mass of *B. brassicae* compared to *M. persicae*, they feed more frequently and therefore accumulate more metabolites than *M. persicae*. This results in increased mortality of the *B. brassicae* aphids compared to *M. Persicae* (Blanch et al., 2012; Pineda et al., 2012). The resistance of *M. persicae* over *B. brassicae* has been shown in several studies where it has been explained that since *M. persicae* can survive at low available food sources (Boquel et al., 2012; Kos et al., 2012) therefore, it is highly likely that before the next feed, the aphid based metabolic system may be able to degrade the ANTs which are more toxic then conjugates themselves. Further studies have supported the existence of detoxifying enzymes (cytochrome P450 monooxygenases, esterases, glutathione S-transferases) in *M. persicae* which actively metabolise the contaminants to enhance the resistance in aphids (Castaneda et al., 2010; Bass and Field, 2011).

When LC₅₀ values obtained for *B. brassicae* and *M. persicae* feeding on treated *A. thaliana* were compared, it was observed that 2,3-DNT is most toxic for the both aphid species and 2,6-DNT was the least toxic (Table 5.1). Among different aphid species, *M. persicae* has a high tolerance to contamination compared to *B. brassicae*. This resistance in *M. persicae* towards various xenobiotics compared to other aphid species has been demonstrated in previous studies (Criniti et al., 2008; Foster et al., 2012; Simpson et al., 2012). The low

binding affinity of radio-labelled imidacloprid to *M. persicae* nicotinic acetylcholine receptor has been shown to induce high resistance to neonicotinoid insecticide compared to other susceptible aphid clones (Bass et al., 2011).

Table 5.1: LC₅₀ for *B. brassicae* and *M. persicae* feeding on treated *A. thaliana*.

Aphids	2,6-DNT	2,4-DNT	2,3-DNT	3,4-DNT
<i>B. brassicae</i>	11.4	0.24	0.08	0.1
<i>M. persicae</i>	13.9	0.343	0.14	0.138

*all the readings were in mg /L of Phytigel media

The MRGR of aphids showed a loss in body mass, which is indicative of the fact that the aphids are starving (due to reversible anti-feeding since their feeding patterns are highly dependent on taste and stylet probing activity) or the DNT metabolites are directly toxic and impact on digestive biochemistry. The reduced plant biomass with the increase in DNT could also be one of the reasons for the weight loss in aphid. The phloem contains sugars and amino acids which aphids need as a food source. This nutrient source becomes limited in case of contaminated and unhealthy plants resulting in aphid to starvation. The results of MRGR showed that *M. persicae* was more resistant than *B. brassicae*. The anti-feeding response of *M. persicae* to imidacloprid (insecticide) has been reported to reduce the MRGR compared to the aphids on untreated plants (Cardoza et al., 2006).

The weight loss was more frequent in *B. brassicae* aphids with the increasing concentration of 2,6-DNTs in the plants compared to *M. persicae* (Figure 5.2). The trend was opposite for 2,4-DNT treated *A. thaliana* infested with both the aphid species where *M. persicae* lost weight more frequently with the increase in concentration of DNT in the media (Figure 5.6). Further *B. brassicae* could not survive at and above 3,4-DNT concentrations of 2.5 mg /L of Phytigel media despite having a reduced rate of weight loss with the increase in 3,4-DNT

concentrations (Figure 5.4). Here this study shows the survival of both aphids varies with the isomers of DNT. It is likely that *M. persicae* aphids were more resistant to the metabolites of certain DNT isomers, unlike *B. brassicae*. The resistance in aphids to different substrates has been demonstrated (Slater et al., 2012) but the no previous study explains specificity for different structural isomers in *M. persicae* and *B. brassicae*. However, carboxylesterase E4 isolated from *M. persicae* or in crude aphid homogenate was able to hydrolyze the stereo isomer- (1*S*)*trans* enantiomer of permethrin (3-phenoxybenzyl (1*RS*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2-dimethylcyclopropanecarboxylate, a pyrethroid, an insecticide) and showed no activity towards other isomers (Dovenshire et al., 2004). These also determine the resistance of aphids towards specific isomers compared to the other where it has been shown that certain enzymes such as Cytochrome P450 in aphids can efficiently metabolise the toxicants (Puinean et al., 2010).

The time course study for aphid weight loss showed that for 2,6-DNT treated *A. thaliana*, the *M. persicae* and *B. brassicae* weight decreased gradually with the increasing concentration and time of exposure (Figure 5.3) which may be again due to anti-feeding response or the toxicants were gradually reducing the cuticular proteins. In a study (Andersen, 2010) the cuticular sclerotization due to hydroxylated phenolic compounds has been shown to affect the development at larval stage. So it was assumed that reduced DNT metabolites might also affect the cuticular sclerotization, therefore affecting the aphid weight. In the case of 2,4-DNT, a sudden weight loss was observed at a 2,4-DNT concentration of 1 mg /L (Figure 5.7), which indicates that the 2,4-DNT at and above this concentration was intolerable for aphids and they were struggling to survive may be due to fact that each ANT isomer induced mortality in aphids. At a 2,3-DNT concentration of 2.5 mg /L of Phytagel, *M. persicae* survived on 2nd day of analysis, which shows that metabolic machinery of aphids was metabolising the compounds on 2nd day (Figure 5.11). However they were observed to die as

the exposure prolonged to 4th and 6th day, which indicates the excessive accumulation of DNT metabolites results in death. No time-course study for reduction in the weight *M. persicae* and *B. brassicae* weight has been reported to date, therefore, the data cannot be compared with previous work.

The findings of the present study reveal that *B. brassicae* aphids have accumulated more metabolites than *M. persicae*. Another important aspect is the reduced availability of ANT_s at high concentration of DNT_s where glucosyl conjugates are more predominant. There are two likely reasons for this variation in metabolites. One is that more glucosyl conjugates could be available at these concentrations compared to the ANT_s in plants which aphids were acquiring. The sequestration of thioglucosides (glucosinolates) by aphids from plants has been reported earlier (Kazana et al., 2007) where these compounds are compartmentalized. Another reason of more glucosyl conjugates compared to ANT_s as obtained from aphids is that the aphid body themselves can conjugate the absorbed ANT_s. Previously Capsaicin β -glucoside has been isolated from the faeces of *Helicoverpa armigera*, *Helicoverpa zea* and *Helicoverpa assulta*, structures confirmed through NMR analysis, when aphids were fed on artificial diet with capsaicin (8-methyl -*N*-vanillyl-6- nonenamide; pest deterrent) (Ahn et al., 2012).

At lower concentrations, the metabolites may be in the intermediate stage of conversion to conjugates and that is why they were available more. Another important thing to note here was that each both *M. persicae* and *B. brassicae* infested on different plants contaminated with different isomers of DNT was observed to have different glycosylated conjugates in them. This shows that either they are acquiring the intense conjugates of plant themselves through phloem feeding or the specific ANT_s only yield those specific conjugates.

Overall this study concludes that survivorship (Figure 5.21), growth and fecundity of *M. persicae* and *B. brassicae* are affected by DNT contaminated food although at less concentration they were able to absorb and metabolise the toxicants. The dead aphids also had DNT based metabolites in them, which shows that DNT metabolites can potential be transported throughout the ecosystem.

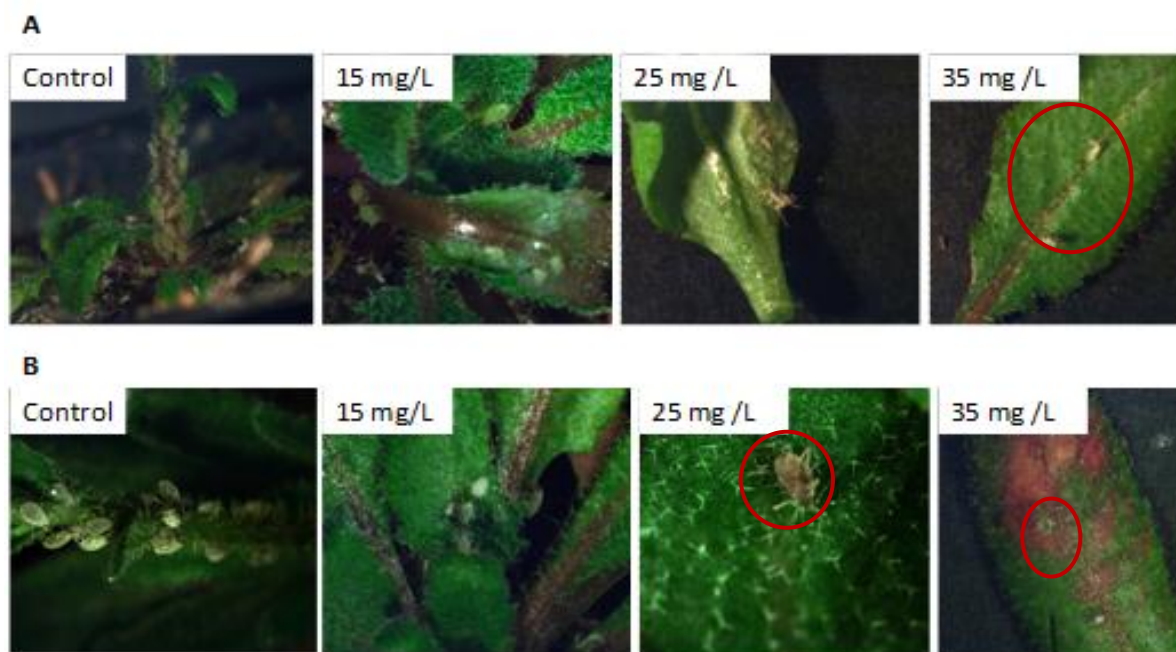


Figure 5.21: (A) *M. persicae* and (B) *B. brassicae* at 14 days old 2,6-DNT treated plants against control. Aphids showed survivorship at *A. thaliana* treated with low concentration of 2,6-DNT and died at high concentrations. Red circle shows the dead aphids.

Chapter 6

General discussion

Studies related to plant toxicity towards nitroaromatics published to date have shown that nitroaromatics follow sequential three phases of detoxification in plant cells with each reaction compartmentalized in the cell and assisted through different plant based enzymes (Mezzari et al., 2005; Gandia-Herrero et al., 2008). The present study was designed to show these phases of detoxification of four different structural isomers of dinitrotoluene (DNT) in the model plant *A. thaliana*. This was achieved using two growth systems. The first involved using Phytigel under sterile growth conditions to avoid external interactions with for example soil microbes. The second system used was hydroponics system, which allowed experiments to be carried out in non axenic environment (potentially lesser external influence than soil based experiments). The uptake, metabolism and conjugation of DNTs in *A. thaliana* with their associated hazardous effects on plants and finally transportation throughout the ecosystem when herbivores are allowed to feed on the contaminated plants through a model system of contaminant, plant and aphid interaction, have been elaborated in the present study. The results indicated that DNT in the plant growth media is absorbed by plants, metabolised conjugated and finally transported to the aphids feeding contaminated plants.

The major phytotoxic activity of DNTs is the inhibition in cell based metabolism and cell division which correlates with the information available in literature (Neuwoehner et al., 2007; Yoon et al., 2007, Rocheleau et al., 2010).

Plant cells possess a battery of enzymes to metabolise xenobiotics in order to achieve tolerance necessary for their survival. Enzymatic reduction mechanisms have been reported as initial steps towards metabolism of nitroaromatics such as DNTs. The metabolism of 2,4-

DNT and 2,6-DNT to monoaminotoluenes has been reported in earlier studies (Yoon et al., 2006 and 2007; Tognetti et al., 2007; Roberts et al., 2007). However the metabolic studies of 2,3-DNT and 3,4-DNT in plants has never been reported.

The present study was designed to observe the toxicities and metabolism of different DNT isomers to *A. thaliana*. Previous studies have demonstrated (Picka and Friedl, 2004, Rocheleau et al., 2006 and 2010; Dokken and Davis, 2011) showed the differences in toxicity of 2,6-DNT and 2,4-DNT towards different plant species. Bacteria can also metabolise both 2,4-DNT and 2,6-DNT (Figure 6.1) and were shown to vary from each other (Spanggord et al., 1991; Nishino et al., 2000; Lewis et al., 2004) in that the former undergoes complete denitration (Figure 6.1 A) prior to ring cleavage and the latter loses one nitro group and then undergoes ring opening (Figure 6.1 B).

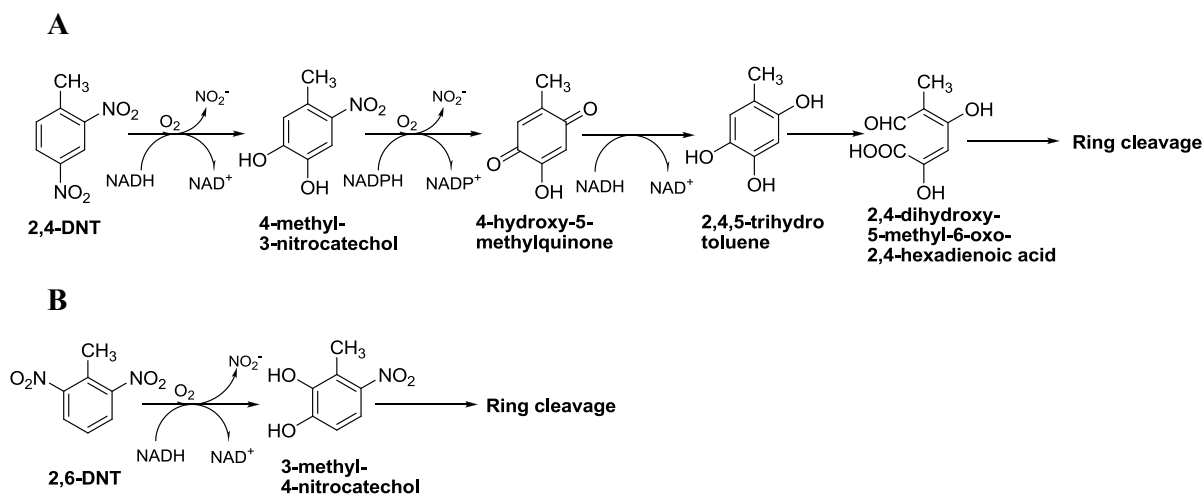


Figure 6.1: Oxygenase-mediated (A) 2,4-DNT and (B) 2,6-DNT metabolism in *Pseudomonas* sp (Spanggord et al., 1991; Lewis et al., 2004).

Though the DNTs transformation in plants and bacteria are not comparable bacteria use DNT as energy source (Figure 6.1) while plants lack the degradative enzymology that would allow ring opening and subsequent release of energy. Thus in contrast to bacteria plants have to

invest a considerable amount of energy into the detoxification processes with the net result a biochemical burden.

6.1 Toxicity of different isomers of DNT

The toxicity data for 2,6-DNT and 2,4-DNT in *A. thaliana* has already been identified, however, 3,4-DNT and 2,3-DNT have never been studied in *A. thaliana*. So the first task was to establish the toxicity data of unknown DNT isomers for *A. thaliana* and for this purpose the EC₅₀ values for all the DNT isomers was determined in Phytigel and hydroponic media through root growth analysis and root and shoot ratio. Finally the time-course study was conducted to show the variation in toxicity over a period of exposure. The Phytigel media was chosen to observe only plant based interactions with DNT and hydroponic media was used to see the variations if the plant is in an non-axenic media (with less microbial interactions than soil).

The results of the present study have shown that the toxicity of all isomers varied in *A. thaliana* (2,6-DNT least toxic and 2,3-DNT most toxic). It was speculated that the toxicity of intermediate products (aminonitrotoluenes) during DNT transformation might be the cause of their variation in toxicity levels. The toxicity of aminonitrotoluenes (ANTs) have been shown in the literature (Picka and Friedl, 2004) where monoaminonitrotoluenes have been shown to be more toxic than the parent DNT molecule while diaminotoluenes have been shown to be the least toxic when tested against wheat (*Triticum aestivum*), mustard (*Sinapis alba*), lettuce (*Lactuca sativa*) and lentil (*Lens culinaris*).

6.2 Identification of DNT based metabolites in *A. thaliana* and their comparison with toxicity data

2,6-DNT and 2,4-DNT based ANTs (2-amino-6-nitrotoluene and 2-amino-4-nitrotoluene, respectively) have been identified from 2,4-DNT and 2,6-DNT fed *A. thaliana* and other

plant species (Tognetti et al., 2007; Yoon et al., 2006). However 2,3-DNT and 3,4-DNT based ANTs have not been reported.

Comparing the EC₅₀ value and rate of metabolites for all DNT isomers, it was observed that the highest concentration of ANTs was slightly higher than the EC₅₀ value when ANTs per g or ANTs per plant were analysed (e.g. the highest concentration of 2A6NT was obtained from the plants grown at a 2,6-DNT concentration of 23 mg /L of Phytagel media, whereas the EC₅₀ for 2,6-DNT was 13 mg /L of Phytagel media). The concentrations of DNT based conjugates were observed to be independent of EC₅₀ value for all DNT isomer (almost all of the 2,6-DNT based conjugates were highest corresponding to 2,6-DNT concentration of 45 mg /L of Phytagel media whereas EC₅₀ was 13 mg /L of Phytagel media), however, they were found to be highly dependent on the concentration of ANTs and DNTs in the treated *A. thaliana*.

6.3 Comparison of concentration of ANTs and DNT based conjugates from *A. thaliana*

The comparison of the concentration of the DNT based conjugates in *A. thaliana* fed with different isomers showed that the total conjugate concentration was far in excess of the concentration of ANTs (2,6-DNT conjugates are almost 70 times more than 2A6NT concentration; Table 6.1). The conjugation of nitroaromatics with glucose and malonate has been observed in several studies (Ekman et al., 2003; Melissa et al., 2005; Gandia-Herrero et al., 2008; Bartha et al., 2010). In case of all DNT isomers the concentration of conjugates was found to be 10² times more than ANTs recovered from same weighed plants treated with similar concentration of DNTs in media. The results indicate that the conjugation reactions play a key role in the detoxification of DNT. Among the different DNT isomers, 2,4-DNT appeared to be readily metabolised according to the findings of Chapter 3 it has been shown to be converted to two metabolites (4A2NT and 2A4NT).

Table 6.1: Range of different DNT based metabolites against the concentrations of DNT in the media.

DNT isomers	Range in the Phytigel /liquid MS media	Range of ANT metabolites	Range of conjugates
2,6-DNT	27.47 - 302.19	3.5 - 34	0.56 – 2.5
2,4-DNT	0.27 - 27.47	0.13 - 1.84	3.2×10^{-2} – 0.14
2,3-DNT	0.27 - 27.47	0.21 - 4.4	3.5×10^{-2} – 0.20
3,4-DNT	0.27 - 27.47	0.27 – 3.7	5.3×10^{-2} – 0.14

*the DNT concentration media is in nM

**the Range of ANT's and conjugates is $\mu\text{mol/g}$ dry tissue wt. of *A. thaliana*

Hydroxylamino based DNT metabolites (HANTs) have never been reported due to their instability. However, it was found that most of the conjugates were associated with hydroxylamino intermediates of DNT compared to ANT's. Since hydroxylamino intermediates of many nitroaromatics are extremely reactive (Ekman et al., 2003; Zhu et al., 2012) then conjugation is an essential mechanism to remove the HANTs. Higher concentrations of conjugates have been reported with other nitroaromatics such as safeners, TNT and RDX compared to their reduced metabolites DeRidder et al., 2002, Nepovim et al., 2004; Melissa et al., 2005; Naumann et al., 2010).

6.4 Possible conjugation of DNT metabolites with GSTs:

The relative concentration of different DNT based conjugates was also very low compared to their concentration in the media. It was considered that the conjugates might have been sequestered into the cell wall of the plant cells limiting their availability through analysis. Another reason could be their conjugation with glutathione. Although nitroamines have been

shown to have limited conjugation with glutathione and prefer conjugation with glucose moiety (UDP- glycosides through glucosyltransferases) and malonic acid (malonate through malonyltransferases) (Brazier-Hicks et al., 2007; Diamon et al., 2010). To observe the conjugation of DNT metabolites with glutathione a preliminary study was conducted through *in situ* labelling of 2,6-DNT treated *A. thaliana* with monochlorobimane (MCB). MCB acts as xenobiotic and combines with glutathione (GSH) to form glutathione γ -S-bimane (GSB) and therefore a competition for GSH to bind with MCB and xenobiotic appears. The dye fluoresces under a multiphoton microscope only when GSB is formed, so reduction in intensity of fluorescence due to competition in MCB and xenobiotics and control can be detected to show GSH conjugation (Melissa et al., 2005).

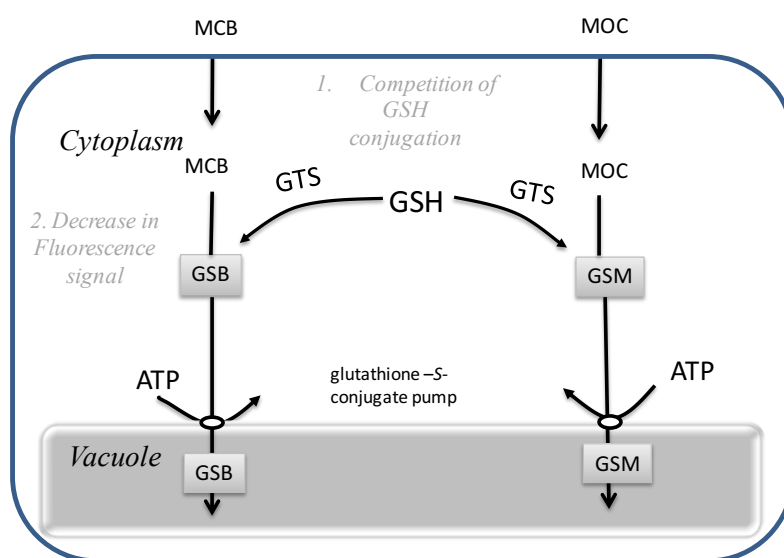


Figure 6.2: Formation of GSB offers a competition for GSH binding to xenobiotics (Melissa et al., 2005).

An intact root of 8 day old *A. thaliana* seedling treated with 5 mg /L of Phytigel media (for 6 days) and intact root of untreated 8 day old *A. thaliana* seedling was placed on a glass slide and transferred to a drop of MCB (100 mM stock prepared in DMSO and diluted to 5 mM through distilled water) with NaN_3 (depletes ATP concentration and avoid GSB vacuolar sequestration). The coverslip was placed gently to avoid root crushing and sealed (with

Vaseline, lanolin, paraffin at 1:1:1 ratio) to avoid evaporation. The fluorescence was measured soon after the treatment with fluorescence of GSB was recorded at $\lambda_{em}=770\text{nm}$. The results showed high intensity colour in controls compared the treated *A. thaliana*, which shows a competition in GSH availability due to MCB and 2,6-DNT. The experiment was conducted for three replicates.

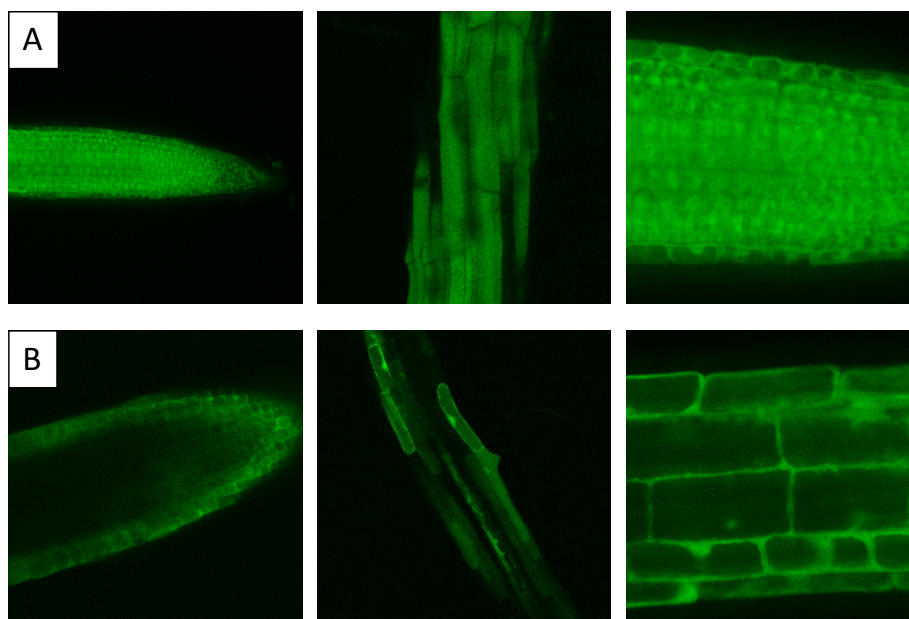


Figure: (A) Images for control (untreated roots) which shows high concentration of GSB due to MCB (5) 5 mg 2,6-DNT /L of Phytigel media treated *A. thaliana* roots showing less intensity of fluorescence due to competition in GSH binding: A preliminary experiment.

6.5 Trophic level interactions of DNT and its metabolites:

A trophic level study was conducted through oligophagus- *Brevicoryne brassicae* and polyphagus *Myzus persicae* to show the impact of DNT treated *A. thaliana* on aphids feeding on them. The results of the study showed that the aphids survival, fecundity and weight was highly compromised due to the contaminated diet further the aphids had both ANT's and DNT based conjugates in their body tissues.

It was considered that EC₅₀ value as calculated through root growth analysis might also impact the aphid survival (LC₅₀). The results suggested that *M. persicae* had an almost similar value of LC₅₀ as EC₅₀ for all DNT isomers (LC₅₀ for *M. persicae* for 2,6-DNT is 13.9 mg /L of Phytigel media and EC₅₀ for *A. thaliana* is 13.3 mg /L of Phytigel media), however the LC₅₀ for *B. brassicae* though less than *M. persicae* but followed the same trend of tolerance to different isomers (2,6-DNT be least toxic and 2,3-DNT highest toxic) (Table 6.2). This shows that at EC₅₀ the amount of toxicant in plant was enough to induce 50 % inhibition in aphids. This shows that aphid survival was highly related to the survival of plants.

Table 6.2: EC₅₀ (DNT isomer mg /L of Phytigel media) for *A. thaliana* and LC₅₀ (mg /L of Phytigel media) for *B. brassicae* and *M. persicae* to different isomers of DNT as calculated through non-linear regression in GraphPad Prism 5.

DNT isomer	EC ₅₀ for <i>A. thaliana</i>	LC ₅₀ for <i>M. persicae</i>	LC ₅₀ for <i>B. brassicae</i>
2,6-DNT	13.3	13.9	11.4
2,4-DNT	0.33	0.34	0.24
3,4-DNT	0.23	0.14	0.10
2,3-DNT	0.13	0.14	0.08

*all readings were in mg /L of Phytigel media

When the concentrations of ANT_s in *A. thaliana* were compared with the concentrations of ANT_s in both *M. persicae* and *B. brassicae*, it was observed that both the aphids had absorbed a small amount of ANT_s from the plants (Figure 6.3). Interestingly the highest concentrations of ANT_s was obtained at 2,6-DNT concentration of 23 and 25mg /L of Phytigel media.

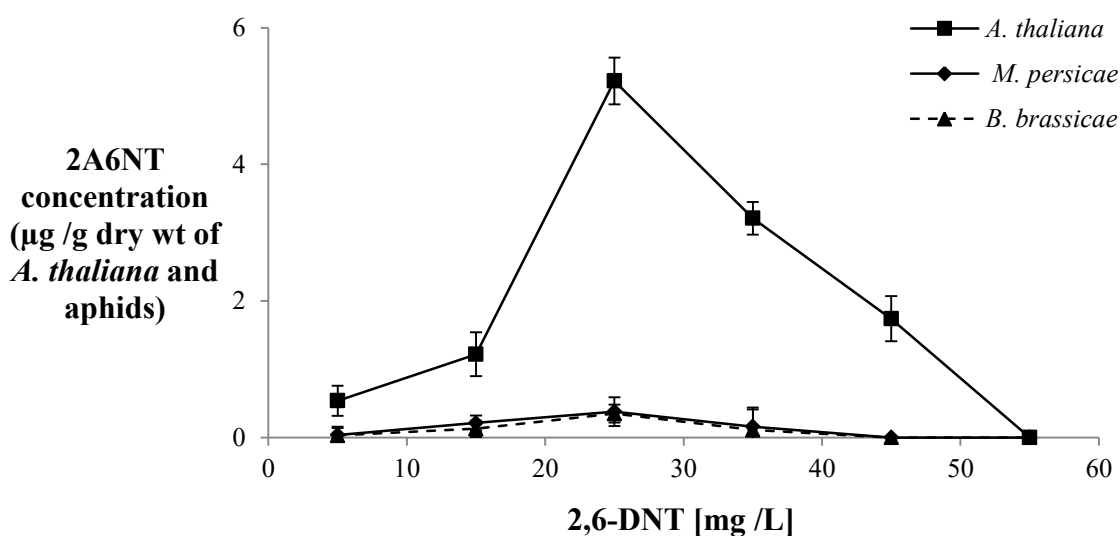


Figure 6.3: Concentration of 2A6NT in *A. thaliana*, *M. persicae* and *B. brassicae* as obtained through GC-MS analysis.

When the relative concentrations of DNT based conjugates was observed among *A. thaliana* and aphids feeding on the treated plants, it was seen that aphids have a similar conjugate profiles as in the plants (with highest glucosides in plants are also highest in aphids) (Table 6.3).

Table 6.3: The highest relative concentrations of first four highest DNT based conjugates as obtained from *A. thaliana*, *M. persicae* and *B. brassicae*

DNT isomer	Glucoside conjugates	Highest concentration in <i>A. thaliana</i>	Highest concentration in <i>M. persicae</i>	Highest concentration in <i>B. brassicae</i>
2,6-DNT	DHAT-O-MG-N-OP (1)	2.51	0.76	0.95
	DHAT-C-MG-N-OP (2)	2.26	0.73	0.94
	HANT-C-MG (4)	2.14	0.71	0.93
	ANT-C-MG (9)	2.05	0.71	0.71

2,3-DNT	DHAT-C-MG-N-OP (12)	204.34	95.33	96.34
	HANT-C-MG-O-M (17)	187.32	93.22	94.33
	HANT-O-MG (15)	182.50	89.12	90.42
	ANT-C-MG (19)	174.33	86.77	89.34
2,4-DNT	HANT-O-MG (25)	202.34	92.33	95.34
	ANT-N-MG (28)	192.34	89.32	91.76
	HANT-C-MG (24)	180.94	86.56	88.22
	DHAT-O-MG-N-OP (22)	156.71	81.62	85.56
3,4-DNT	HANT-C-MG (34)	197.68	91.78	92.34
	ANT-C & N- DG (40)	189.79	86.02	91.21
	ANT-N-MG (38)	185.01	84.32	85.11
	HANT-O-MG-O-M (36)	176.61	75.96	83.45

*all the 2,6-DNT based glucosides are in $\mu\text{mol/g}$ dry tissue wt. of *A. thaliana*

** the rest of the glucosides are in nmol/g dry tissue wt. of *A. thaliana*

This shows that either aphids are acquiring the plant based metabolites or the respective glucosides are based on different isomers as they undergo the specific conjugation. The glucosylation of capsaicin (Capsaicin β -glucoside) isolated from *Helicoverpa armigera*, *Helicoverpa zea* and *Helicoverpa assulta* which confirmed the glucosylation activity in aphids (Ahn et al., 2011).

The present study therefore concludes the overall pathway of DNT metabolism in plant cells with the enzymatic interactions as extracted from available literature (Colemann et al., 1997; Melissa et al., 2005; Hannink et al., 2007) as described in the Figure 6.5.

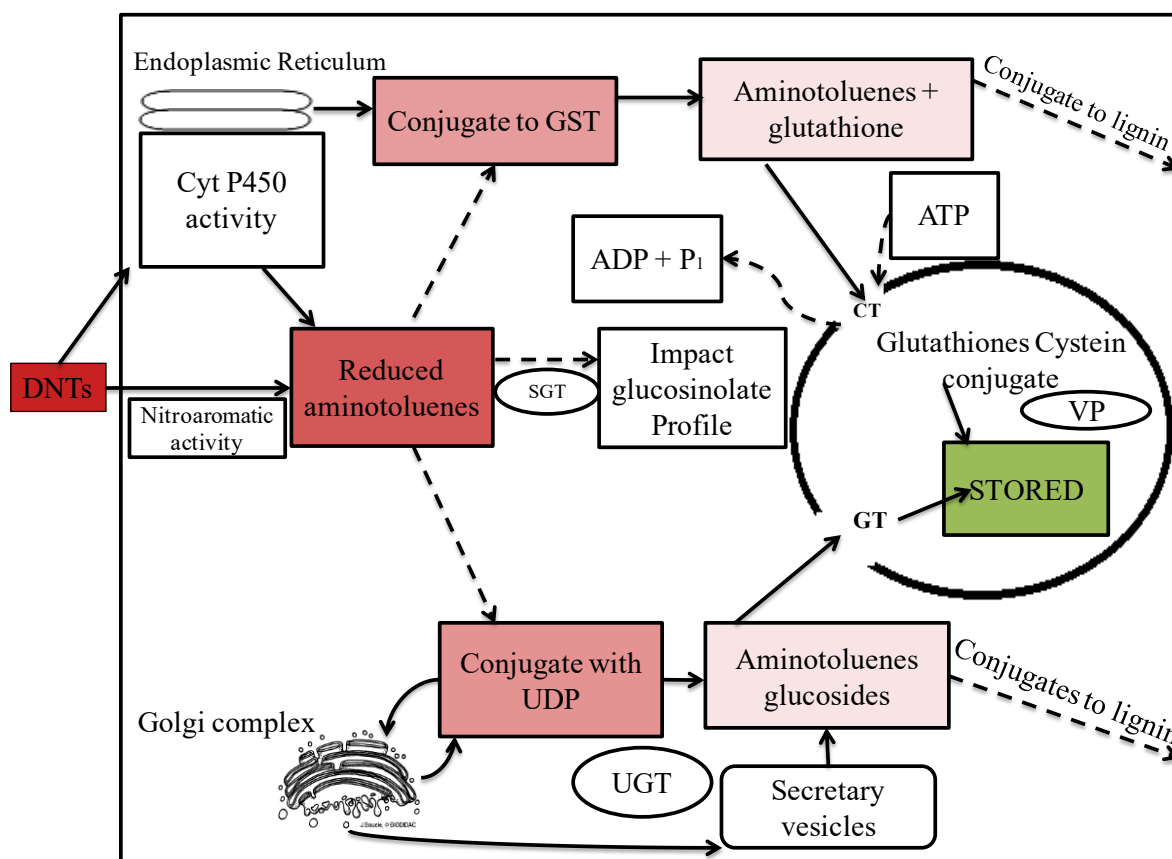


Figure 6.5: Detailed proposed pathway of DNT transformation, conjugation and storage in the plant cell with the involvement of different cell organelles and enzymes as extracted from present study and available literature. The dotted arrows show the possible interactions. The reduction in colour shows their increase in polarity and decrease in toxicity. Finally green colour shows the stored form. Abbreviations: SGT- S-glucosyltransferase; UGT- Uridine diphosphate glucosyltransferase; GT- ATP dependent glucoside conjugate transporter; CT- glutathione conjugate transporter; VP- vacuoler peptidase (Cole et al.,1987; Sun et al.,2012; Herrero et al., 2008).

Phytoremediation is most preferred approach to reduce the contamination of the environmental resources where plants are used to absorb and detoxify the hazardous pollutants. It avoids the use of complex reduction and oxidation processes involved in chemical and physical remediation systems. Through the present study a model plant *A. thaliana* was studied to see the possible interaction and fate of a resistant nitroaromatic compound in the environment, if Phytoremediation is considered as an approach to remove this contamination. It was found that DNT can be absorbed and detoxified to more polar and less hazardous metabolites (aminotoluenes and conjugates) and gets incorporated into the

plant body. Although the percentage of this absorption and metabolism is low, however, this can be tackled through the use of higher plants, which offer high absorption and metabolism. Therefore Phytoremediation is very useful to remove DNTs from the contaminated environmental resources.

In conclusion, DNTs are absorbed and transformed to reduced and conjugated metabolites in *A. thaliana* and are further transported to herbivore feeders. This shows that if Phytoremediation of DNTs is considered, there is likelihood of transport of DNT based metabolites throughout the ecosystem. Keeping in view the results from the present study future aspects could be:

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- Lignin contents should be analysed to identify incorporation of DNT based conjugates in the plant cell wall. This can be achieved through specific lignin staining or acid-base hydrolysis.
- Transport of DNT based metabolites through Tritrophic study using lady bird (*Adalia punctata*) and analysing both DNT based glycosides and ANTs.
- Honey dew analysis for DNT based metabolites obtained from aphids feeding on DNT treated plants as they could possible source of ants and hence a source of contamination transport.
- Since *A. thaliana* is a weed and has limited metabolism pool therefore the toxicity of DNTs should be analysed in higher plants to analyse the difference in tolerance level and therefore effectiveness in phytoremediation.

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Appendix I

(Chapter 2 data)

(i) Root length Analysis

Phytigel media:

Table 1: Toxicity analysis of 2,6-DNT in *A. thaliana* through root growth in Phytigel media. The data are means of length from 7 day (post cultivation) old plant (15 replicates) grown at 20 ± 3 °C temperature and 16:8 h day night period.

2,6-DNT Concentration (mg /L)	Root length (cm)
Control	6.4± 0.27
0.25% Ethanol Control	6.37±0.22
5	5.43± 0.16
10	3.83± 0.21
15	2.7± 0.18
20	1.72± 0.14
25	1.04± 0.027
30	0.77± 0.016
35	0.34± 0.021
40	0.15± 0.014
45	0.03± 0.017
50	0

Table 2: Toxicity analysis of 2,3-DNT, 2,4-DNT and 3,4-DNT in *A. thaliana* through root growth in Phytigel media. The data are means of length from 7 day (post cultivation) old plant (15 replicates) grown at 20 ± 3 °C temperature and 16:8 h day night period.

Concentrations (mg /L of Phytigel media)	Root length (cm) of <i>A. thaliana</i> in 3,4-DNT	Root length (cm) of <i>A. thaliana</i> in 2,4-DNT	Root length (cm) of <i>A. thaliana</i> in 2,3-DNT
Control	6.6 $\pm$ 0.91	7.06 $\pm$ 0.92	6.87 $\pm$ 0.92
0.25% Ethanol control	6.42 $\pm$ 0.81	6.71 $\pm$ 0.93	6.17 $\pm$ 0.81
0.05	5.63 $\pm$ 1.03	5.93 $\pm$ 0.91	4.7 $\pm$ 0.88
0.1	4.7 $\pm$ 1.04	4.82 $\pm$ 1.02	3.75 $\pm$ 0.92
0.25	3.24 $\pm$ 0.22	3.75 $\pm$ 0.28	2.75 $\pm$ 0.17
0.5	1.83 $\pm$ 0.26	2.78 $\pm$ 0.22	1.94 $\pm$ 0.028
1	1.32 $\pm$ 0.23	2.02 $\pm$ 0.21	1.21 $\pm$ 0.021
1.5	0.66 $\pm$ 0.017	1.07 $\pm$ 0.033	0.53 $\pm$ 0.018
2	0.47 $\pm$ 0.017	0.72 $\pm$ 0.035	0.24 $\pm$ 0.033
2.5	0.233 $\pm$ 0.032	0.411 $\pm$ 0.027	0.122 $\pm$ 0.03
3	0.188 $\pm$ 0.33	0.3 $\pm$ 0.028	0.08 $\pm$ 0.03
3.5	0.1 $\pm$ 0.018	0.23 $\pm$ 0.031	0.05 $\pm$ 0.02
4	0.05 $\pm$ 0.014	0.17 $\pm$ 0.033	0.02 $\pm$ 0.01
4.5	0.02 $\pm$ 0.01	0.07 $\pm$ 0.01	0
5	0	0.06 $\pm$ 0.03	0
5.5	0	0.04 $\pm$ 0.02	0
6	0	0	0

Hydroponic media

Table 3: Toxicity analysis of 2,6-DNT in *A. thaliana* through root growth in hydroponic media. The data are means of length from 7 day (post cultivation) old plant (15 replicates) grown at 20 ± 3 °C temperature and 16:8 h day night period.

2,6-DNT Concentration (mg /L)	Root Length (cm) of <i>A. thaliana</i>
Control	12.3 $\pm$ 1.32
0.25% Ethanol Control	11.9 $\pm$ 1.22
5	9.82 $\pm$ 1.12
10	8.79 $\pm$ 1.24
15	8.02 $\pm$ 1.16
20	7.88 $\pm$ 1.31
25	6.45 $\pm$ 1.11
30	5.76 $\pm$ 1.37
35	3.56 $\pm$ 0.21
40	2.67 $\pm$ 0.19
45	1.22 $\pm$ 0.08
50	0.34 $\pm$ 0.01
55	0
60	0
65	0

Table 4: Toxicity analysis of 2,3-DNT, 3,4-DNT and 2,4-DNT in *A. thaliana* through root growth in hydroponic media. The data are means of length from 7 day (post cultivation) old plant (15 replicates) grown at 20 ± 3 °C temperature and 16:8 h day night period.

Concentration (mg /L)	3,4-DNT	2,4-DNT	2,3-DNT
Control	12.34 $\pm$ 1.32	12.45 $\pm$ 1.31	12.67 $\pm$ 1.31
0.25% Ethanol control	12.03 $\pm$ 1.12	12.13 $\pm$ 1.30	12.14 $\pm$ 1.33
0.05	10.06 $\pm$ 1.02	10.45 $\pm$ 1.32	8.71 $\pm$ 1.12
0.1	8.79 $\pm$ 1.31	9.89 $\pm$ 1.22	8.45 $\pm$ 1.03
0.25	8.02 $\pm$ 1.09	9.01 $\pm$ 1.12	7.82 $\pm$ 1.03
0.5	7.88 $\pm$ 1.12	8.65 $\pm$ 1.23	7.06 $\pm$ 1.23
1	6.45 $\pm$ 1.22	7.67 $\pm$ 1.21	5.98 $\pm$ 1.12
1.5	6.02 $\pm$ 1.21	6.01 $\pm$ 1.03	5.08 $\pm$ 1.25
2	5.21 $\pm$ 1.12	5.87 $\pm$ 1.12	4.44 $\pm$ 0.11
2.5	4.89 $\pm$ 0.23	5.03 $\pm$ 1.15	3.65 $\pm$ 0.15
3	3.78 $\pm$ 0.22	4.78 $\pm$ 0.11	3.08 $\pm$ 0.12
3.5	2.45 $\pm$ 0.11	4.01 $\pm$ 0.09	3.01 $\pm$ 0.11
4	1.76 $\pm$ 0.12	2.02 $\pm$ 0.02	2.77 $\pm$ 0.01
4.5	0.78 $\pm$ 0.01	1.44 $\pm$ 0.1	2.01 $\pm$ 0.2
5	0.34 $\pm$ 0.02	0.54 $\pm$ 0.02	1.33 $\pm$ 0.07
5.5	0.22 $\pm$ 0.01	0.33 $\pm$ 0.01	0.34 $\pm$ 0.02
6	0	0	0

(ii) Root and Shoot biomass analysis

Phytigel media

Table 5: Toxicity analysis of 2,6-DNT in *A. thaliana* through root shoot dried weight compared to control. The data are means of weight from 12 (14 days old) seedlings (6 replicates) grown at 20±3 °C temperature and 16:8 h day night period.

2,6-DNT Concentration (mg /L)	Dried wt. (mg) of roots of <i>A. thaliana</i> grown in Phytigel media	Dried wt. (mg) of shoots of <i>A. thaliana</i> grown in Phytigel media
Control	5.5 ± 0.22	17.22 ± 0.24
0.25% Ethanol Control	5.4 ± 0.45	17.14 ± 0.34
5	4.7 ± 0.44	15.22 ± 0.31
10	4.2 ± 0.65	14.98 ± 0.66
15	3.44 ± 0.23	14.33 ± 0.54
20	3.02 ± 0.32	12.03 ± 0.32
25	2.01 ± 0.44	11.97 ± 0.23
30	1.77 ± 0.065	9.65 ± 0.33
35	1.02 ± 0.034	7.22 ± 0.34
40	0.77 ± 0.034	5.46 ± 0.34
45	0.4 ± 0.054	3.98 ± 0.54
50	0	1.65 ± 0.072
55	0	0.34 ± 0.034
60	0	0
65	0	0

Table 6: Toxicity analysis of 2,4-DNT in *A. thaliana* through root shoot dried weight grown in Phytigel media. The data are means of weight from 12 (14 days old) seedlings (6 replicates) grown at 20±3 °C temperature and 16:8 h day night period.

2,4-DNT Concentration (mg /L)	Dried wt. (mg) of roots of <i>A. thaliana</i> grown in Phytigel media	Dried wt. (mg) of shoots of <i>A. thaliana</i> grown in Phytigel media
Control	5.7 ± 0.31	17.34 ± 0.22
0.25% Ethanol control	5.34 ± 0.23	17.12 ± 0.34
0.05	4.88 ± 0.44	15.22 ± 0.44
0.1	4.02 ± 0.34	15.04 ± 0.65
0.25	3.87 ± 0.56	13.02 ± 0.54
0.5	3.45 ± 0.55	12.75 ± 0.56
1	3.01 ± 0.44	11.04 ± 0.34
1.5	2.77 ± 0.65	10.23 ± 0.33
2	2.34 ± 0.43	8.99 ± 0.54
2.5	2.01 ± 0.34	8.02 ± 0.45
3	1.88 ± 0.65	5.92 ± 0.55
3.5	1.56 ± 0.43	5.02 ± 0.34
4	1.23 ± 0.23	4.33 ± 0.36
4.5	0.98 ± 0.061	3.23 ± 0.22
5	0.56 ± 0.048	2.98 ± 0.082
5.5	0.33 ± 0.033	1.22 ± 0.029
6	0	0.23 ± 0.012

Table 7: Toxicity analysis of 3,4-DNT in *A. thaliana* through root shoot dried weight in Phytigel media. The data are means of weights from 12 (14 days old) seedlings (6 replicates) grown at 20±3 °C temperature and 16:8 h day night period.

3,4-DNT Concentration (mg /L)	Dried wt. (mg) of roots of <i>A. thaliana</i> grown in Phytigel media	Dried wt. (mg) of shoots of <i>A. thaliana</i> grown in Phytigel media
Control	5.7±0.33	15.67±0.29
0.25% Ethanol control	5.34±0.23	15.02±0.34
0.05	4.66±0.45	12.33±0.41
0.1	4.42±0.34	11.34±0.65
0.25	4.12±0.56	11.02±0.54
0.5	4.02±0.55	9.75±0.56
1	3.81±0.44	9.04±0.34
1.5	3.47±0.65	8.23±0.33
2	3.03±0.43	7.99±0.54
2.5	2.98±0.34	7.02±0.45
3	2.66±0.65	5.92±0.55
3.5	2.03±0.43	5.32±0.34
4	1.67±0.082	4.11±0.31
4.5	1.23±0.065	3.23±0.22
5	0.56±0.044	2.18±0.12
5.5	0.15±0.033	1.84±0.025
6	0	0.13±0.012

Table 8: Toxicity analysis of 2,3-DNT in *A. thaliana* through root shoot dried weight in Phytigel media. The data are means of weights from 12 (14 days old) seedlings (6 replicates) grown at 20±3 °C temperature and 16:8 h day night period.

2,3-DNT Concentration (mg /L)	Dried wt. (mg) of roots of <i>A. thaliana</i> grown in Phytigel media	Dried wt. (mg) of shoots of <i>A. thaliana</i> grown in Phytigel media
Control	5.45 ± 0.33	17.67 ± 0.22
0.25% Ethanol control	5.22 ± 0.23	17.02 ± 0.34
0.05	4.22 ± 0.40	15.33 ± 0.38
0.1	3.23 ± 0.34	14.34 ± 0.65
0.25	3.11 ± 0.56	13.95 ± 0.54
0.5	2.89 ± 0.57	13.65 ± 0.56
1	2.45 ± 0.44	12.87 ± 0.38
1.5	2.11 ± 0.65	8.23 ± 0.33
2	1.98 ± 0.43	5.21 ± 0.54
2.5	1.56 ± 0.31	3.98 ± 0.45
3	1.32 ± 0.65	3.02 ± 0.55
3.5	0.97 ± 0.043	1.77 ± 0.034
4	0.18 ± 0.013	1.54 ± 0.034
4.5	0	0.98 ± 0.022
5	0	0
5.5	0	0
6		0

Hydroponic media

Table 9: Toxicity analysis of 2,6-DNT in *A. thaliana* grown in hydroponic media through root shoot dried weight compared to control. The data are means of weights from 12 (14 days old) seedlings (6 replicates) grown at 20±3 °C temperature and 16:8 h day night period.

2,6-DNT Concentration (mg /L)	Dried wt. (mg) of roots of <i>A. thaliana</i> grown in hydroponic media	Dried wt. (mg) of shoots of <i>A. thaliana</i> grown in hydroponic media
Control	10.11 ± 0.45	17.16 ± 0.44
0.25% Ethanol Control	10.09 ± 0.47	17.01 ± 0.41
5	9.88 ± 0.56	16.15 ± 0.29
10	7.67 ± 0.38	16.01 ± 0.34
15	6.83 ± 0.56	14.22 ± 0.59
20	5.77 ± 0.55	9.98 ± 0.55
25	4.78 ± 0.33	9.02 ± 0.67
30	3.45 ± 0.65	8.77 ± 0.57
35	2.33 ± 0.31	7.62 ± 0.65
40	1.64 ± 0.074	6.22 ± 0.33
45	0.76 ± 0.054	4.33 ± 0.54
50	0.31 ± 0.023	3.32 ± 0.045
55	0.08 ± 0.012	1.76 ± 0.034
60	0	0.34 ± 0.011
65	0	0

Table 10: Toxicity analysis of 2,4-DNT in *A. thaliana* grown in hydroponic media through root shoot dried weight compared to control. The data are means of weights from 12 (14 days old) seedlings (6 replicates) grown at 20±3 °C temperature and 16:8 h day night period.

2,4-DNT Concentration (mg /L)	Dried wt. (mg) of roots of <i>A. thaliana</i> grown in hydroponic media	Dried wt. (mg) of shoots of <i>A. thaliana</i> grown in hydroponic media
Control	10.22 ± 0.34	17.89 ± 0.44
0.25% Ethanol control	10.23 ± 0.34	17.67 ± 0.45
0.05	8.96 ± 0.56	15.34 ± 0.67
0.1	8.04 ± 0.34	15.22 ± 0.67
0.25	7.65 ± 0.67	14.89 ± 0.54
0.5	6.09 ± 0.66	12.33 ± 0.32
1	4.78 ± 0.33	10.12 ± 0.67
1.5	4.32 ± 0.65	9.98 ± 0.33
2	3.78 ± 0.54	7.88 ± 0.65
2.5	3.76 ± 0.34	6.76 ± 0.33
3	3.44 ± 0.54	4.33 ± 0.66
3.5	2.06 ± 0.23	3.32 ± 0.45
4	1.34 ± 0.22	1.76 ± 0.34
4.5	0.34 ± 0.45	1.12 ± 0.43
5	0.14 ± 0.55	0.88 ± 0.18
5.5	0.11 ± 0.08	0.67 ± 0.15
6	0	0.21 ± 0.11

Table 11: Toxicity analysis of 3,4-DNT in *A. thaliana* grown in hydroponic media through root shoot dried weight compared to control. The data are means of weights from 12 (14 days old) seedlings (6 replicates) grown at 20±3 °C temperature and 16:8 h day night period.

3,4-DNT Concentration	Dried wt. (mg) of roots of <i>A. thaliana</i> grown in hydroponic media	Dried wt. (mg) of shoots of <i>A. thaliana</i> grown in hydroponic media
Control	10.34±0.45	17.44±0.43
0.25% Ethanol control	10.22±0.49	17.22±0.45
0.05	8.9± 0.65	16.89±0.33
0.1	8.88±0.55	16.01±0.61
0.25	7.89±0.45	14.22±0.58
0.5	7.23±0.55	9.98±0.45
1	5.67±0.22	9.02±0.675
1.5	4.98±0.69	8.77±0.43
2	4.33±0.37	7.62±0.34
2.5	3.67±0.22	6.98±0.41
3	3.11±0.67	4.53±0.57
3.5	2.78±0.41	3.66±0.45
4	2.44±0.45	3.01±0.32
4.5	1.89±0.23	2.34±0.42
5	1.22±0.049	1.98±0.023
5.5	0.33±0.054	1.56±0.022
6	0	0.22±0.038

Table 12: Toxicity analysis of 2,3-DNT in *A. thaliana* grown in hydroponic media through root shoot dried weight compared to control. The data are means of weights from 12 (14 days old) seedlings (6 replicates) grown at 20±3 °C temperature and 16:8 h day night period.

2,3-DNT Concentration (mg /L)	Dried wt. (mg) of roots of <i>A. thaliana</i> grown in hydroponic media	Dried wt. (mg) of shoots of <i>A. thaliana</i> grown in hydroponic media
Control	10.54 ± 0.41	17.22 ± 0.44
0.25% Ethanol control	10.12 ± 0.65	17.11 ± 0.45
0.05	8.02 ± 0.56	16.05 ± 0.54
0.1	7.88 ± 0.44	15.67 ± 0.66
0.25	6.57 ± 0.56	13.21 ± 0.54
0.5	5.87 ± 0.66	10.34 ± 0.51
1	5.01 ± 0.39	9.33 ± 0.67
1.5	4.98 ± 0.67	8.77 ± 0.59
2	4.52 ± 0.58	7.62 ± 0.65
2.5	3.22 ± 0.34	6.32 ± 0.38
3	2.89 ± 0.66	4.33 ± 0.54
3.5	2.32 ± 0.023	3.42 ± 0.45
4	1.24 ± 0.064	1.76 ± 0.055
4.5	0.65 ± 0.045	0.34 ± 0.016
5	0	0
5.5	0	0
6	0	

(iii) Time course root length analysis

Hydroponic media only

Table 13: Time course toxicity analysis of 2,6-DNT in *A. thaliana* root growth (cm) in hydroponic media. The data points are mean of technical duplicates (root and shoot lengths of 36 seedlings) of the 28 day old (post cultivation) plants treated with different concentrations of 2,4-DNT grown at 20 ± 3 °C temperature and 16:8 h day night period.

2,6-DNT Concentration (mg /L)	7 th Day	14 th Day	21 st Day	28 th Day
Control	8.74±1.56	12.3±1.64	17.72±1.91	21.34±1.25
0.25% Ethanol Control	8.55±1.55	12.01±1.44	17.65±1.67	21.12±1.32
5	7.32±1.66	9.82±1.91	8.56±1.31	7.88±1.21
10	7.21±1.32	8.79±1.67	8.23±1.25	6.34±1.91
15	6.23±1.45	8.02±1.31	7.86±1.28	5.89±0.67
20	6.01±1.44	7.88±1.31	7.22±1.32	5.11±0.22
25	5.67±1.23	6.45±1.25	6.01±1.21	4.21±0.34
30	5.23±1.16	5.76±1.56	5.02±0.45	2.33±0.25
35	5.01±0.88	5.21±1.43	4.98±0.12	1.23±0.032
40	3.44±0.32	4.22±0.44	3.01±0.67	0.34±0.03
45	2.22±0.23	0	0	0
50	1.28±0.33	0	0	0
55	0.98±0.23	0	0	0
60	0	0	0	0
65	0	0	0	0

Table 14: Time course toxicity analysis of 2,4-DNT in *A. thaliana* root growth (cm) in hydroponic media. The data points are mean of technical duplicates (root and shoot lengths of 36 seedlings) of the 28 day old (post cultivation) plants treated with different concentrations of 2,4-DNT grown at 20 ± 3 °C temperature and 16:8 h day night period.

2,4-DNT Concentration (mg /L)	7th Day	14th Day	21st Day	28th Day
Control	8.33±1.22	12.34±4.43	17.78±4.32	21.22±1.11
0.25% Ethanol Control	8.02±1.45	12.03±4.32	17.34±4.98	21.13±1.35
0.05	7.75±1.32	10.06±3.23	8.23±4.12	5.22±1.45
0.1	7.33±1.43	8.79±4.55	8.01±4.11	5.01±1.21
0.25	7.02±1.33	8.02±1.14	7.66±1.78	4.97±1.34
0.5	6.34±1.43	7.88±1.32	7.43±1.45	4.56±0.83
1	5.01±1.65	6.45±1.23	5.44±1.21	3.22±0.81
1.5	4.99±1.53	6.02±1.32	5.12±1.34	3.11±0.44
2	4.122±1.4	5.21±1.65	4.78±1.33	2.98±0.11
2.5	3.67±0.44	4.89±1.56	4.01±1.45	2.78±0.21
3	3.01±0.32	3.78±1.23	3.3±0.23	2.01±0.61
3.5	1.77±0.11	2.45±1.11	2.01±0.55	1.55±0.6
4	0.34±0.06	1.76±0.12	1.22±0.14	0.98±0.06
4.5	0.23±0.04	0.78±0.089	0.45±0.032	0.23±0.08
5	0.11±0.03	0.34±0.08	0.22±0.023	0.1±0.02
5.5	0	0.22±0.041	0.12±0.032	0
6	0	0	0	0

Table 15: Time course toxicity analysis of 3,4-DNT in *A. thaliana* root growth (cm) in hydroponic media. The data points are mean of technical duplicates (root and shoot lengths of 36 seedlings) of the 28 day old (post cultivation) plants treated with different concentrations of 2,4-DNT grown at 20 ± 3 °C temperature and 16:8 h day night period.

2, 3-DNT Concentration (mg /L)	7th Day	14th Day	21st Day	28th Day
Control	8.12±0.77	12.45±1.45	11.2±0.43	10.22±2.12
0.25% Ethanol Control	8.02±0.76	12.13±1.32	10.4±0.65	9.13±1.39
0.05	7.66±0.86	8.56±0.43	7.34±0.53	7.01±2.25
0.1	5.67±0.77	7.89±0.33	6.42±0.42	6.29±3.02
0.25	5.02±0.78	7.01±0.43	5.99±0.45	5.62±2.31
0.5	4.89±0.97	6.65±4.65	4.89±0.32	4.54±0.61
1	4.45±1.12	5.67±2.53	4.67±0.43	4.41±0.53
1.5	3.66±0.08	5.01±1.42	4.1±0.33	3.97±0.08
2	2.89±0.02	4.87±0.86	3.8±0.43	2.44±1.02
2.5	2.02±0.8	4.03±0.77	3.65±0.5	2.58±1.7
3	1.98±0.1	3.78±0.3	3.23±0.01	2.42±1.09
3.5	1.55±0.5	3.01±0.23	2.45±0.09	1.98±0.096
4	0.89±0.06	2.02±0.11	1.32±0.1	1.07±0.3
4.5	0.76±0.03	1.44±0.07	1.41±0.04	0.29±0.01
5	0.22±0.01	0.54±0.02	0.33±0.01	0
5.5	0	0.22±0.01	0	0
6	0	0	0	0

Table 16: Time course toxicity analysis of 2,3-DNT in *A. thaliana* root growth (cm) in hydroponic media. The data points are mean of technical duplicates (root and shoot lengths of 36 seedlings) of the 28 day old (post cultivation) plants treated with different concentrations of 2,3-DNT grown at $20\pm 3^{\circ}\text{C}$ temperature and 16:8 h day night period.

2,3-DNT Concentration (mg /L)	7th Day	14th Day	21st Day	28th Day
Control	8.66 $\pm$ 1.45	12.67 $\pm$ 1.23	10.29 $\pm$ 2.45	9.98 $\pm$ 1.23
0.25% Ethanol Control	8.43 $\pm$ 1.44	12.14 $\pm$ 1.42	10.03 $\pm$ 2.05	8.43 $\pm$ 2.09
0.05	7.43 $\pm$ 1.01	10.99 $\pm$ 1.42	9.44 $\pm$ 1.83	7.91 $\pm$ 1.45
0.1	7.64 $\pm$ 1.09	10.45 $\pm$ 2.9	8.02 $\pm$ 2.42	6.89 $\pm$ 1.22
0.25	6.23 $\pm$ 1.12	9.02 $\pm$ 2.34	7.22 $\pm$ 1.95	5.95 $\pm$ 1.34
0.5	5.67 $\pm$ 2.89	8.56 $\pm$ 1.22	6.29 $\pm$ 1.32	5.67 $\pm$ 1.12
1	4.56 $\pm$ 1.32	7.98 $\pm$ 1.09	5.17 $\pm$ 2.09	4.92 $\pm$ 1.6
1.5	3.98 $\pm$ 1.44	6.88 $\pm$ 2.06	4.91 $\pm$ 1.23	4.87 $\pm$ 2.11
2	2.66 $\pm$ 0.12	5.44 $\pm$ 1.34	3.53 $\pm$ 1.26	3.44 $\pm$ 1.92
2.5	2.02 $\pm$ 0.098	4.65 $\pm$ 0.71	3.05 $\pm$ 0.05	2.88 $\pm$ 0.07
3	1.77 $\pm$ 0.077	3.98 $\pm$ 0.62	1.93 $\pm$ 0.06	1.62 $\pm$ 0.29
3.5	1.02 $\pm$ 0.09	3.01 $\pm$ 0.09	1.45 $\pm$ 0.05	1.45 $\pm$ 0.06
4	0.87 $\pm$ 0.02	2.77 $\pm$ 0.051	0.92 $\pm$ 0.02	0.58 $\pm$ 0.03
4.5	0.76 $\pm$ 0.03	2.01 $\pm$ 0.08	0.41 $\pm$ 0.06	0.32 $\pm$ 0.01
5	0.22 $\pm$ 0.01	1.33 $\pm$ 0.02	0.33 $\pm$ 0.07	0
5.5	0	0.34 $\pm$ 0.09	0	0
6	0	0	0	0

Appendix II

(Chapter 3 data)

i) Uptake and metabolism of 2,6-DNT in *A. thaliana* grown in Phytigel media

Table 1: GC-MS peaks for 2,6-DNT standards

Conc. of 2,6-DNT (mg /L)	Area of peaks from GC-MS			
	1	2	3	Average
0.5	2772	2779	2773	2774.667
1	10760	10723	10790	10757.67
5	20869	20836	20876	20860.33
10	41574	42465	42780	42273
20	88905	88189	88760	88618
30	135344	135935	135345	135541.3
40	179745	179215	179615	179525
50	224406	224412	224397	224405

Table 2: GC-MS peaks for 2A6NT standards

Conc. of 2A6NT (mg /L)	Area of peaks from GC-MS			
	1	2	3	Average
0.5	1389	1381	1387	1039.375
1	2744	2743	2789	2069.25
5	13741	13728	13769	10310.75
10	21691	21687	21696	16271
20	53382	53387	53376	40041.25

30	83073	83071	83076	62312.5
40	111764	112761	113768	84583.25
50	138455	138465	138440	103852.5

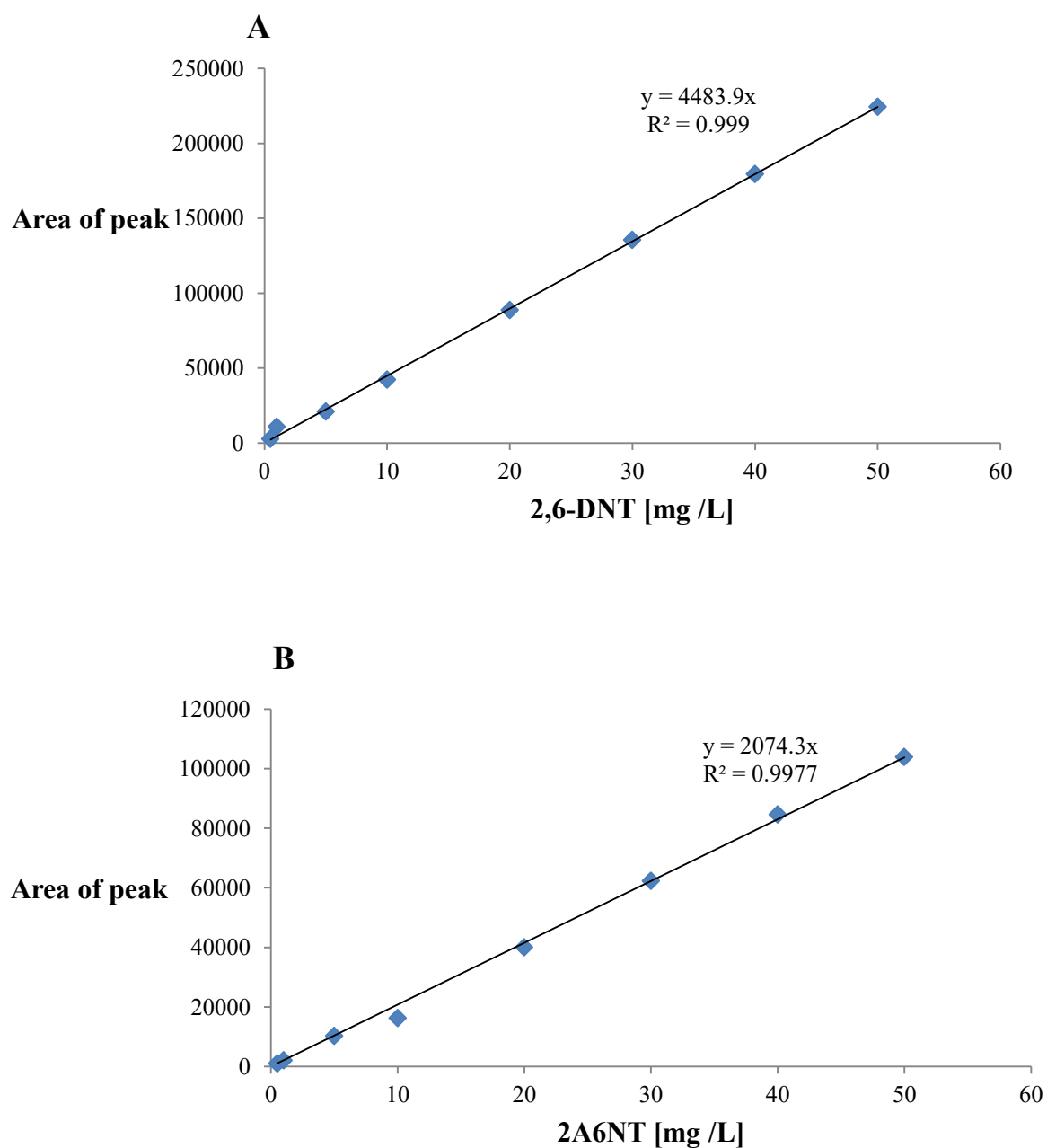


Figure 1: Calibration graphs of (A) 2,6-DNT and (B) 2A6NT

Table 3: 2,6-DNT and 2A6NT concentrations recovered from 18 days (post cultivation) old *A. thaliana* plants grown in different concentrations of 2,6-DNT in sterile Phytigel media (growth temperature was 20±3°C for 16:8 h day:night period) and analysed by GC-MS.

2,6-DNT concentration (mg /L)	2A6NT and 2,6-DNT (µg /g dry tissue wt)		2A6NT and 2,6-DNT (µg /single plant of <i>A. thaliana</i>)	
	2A6NT	Untransformed 2,6-DNT	2A6NT	Untransformed 2,6-DNT
Control	0	0	0	0
0.25% Ethanol Control	0	0	0	0
3	0.22±0.05	0	0.064±0.01	0
13	1.22±0.12	0.22±0.056	0.34±0.025	0
23	5.23±0.18	0.236±0.066	0.87±0.03	0.039±0.016
33	3.22±0.27	0.346±0.173	0.45±0.054	0.057±0.0347
43	1.74±0.18	1.22±0.15	0.24±0.036	0.204±0.029
53	0	0	0	0

ii) Uptake and metabolism of 2,4-DNT in *A. thaliana* grown in Phytigel media

Table 4: GC-MS peaks for 2,4-DNT standards

Conc. of 2,4-DNT (mg /L)	Area of peaks from GC-MS			
	1	2	3	Average
1	2209	2207	2212	2209.33
5	23855	23852	23857	23854.7
10	38960	38957	38963	38960
20	72291	72289	72293	72291
30	108615	108615	108617	108616
40	142926	142928	142923	142926
50	188166	188162	188169	188166

Table 5: GC-MS peaks for 2A4NT standards

Conc. of 2A4NT (mg /L)	Area of peaks from GC-MS			
	1	2	3	Average
1	4712	4715	4711	4712.67
5	24560	24553	24573	24562
10	46120	46117	46124	46120.3
20	96220	98240	98269	97576.3
30	141360	141371	141351	141361
40	198480	198492	189473	195482
50	4712	4715	4711	4712.67

Table 6: GC-MS peaks for 4A2NT standards

Conc. of 4A2NT (mg /L)	Area of peaks from GC-MS			
	1	2	3	Average
1	5712	5724	5721	5719
5	28560	28551	28560	28557
10	57120	57112	57134	57122
20	104240	104232	104245	104239
30	171360	141371	141351	151361
40	228480	198492	189473	205482
50	5712	5724	5721	5719

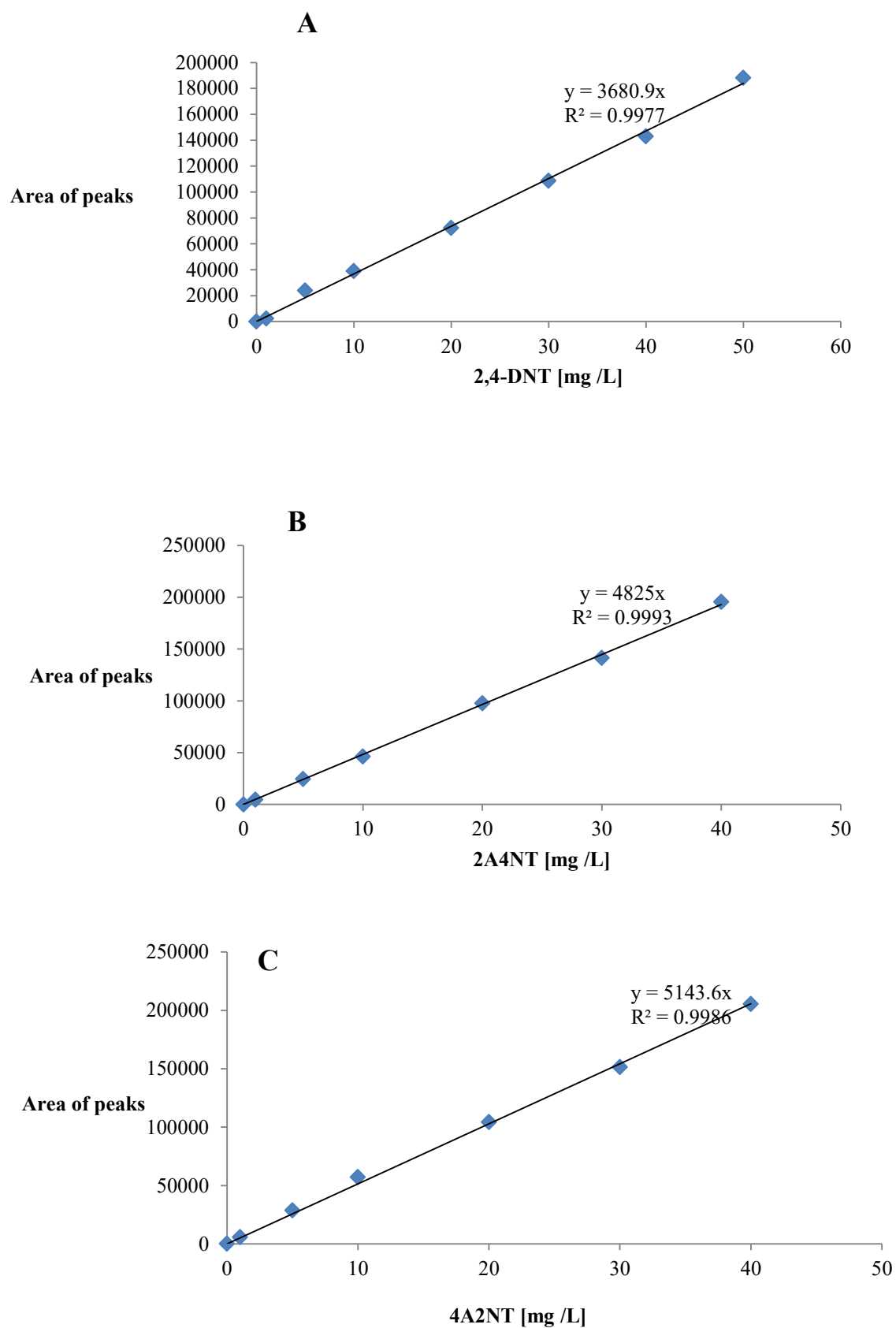


Figure 2: Calibration graphs of (A) 2,4-DNT, (B) 2A4NT and (C) 4A2NT.

Table 7: 2,4-DNT, 2A4NT and 4A2NT concentrations recovered from 18 days (post cultivation) old *A. thaliana* plants grown in different concentrations of 2,6-DNT in sterile Phytigel media (growth temperature was $20\pm 3^{\circ}\text{C}$ for 16:8 h day:night period) and analysed by GC-MS.

2,4-DNT concentration (mg /L)	2A4NT, 4A2NT and 2,4-DNT (ng /g dry tissue wt)			2A4NT, 4A2NT and 2,4-DNT (ng /single plant of <i>A. thaliana</i>)		
	2A4NT	4A2NT	Untransformed 2,4-DNT	2A4NT	4A2NT	Untransformed 2,4-DNT
Control	0	0	0	0	0	0
0.25% Ethanol Control	0	0	0	0	0	0
0.03	0	11.34 $\pm$ 18.26	0	0	10.91 $\pm$ 4.38	0
0.1	45.42 $\pm$ 12.66	80.68 $\pm$ 14.20	0	33.33 $\pm$ 1.23	37.22 $\pm$ 3.40	0
0.3	134.19 $\pm$ 25.8	204.13 $\pm$ 20.85	0	106.66 $\pm$ 6.35	108.84 $\pm$ 5.01	0
0.9	280.31 $\pm$ 26.6	508.4 $\pm$ 43.99	0	93.43 $\pm$ 6.56	183.24 $\pm$ 10.55	0
1.8	258.37 $\pm$ 7.0	434.68 $\pm$ 32.09	44.15 $\pm$ 8.71	86.12 $\pm$ 6.13	155.95 $\pm$ 7.70	10.22 $\pm$ 1.74
2.7	240.76 $\pm$ 18.81	327.26 $\pm$ 25.76	56.15 $\pm$ 10.86	80.25 $\pm$ 3.722	118.17 $\pm$ 6.18	12.23 $\pm$ 2.17
3.6	60.10 $\pm$ 18.81	95.58 $\pm$ 15.58	311.821 $\pm$ 25.45	20.03 $\pm$ 4.06	45.2 $\pm$ 3.74	24.13 $\pm$ 4.87
4.5	0	0	0	0	0	0

iii) **Uptake and metabolism of 2,3-DNT in *A. thaliana* grown in Phytigel media**

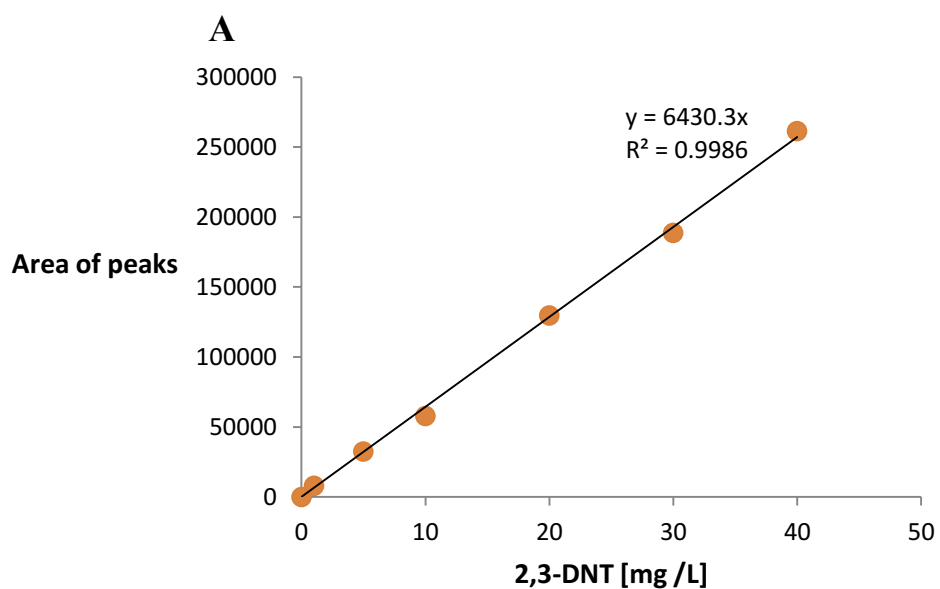
Table 8: GC-MS peaks for 2,3-DNT standards

Conc. of 2,3-DNT (mg /L)	Area of peaks from GC-MS			
	1	2	3	Average
1	7886	7888	7886	7886.667
5	32430	32429	32442	32433.67
10	57860	57859	57870	57863
20	129720	129712	129730	129720.7

30	188580	188578	188590	188582.7
40	261440	261432	261452	261441.3
50	314300	314278	314321	314299.7

Table 9: GC-MS peaks for 2A3NT standards

Conc. of 2A3NT (mg /L)	Area of peaks from GC-MS			
	1	2	3	Average
1	19420	19403	19457	19426.67
5	34840	34803	34876	34839.67
10	74594	74534	74532	74553.33
20	113520	113510	113524	113518
30	155360	155362	155384	155368.7
40	3884	3879	3892	3885
50	19420	19403	19457	19426.67



B

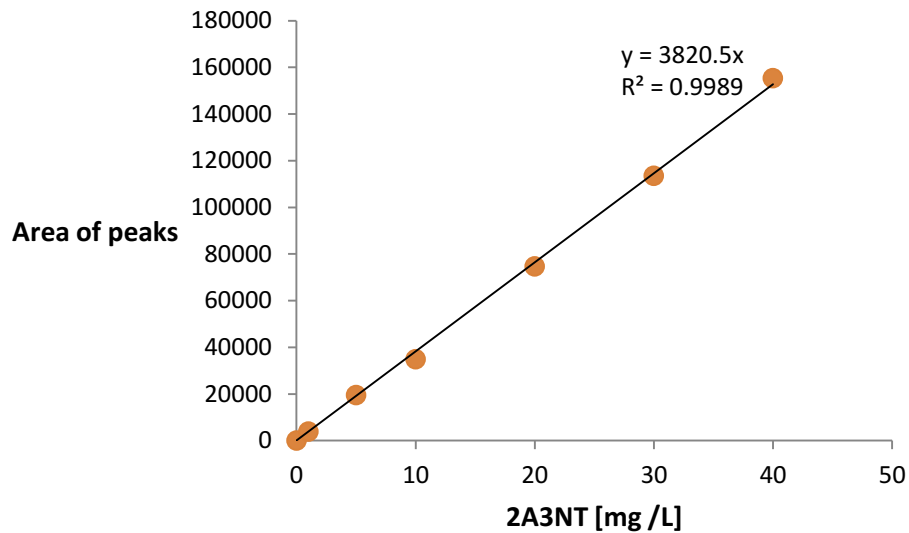


Figure 3: Calibration graphs of (A) 2,6-DNT and (B) 2A6NT

Table 10: 2,3-DNT and 2A3NT concentrations recovered from 18 days (post cultivation) old *A. thaliana* plants grown in different concentrations of 2,3-DNT in sterile Phytigel media (growth temperature was $20 \pm 3^{\circ}\text{C}$ for 16:8 h day:night period) and analysed by GC-MS.

2,3-DNT concentration (mg /L)	2A3NT and 2,3-DNT (ng /g dry tissue wt)		2A3NT and 2,3-DNT (ng /single plant of <i>A. thaliana</i>)	
	2A3NT	Untransformed 2,3- DNT	2A3NT	Untransformed 2,3- DNT
Control	0	0	0	0
0.25% Ethanol Control	0	0	0	0
0.05	32.8+13.17	0	32.21+2.22	0
0.1	66.8+13.60	13.1+6.55	65.43+3.34	0
0.25	134+23.33	24.2+9.21	86.54+5.32	0
0.5	320+21.24	89.9+8.76	60.44+5.41	0
1	675.22+18.13	90.23+15.55	54.32+3.25	24.32+1.67
1.5	370.22+18.33	141+19.87	43.78+4.32	28.2+2.11
2	290.23+25.28	154+13.45	34.56+5.44	30.8+2.34

2.5	103.2+21.48	162+21.22	22.33+4.53	45.66+3.32
5	0	0	0	0

iv) **Uptake and metabolism of 3,4-DNT in *A. thaliana* grown in Phytigel media**

Table 11: GC-MS peaks for 3,4-DNT standards

Conc. of 3,4-DNT (mg /L)	Area of peaks from GC-MS			
	1	2	3	Average
1	7886	7888	7886	7886.66
5	32430	32429	32442	32433.67
10	57860	57859	57870	57863
20	129720	129712	129730	129720.7
30	188580	188578	188590	188582.7
40	261440	261432	261452	261441.3
50	314300	314278	314321	314299.7

Table 12: GC-MS peaks for 3A4NT standard

Conc. of 3A4NT (mg /L)	Area of peaks from GC-MS			
	1	2	3	Average
1	15420	15448	15677	15515
5	31030	31026	31035	31030.33
10	72365	72344	72367	72358.67
20	113870	113760	113763	113797.7
30	155354	155363	155371	155362.7
40	3774	3777	3797	3782.667
50	15420	15448	15677	15515

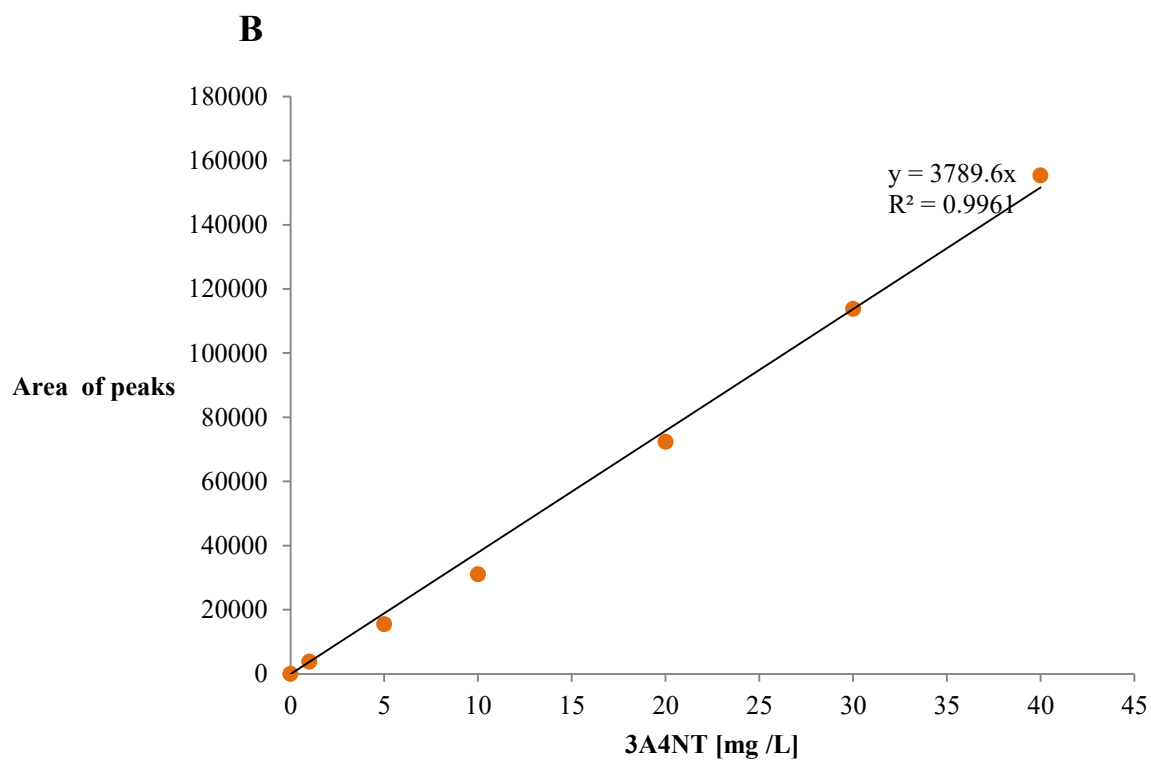
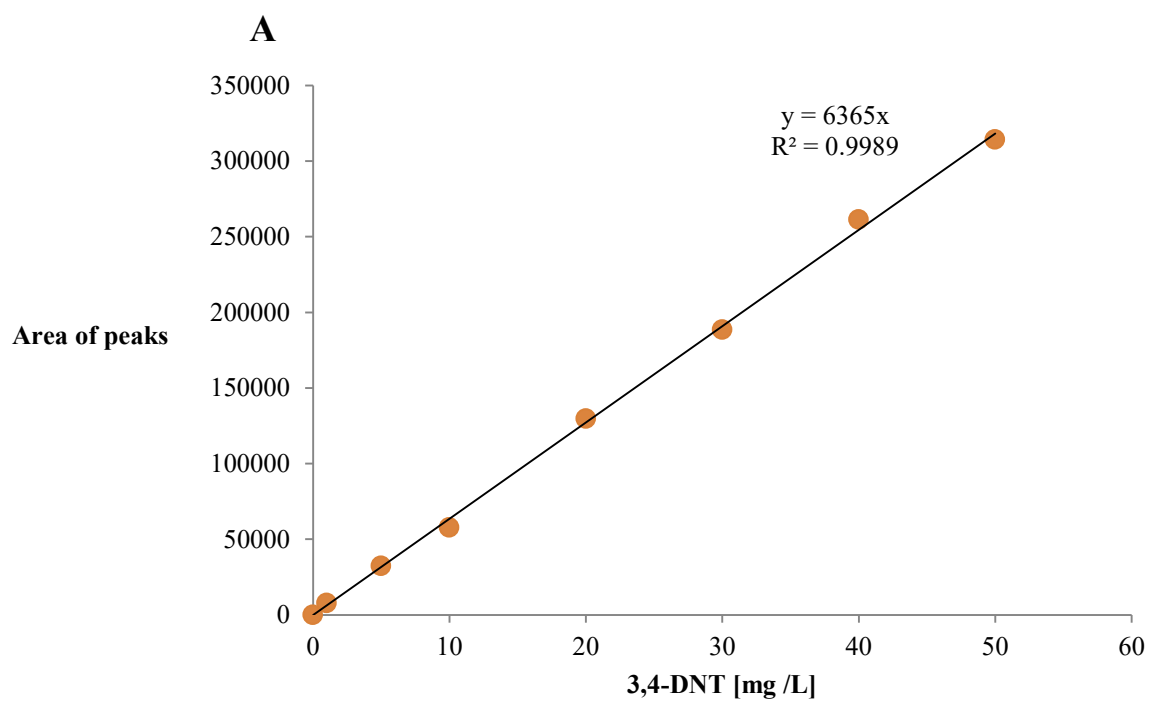


Figure 4: Calibration graphs of (A) 3,4-DNT and (B) 3A4NT

Table 13: 3,4-DNT and 3A4NT concentrations recovered from 18 days (post cultivation) old *A. thaliana* plants grown in different concentrations of 3,4 -DNT in sterile Phytigel media (growth temperature was $20\pm 3^{\circ}\text{C}$ for 16:8 h day:night period) and analysed by GC-MS.

3,4-DNT concentration (mg /L)	3A4NT and 3,4-DNT (ng /g dry tissue wt)		3A4NT and 3,4-DNT (ng /single plant of <i>A. thaliana</i>)	
	3A4NT	Untransformed 3,4-DNT	3A4NT	Untransformed 3,4-DNT
Control	0	0	0	0
0.25% Ethanol Control	0	0	0	0
0.02	0	0	0	0
0.2	90.2+6.65	0	32.56+8.99	0
0.4	278.4+8.87	18.2+12.33	100.5+10.12	0
0.8	564.32+13.45	89+17.34	203.72+18.92	4.23+1.22
1.6	324.4+17.82	102.2+23.33	117.11+8.79	8.7+2.21
3.2	73.69+17.98	130.32+13.45	28.4+9.91	10.45+3.12
4.6	41.23+11.32	170.2+13.34	14.88+11.32	22.32+6.72
6.4	0	0	0	0

Uptake and metabolism of 2,6-DNT in *A. thaliana* grown in hydroponic media (Time course study)

Table 14: The amount (mean of four experimental replicates, n=4) of 2A6NT and untransformed 2,6-DNT recovered from *A. thaliana* plants grown for 28 days post cultivation ($20\text{--}23^{\circ}\text{C}$ temperature, $120\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 2,6-DNT analysed by GC-MS.

Days of observation	2,6-DNT (mg /L of hydroponic media)	Transformed 2A6NT (μg /L of hydroponic media)	Untransformed 2,6-DNT (μg /L of hydroponic media)

7 th day	5	1.45±0.22	0.54±0.02
	15	5.33±0.18	1.25±0.23
	25	3.47±0.16	5.64±0.35
	35	0.944±0.17	6.11±0.34
	45	0	7.84±0.34
	55	0	9.22±0.34
14 th day	5	2.14±0.24	0
	15	6.75±0.27	0
	25	4.25±0.23	0.23±0.15
	35	1.23±0.16	0.33±0.23
	45	0	1.11±0.34
	55	0	3.29±0.36
21 st day	5	1.81±0.24	0
	15	6.55±0.24	1.58±0.38
	25	3.67±0.22	7.62±0.41
	35	1.04±0.18	8.25±0.28
	45	0	10.25±0.34
	55	0	12.23±0.23
28 th day	5	0.84±0.14	3.22±0.26
	15	3.45±0.17	4.23±0.27
	25	2.608±0.13	8.45±0.25
	35	0.289±0.12	9.45±0.26
	45	0	12.75±0.22
	55	0	22.23±0.25

Table 15: The amount (mean of four experimental replicates, n=4) of 2A4NT and recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23°C temperature, 120 µmol m⁻² s⁻¹ and 16:8 h day:night cycle) in hydroponic media with different concentration of 2,4-DNT analysed by GC-MS.

2,4-DNT (mg /L of hydroponic media)	Transformed 2A4NT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	0	0	0	0
0.1	76.35±5.42	91.23±5.23	77.88±4.52	41.23±3.37
0.25	95.17±4.5	117.67±3.36	97.66±4.23	64.47±3.34
0.5	111.95±4.4	137.34±4.25	122.58±6.57	98.74±4.54
1	66.39±9.84	110.17±4.55	86.75±5.23	45.22±4.67
2.5	72.33±3.56	88.76±5.43	67.85±3.68	30.11±4.55
5	65.43±4.34	76.44±5.33	45.64±4.32	0

Table 16: The amount (mean of four experimental replicates, n=4) of 4A2NT and untransformed 2,4-DNT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23⁰C temperature, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 2,4-DNT analysed by GC-MS.

2,4-DNT (mg /L of hydroponic media)	Transformed 4A2NT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	76.61±6.45	91.22±6.78	75.51±5.66	58.91±8.76
0.1	100.34±7.65	113.02±5.67	103.28±7.89	83.44±8.65
0.25	102.33±5.45	160.45±6.78	122.31±7.56	98.13±8.76
0.5	120.29±8.76	183.45±6.78	172.33±8.44	112.38±9.45
1	98.76±8.71	102.34±7.88	83.44±7.23	77.82±4.67
2.5	78.33±8.65	81.34±6.57	61.03±7.22	52.34±6.22
5	65.45±5.44	76.54±5.33	52.31±7.88	45.66±6.55

Table 17: The amount (mean of four experimental replicates, n=4) untransformed 2,4-DNT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23°C temperature, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 2,4-DNT analysed through GC-MS.

2,4-DNT (mg /L of hydroponic media)	Untransformed 2,4-DNT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	0	0	3.22±0.54	4.21±0.98
0.1	0	3.24±0.56	5.83±1.69	6.24±1.63
0.25	0	4.58±0.74	7.32±1.89	8.22±2.56
0.5	0	6.77±1.58	8.01±1.65	10.25±2.45
1	0	7.28±1.94	8.92±1.23	24.53±4.65
2.5	4.27±0.22	8.31±2.45	19.52±2.53	48.22±4.57
5	9.3±1.67	14.12±5.85	21.58±2.74	56.28±6.87

Table 18: The amount (mean of four experimental replicates, n=4) 2A3NT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23°C temperature, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 2,3-DNT analysed by GC-MS.

2,3-DNT (mg /L of hydroponic media)	Transformed 2A3NT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	18.22±6.56	21.26±4.33	15.26±5.67	7.87±5.51
0.1	35.45±3.76	67.45±4.87	24.24±4.32	14.45±5.44
0.25	73.45±9.87	158.74±18.94	61.27±12.32	24.56±7.89
0.5	81.95±14.56	377.48±18.56	175.27±16.54	98.74±11.32
1	66.39±15.56	273.45±7.91	153.32±21.25	42.87±6.77
2.5	31.27±3.44	112.27±7.48	57.88±7.69	5.56±1.23
5	28.77±7.89	62.34±8.49	21.45±1.33	0

Table 19: The amount (mean of four experimental replicates, n=4) untransformed 2,3-DNT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23⁰C temperature, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 2,3-DNT analysed by GC-MS.

2,3-DNT (mg /L of hydroponic media)	Untransformed 2,3-DNT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	0	0	0	42.47 $\pm$ 5.45
0.1	0	0	40.47 $\pm$ 2.57	56.47 $\pm$ 2.67
0.25	0	0	41.25 $\pm$ 4.67	58.74 $\pm$ 5.65
0.5	0	0	44.01 $\pm$ 5.74	69.87 $\pm$ 6.67
1	0	40.74 $\pm$ 5.45	55.45 $\pm$ 6.43	72.49 $\pm$ 6.87
2.5	0	55.27 $\pm$ 4.44	70.28 $\pm$ 5.52	84.28 $\pm$ 6.78
5	0	67.41 $\pm$ 7.23	88.79 $\pm$ 6.98	105.45 $\pm$ 5.75

Table 20: The amount (mean of four experimental replicates, n=4) 3A4NT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23⁰C temperature, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 3,4-DNT analysed by GC-MS.

3,4-DNT (mg /L of hydroponic media)	Transformed 3A4NT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	0	0	0	0
0.1	45.45 $\pm$ 8.97	65.48 $\pm$ 11.2	44.31 $\pm$ 9.07	38.12 $\pm$ 4.13
0.25	65.45 $\pm$ 7.88	106.53 $\pm$ 7.66	61.27 $\pm$ 8.54	54.56 $\pm$ 8.12
0.5	77.86 $\pm$ 9.87	145.32 $\pm$ 9.23	112.98 $\pm$ 12.21	92.61 $\pm$ 11.43
1	96.64 $\pm$ 8.34	178.44 $\pm$ 7.87	123.32 $\pm$ 6.45	97.66 $\pm$ 8.56
2.5	65.66 $\pm$ 8.81	134.98 $\pm$ 9.71	77.88 $\pm$ 8.21	65.56 $\pm$ 9.44
5	41.23 $\pm$ 7.28	60.67 $\pm$ 8.23	11.48 $\pm$ 4.23	0

Table 21: The amount (mean of four experimental replicates, n=4) untransformed 3,4-DNT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23°C temperature, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 3,4-DNT analysed by GC-MS.

3,4-DNT (mg /L of hydroponic media)	Untransformed 3,4-DNT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	0	0	0	0
0.1	0	27.84 $\pm$ 5.43	31.61 $\pm$ 5.21	38.22 $\pm$ 4.23
0.25	21.31 $\pm$ 1.22	32.6 $\pm$ 5.44	38.77 $\pm$ 6.34	41.23 $\pm$ 5.77
0.5	23.41 $\pm$ 2.34	35.66 $\pm$ 8.56	45.63 $\pm$ 6.75	51.54 $\pm$ 5.78
1	29.89 $\pm$ 2.13	44.23 $\pm$ 9.87	49.29 $\pm$ 7.89	58.77 $\pm$ 2.44
2.5	32.34 $\pm$ 3.44	48.32 $\pm$ 11.56	52.95 $\pm$ 13.44	62.13 $\pm$ 6.54
5	38.77 $\pm$ 3.21	51.73 $\pm$ 10.25	76.44 $\pm$ 11.29	113.41 $\pm$ 12.32

Table 22: The amount (mean of four experimental replicates, n=4) transformed 2,6-DNT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23°C temperature, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 2,6-DNT analysed by GC-MS.

2,6-DNT (mg /L of hydroponic media)	Transformed 2A6NT (μg /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
5	22.23 $\pm$ 2.33	34.54 $\pm$ 4.45	24.97 $\pm$ 3.22	0
15	31.54 $\pm$ 2.11	42.78 $\pm$ 2.12	35.67 $\pm$ 2.32	15.23 $\pm$ 2.21
25	38.86 $\pm$ 2.56	45.66 $\pm$ 2.55	42.44 $\pm$ 2.98	23.22 $\pm$ 2.88
35	27.84 $\pm$ 1.98	38.33 $\pm$ 3.22	29.88 $\pm$ 3.22	0
45	18.45 $\pm$ 3.44	22.21 $\pm$ 2.45	0	0
55	0	0	0	0

Table 23: The amount (mean of four experimental replicates, n=4) untransformed 2,6-DNT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23°C

temperature, $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 2,6-DNT analysed by GC-MS.

2,6-DNT (mg /L of hydroponic media)	Untransformed 2,6-DNT (μg /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
5	31.44 $\pm$ 2.84	38.32 $\pm$ 2.22	53.43 $\pm$ 1.94	53.45 $\pm$ 2.88
15	35.63 $\pm$ 1.83	52.46 $\pm$ 2.22	59.54 $\pm$ 1.94	61.22 $\pm$ 3.21
25	33.23 $\pm$ 1.45	38.97 $\pm$ 4.33	53.34 $\pm$ 3.23	54.56 $\pm$ 2.32
35	31.45 $\pm$ 2.22	34.56 $\pm$ 2.11	41.32 $\pm$ 1.87	51.44 $\pm$ 1.56
45	30.22 $\pm$ 1.21	33.28 $\pm$ 1.78	41.22 $\pm$ 1.87	48.34 $\pm$ 1.99
55	0	0	0	0

Table 24: The amount (mean of four experimental replicates, n=4) transformed 2A4NT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23⁰C temperature, $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 2,4-DNT analysed by GC-MS.

2, 4-DNT (mg /L of hydroponic media)	Transformed 2A4NT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	29.51 $\pm$ 2.45	33.34 $\pm$ 2.67	27.89 $\pm$ 3.48	19.89 $\pm$ 2.21
0.1	35.44 $\pm$ 2.5	41.29 $\pm$ 2.12	32.44 $\pm$ 3.12	21.98 $\pm$ 1.98
0.25	38.76 $\pm$ 2.4	48.11 $\pm$ 3.36	41.13 $\pm$ 2.23	25.64 $\pm$ 1.76
0.5	31.23 $\pm$ 2.4	40.13 $\pm$ 2.25	28.74 $\pm$ 3.6	20.65 $\pm$ 1.66
1	27.64 $\pm$ 2.22	32.12 $\pm$ 2.45	25.43 $\pm$ 2.23	18.81 $\pm$ 1.76
2.5	22.37 $\pm$ 1.98	29.93 $\pm$ 1.99	21.32 $\pm$ 2.68	0
5	18.91 $\pm$ 2.34	21.22 $\pm$ 2.33	16.78 $\pm$ 2.32	0

Table 25: The amount (mean of four experimental replicates, n=4) transformed 4A2NT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23⁰C temperature, $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 2,4-DNT analysed by GC-MS.

2, 4-DNT (mg /L of hydroponic media)	Transformed 2A4NT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	30.51±2.34	34.56±1.98	23.21±2.23	18.76±1.88
0.1	35.45±2.67	45.54±1.89	38.76±2.12	32.11±1.21
0.25	41.18±2.11	47.64±1.98	40.67±2.09	35.44±2.55
0.5	39.87±2.68	42.33±2.78	37.65±2.11	27.74±2.65
1	31.21±2.22	38.42±2.11	29.87±1.98	19.78±2.76
2.5	22.37±1.95	29.14±2.09	22.43±2.11	16.78±1.66
5	18.91±1.78	24.56±2.67	18.78±2.11	0

Table 26: The amount (mean of four experimental replicates, n=4) untransformed 2,4-DNT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23⁰C temperature, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 2,4-DNT analysed by GC-MS.

2, 4-DNT (mg /L of hydroponic media)	Untransformed 2,4-DNT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	24.44±2.31	31.54±2.98	38.87±3.34	41.23±2.08
0.1	31.54±2.12	42.78±1.76	48.77±2.4	55.67±3.42
0.25	37.86±4.09	48.77±3.87	54.42±3.77	62.94±3.65
0.5	31.23±3.28	38.33±3.78	42.76±2.87	55.41±3.29
1	28.76±2.12	35.46±1.98	40.12±4.11	43.23±4.67
2.5	22.37±1.88	28.76±2.34	34.54±4.12	39.76±3.45
5	17.86±3.98	21.33±2.31	26.76±1.87	34.21±3.11

Table 27: The amount (mean of four experimental replicates, n=4) transformed 3A4NT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23⁰C temperature, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 3,4-DNT analysed by GC-MS.

3,4-DNT (mg /L of hydroponic media)	Transformed 3A4NT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	27.67±2.33	32.42±2.76	25.14±2.04	18.98±3.67
0.1	34.56±2.99	40.98±3.12	31.27±3.56	20.99±3.31
0.25	38.77±3.56	49.76±2.23	39.84±3.99	25.66±3.34
0.5	29.67±3.21	40.13±3.6	31.22±2.11	17.67±1.98
1	26.5±2.84	31.22±2.23	22.34±2.11	0
2.5	21.33±3.56	28.76±2.68	21.87±2.65	0
5	17.67±3.97	21.22±3.45	16.78±2.76	0

Table 28: The amount (mean of four experimental replicates, n=4) untransformed 3,4-DNT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23⁰C temperature, 120 µmol m⁻² s⁻¹ and 16:8 h day:night cycle) in hydroponic media with different concentration of 3,4-DNT analysed by GC-MS.

3,4-DNT (mg /L of hydroponic media)	Untransformed 3,4-DNT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	20.98±2.45	28.76±3.11	31.23±1.98	35.44±2.34
0.1	28.76±2.33	31.23±1.67	36.75±1.9	40.32±2.33
0.25	35.53±3.01	39.76±2.87	42.31±2.55	49.55±2.13
0.5	31.56±1.98	38.45±1.22	41.21±2.13	44.53±2.11
1	29.56±1.45	34.55±2.78	38.76±2.97	40.32±2.67
2.5	25.46±3.56	28.77±3.55	34.32±3.68	37.5±3.54
5	18.91±3.54	24.56±2.34	28.76±4.32	31.14±3.22

Table 29: The amount (mean of four experimental replicates, n=4) transformed 3A4NT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23⁰C temperature, 120 µmol m⁻² s⁻¹ and 16:8 h day:night cycle) in hydroponic media with different concentration of 3,4-DNT analysed by GC-MS.

2,3-DNT (mg /L of hydroponic media)	Transformed 2A3NT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	27.67±2.78	30.98±2.23	24.48±2.31	16.53±1.87
0.1	32.55±3.01	41.32±2.12	31.76±1.98	18.94±1.66
0.25	35.46±2.76	45.71±2.44	34.62±1.98	21.34±2.01
0.5	30.11±2.11	40.13±2.12	31.22±1.98	16.58±2.22
1	23.98±2.56	29.42±2.62	22.34±2.41	0
2.5	21.33±1.98	25.37±2.13	20.19±2.33	0
5	0	0	0	0

Table 30: The amount (mean of four experimental replicates, n=4) untransformed 2,3-DNT recovered from *A. thaliana* plants grown for 28 days post cultivation (20-23⁰C temperature, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16:8 h day:night cycle) in hydroponic media with different concentration of 2,3-DNT analysed by GC-MS.

2,3-DNT (mg /L of hydroponic media)	Untransformed 2,3-DNT (ng /g dry wt of <i>A. thaliana</i>)			
	7 th day	14 th day	21 st day	28 th day
0.05	19.2±2.34	26.73±3.32	31.23±2.44	35.44±1.98
0.1	28.76±2.34	30.23±2.44	36.75±1.98	40.32±1.99
0.25	35.53±2.56	37.74±2.43	42.31±2.44	49.11±2.12
0.5	30.77±2.14	35.67±2.13	37.89±2.12	40.97±2.11
1	27.89±3.22	32.12±2.22	36.75±2.45	39.78±2.41
2.5	16.78±2.56	21.33±2.32	23.44±2.68	0
5	0	0	0	0

Appendix III

(Chapter 4 data)

Table 1: Conjugates of reduced 2,6-DNT metabolites recovered from an homogenous mixture of ten 14 day (post cultivation) old *A. thaliana* plant grown in different concentrations of 2,6-DNT in sterile liquid MS media and analysed by LC-MS/MS

Concentration of 2, 6 DNT	DHAT- <i>O</i> - MG- <i>N</i> -OP (1)	DHAT- <i>C</i> - MG - <i>N</i> -OP (2)	HANT- <i>C</i> - MG - <i>N</i> -OB (3)	HANT- <i>C</i> - MG (4)	HANT- <i>O</i> - MG (5)	HANT- <i>O</i> - MG- <i>O</i> -M (6)	HANT- <i>C</i> - MG- <i>O</i> -M (7)	ANT- <i>N</i> - MG (8)	ANT- <i>C</i> - MG (9)	ANT- <i>C</i> & <i>N</i> - DG (10)
5	0.98±0.12	0.76±0.12	0.86±0.024	0.76±0.14	0.66±0.086	0.56±0.065	0.56±0.087	0.87±0.13	0.65±0.082	0.65±0.065
15	1.45±0.14	1.32±0.098	0.97±0.085	0.97±0.078	0.87±0.11	0.76±0.09	0.87±0.09	1.21±0.087	0.88±0.055	0.75±0.067
25	1.67±0.09	1.43±0.13	1.22±0.12	1.54±0.12	1.21±0.12	0.98±0.08	1.21±0.08	1.34±0.16	1.54±0.045	1.15±0.078
35	2.24±0.087	2.03±0.14	1.67±0.14	1.86±0.076	1.67±0.14	1.1±0.087	1.24±0.14	1.65±0.089	1.87±0.076	1.34±0.12
45	2.5±0.098	2.26±0.16	1.9±0.098	2.14±0.14	1.76±0.098	1.21±0.098	1.34±0.098	1.92±0.14	2.05±0.098	1.65±0.098
55	2.25±0.076	2.14±0.076	1.78±0.14	2.11±0.093	1.65±0.076	1.01±0.078	1.01±0.076	1.78±0.12	1.99±0.1	1.61±0.076

Table 2: Conjugates of reduced 2,3-DNT metabolites recovered from homogenous mixture of ten 14 day (post cultivation) old *A. thaliana* plant grown in different concentrations of 2,3-DNT in sterile liquid MS media and analysed by LC-MS/MS (growth temperature was 20±3⁰C for 16:8 h day:night period; constant speed 130 rpm). Data was plotted as mean of four replicates (ten plants from one container were freeze dried and homogenized to give one replicate).

Concentration of 2, 3 DNT	DHAT- <i>O</i> - MG- <i>N</i> -OP (11)	DHAT- <i>C</i> - MG- <i>N</i> -OP (12)	HANT- <i>C</i> - MG- <i>N</i> -OB (13)	HANT- <i>C</i> - MG (14)	HANT- <i>O</i> - MG (15)	HANT- <i>O</i> - MG- <i>O</i> -M (16)	HANT- <i>C</i> - MG- <i>O</i> -M (17)	ANT- <i>N</i> -MG (18)	ANT- <i>C</i> -MG (19)	ANT- <i>C</i> & <i>N</i> - DG (20)
0.05	56.43±12.33	68.45±12.31	48.54±5.67	38.77±9.87	50.54±12.31	32.45±3.44	60.56±12.31	45.07±5.72	32.45±12.33	43.56±5.72
0.1	67.55±9.87	98.78±3.44	59.66±9.87	47.55±5.64	75.43±12.34	43.22±4.52	91.89±12.34	43.25±9.87	86.55±9.87	52.34±9.87
0.25	76.54±6.78	121.45±5.62	92.34±5.64	58.76±5.72	89.75±9.1	67.54±7.84	115.56±9.1	76.73±5.64	97.64±6.78	65.77±5.64
0.5	109.65±5.66	143.53±8.92	101.76±8.74	77.45±9.87	118.43±12.31	72.33±7.64	135.64±12.31	87.65±8.74	123.41±5.66	84.19±8.97
1	137.987±10.23	167.83±10.22	130.097±10.23	93.45±5.64	145.32±5.67	81.22±7.66	162.94±5.67	131.22±6.78	145.6±10.23	118.79±6.78
2.5	145.6±12.33	204.34±5.67	137.71±6.77	97.85±8.74	182.538±7.81	87.43±7.82	187.32±7.81	134.24±12.34	174.148±12.3	120.45±7.89
5	103.2±9.78	185.46±7.83	130.21±6.75	91.23±4.52	145.2±5.67	36.45±6.77	177.57±5.67	67.54±6.75	166.54±9.78	112.3±12.34

Table 3: Conjugates of reduced 2,4-DNT metabolites recovered from homogenous mixture of ten 14 day (post cultivation) old *A. thaliana* plant grown in different concentrations of 2,4-DNT in sterile liquid MS media and analysed by LC-MS/MS (growth temperature was 20±3⁰C for 16:8 h day:night period; constant speed 130 rpm). Data was plotted as mean of four replicates (ten plants from one container were freeze dried and homogenized to give one replicate).

Concentration of 3, 4 DNT	DHAT- <i>O</i> - MG- <i>N</i> -OP (21)	DHAT- <i>C</i> - MG- <i>N</i> -OP (22)	HANT- <i>C</i> - MG- <i>N</i> - OB (23)	HANT- <i>C</i> - MG (24)	HANT- <i>O</i> - MG (25)	HANT- <i>O</i> - MG- <i>O</i> -M (26)	HANT- <i>C</i> - MG- <i>O</i> -M (27)	ANT- <i>N</i> -MG (28)	ANT- <i>C</i> -MG (29)	ANT- <i>C</i> & <i>N</i> -DG (30)
0.05	57.82±18.23	61.45±18.23	0	69.45±12.34	67.54±9.67	57.45±2.22	55.46±2.31	76.45±2.34	56.72±3.98	51.9±9.2
0.1	79.45±12.22	79.76±12.22	35.46±6.78	84.76±11.21	110.34±15.33	75.76±12.43	72.09±3.42	98.76±30.4	67.72±13.67	68.53±10.3
0.25	102.34±9.87	105.79±9.87	67.18±5.78	113.79±35.67	133.21±11.2	101.79±10.98	94.98±6.32	118.79±12.33	97.29±12.9	80.46±12.98
0.5	118.24±8.79	121.35±8.79	82.7±10.21	128.35±24.4	142.3±12.21	116.35±11.32	110.88±11.3	123.35±23.33	103.45±22.21	96.36±11.09
1	121.34±12.31	130.3±12.31	86.96±8.77	142.3±13.43	187.23±12.3	123.3±10.31	113.72±9.87	145.3±11.98	118.8±19.54	99.2±18.02
2.5	132.45±9.76	156.7±9.76	98.07±7.65	178.34±11.31	202.34±44.1	141.04±12.33	124.83±2.13	192.34±22.09	134.54±11.34	110.31±22.43
5	129.76±6.78	148.23±6.78	95.52±6.78	180.94±21.09	193.42±12.33	133.98±9.87	112.48±4.65	187.94±12.87	130.21±21.98	108.76±19.09

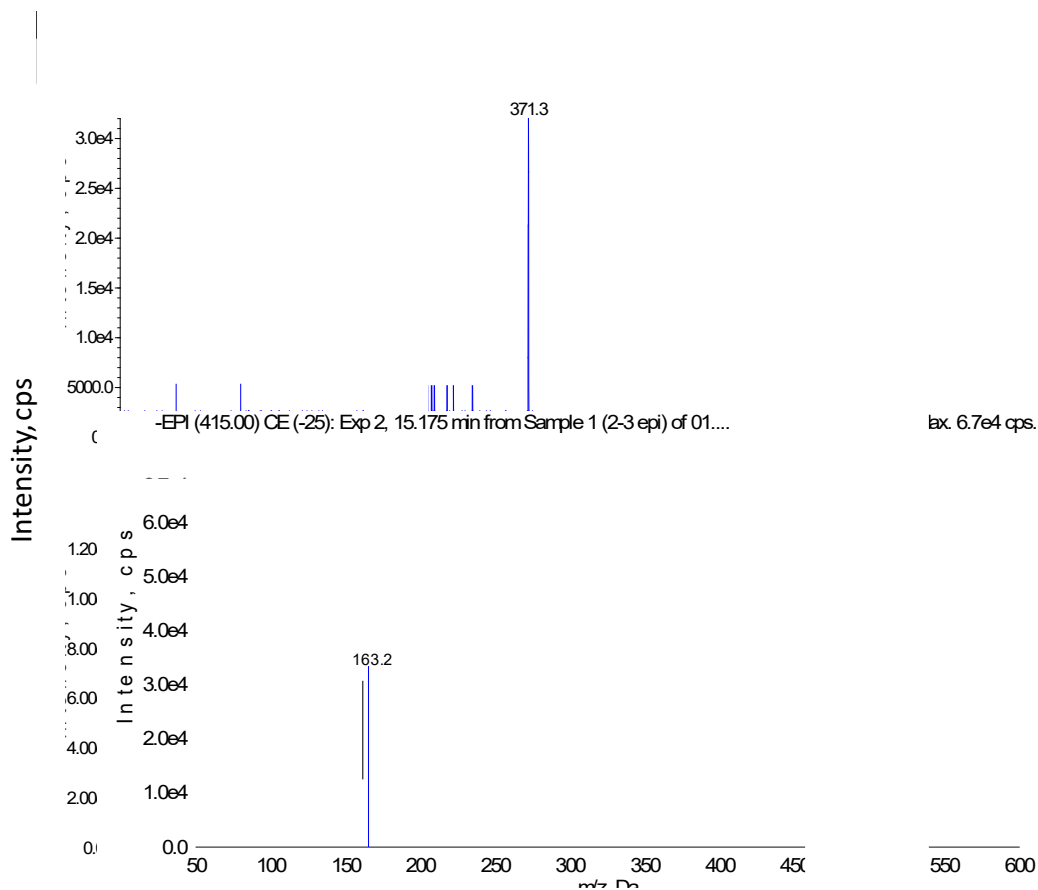
Table 4: Conjugates of reduced 3,4-DNT metabolites recovered from homogenous mixture of ten 14 day (post cultivation) old *A. thaliana* plant grown in different concentrations of 3,4-DNT in sterile liquid MS media and analysed by LC-MS/MS (growth temperature was 20±3⁰C for 16:8 h day:night period; constant speed 130 rpm). Data was plotted as mean of four replicates (ten plants from one container were freeze dried and homogenized to give one replicate).

Concentration of 3, 4 DNT	DHAT- <i>O</i> - MG- <i>N</i> -OP (31)	DHAT- <i>C</i> - MG - <i>N</i> -OP (32)	HANT- <i>C</i> - MG- <i>N</i> -OB (33)	HANT- <i>C</i> - MG (34)	HANT- <i>O</i> - MG (35)	HANT- <i>O</i> - MG- <i>O</i> -M (36)	HANT- <i>C</i> - MG - <i>O</i> -M (37)	ANT- <i>N</i> -MG (38)	ANT- <i>C</i> -MG (39)	ANT- <i>N</i> & <i>C</i> - MG (40)
0.05	39.775±12.33	28.415±5.72	31.885±5.67	68.45±12.31	0	47.388±12.33	20.165±0.03	55.778±12.31	24.955±5.72	60.56±12.31
0.1	70.105±9.87	43.25±9.87	62.215±9.87	98.78±3.44	0	77.718±9.87	35±0.04	86.108±12.34	39.79±9.87	90.89±12.34
0.25	76.54±6.78	76.73±5.64	92.34±5.64	121.45±5.62	53.13±7.84	100.388±6.78	58.76±0.03	108.778±9.1	73.27±5.64	113.56±9.1
0.5	115.797±5.66	93.41±8.74	107.907±8.74	143.53±8.92	65.44±5.63	123.41±5.66	77.45±0.04	112.34±12.31	89.95±8.97	135.64±12.31
1	137.987±10.23	126.627±6.78	130.097±10.23	167.83±10.22	72.34±4.55	145.6±10.23	93.45±0.08	123.45±5.67	118.79±6.78	159.94±5.67
2.5	145.6±12.33	134.24±12.34	137.71±6.77	197.68±5.67	87.65±5.43	176.618±12.33	97.85±0.04	185.008±7.81	120.45±7.89	189.79±7.81
5	114.5±9.78	87.65±6.75	130.21±6.75	185.46±7.83	0	164.398±9.78	91.23±0.05	172.788±5.67	112.3±12.34	177.57±5.67

Due to large number of pages and repetition in information, only 2,3-DNT based glucosyl conjugate fragmentation is given, the rest of the isomers had same pattern of fragmentation. The basis of structure and structures are as explained in the chapter.

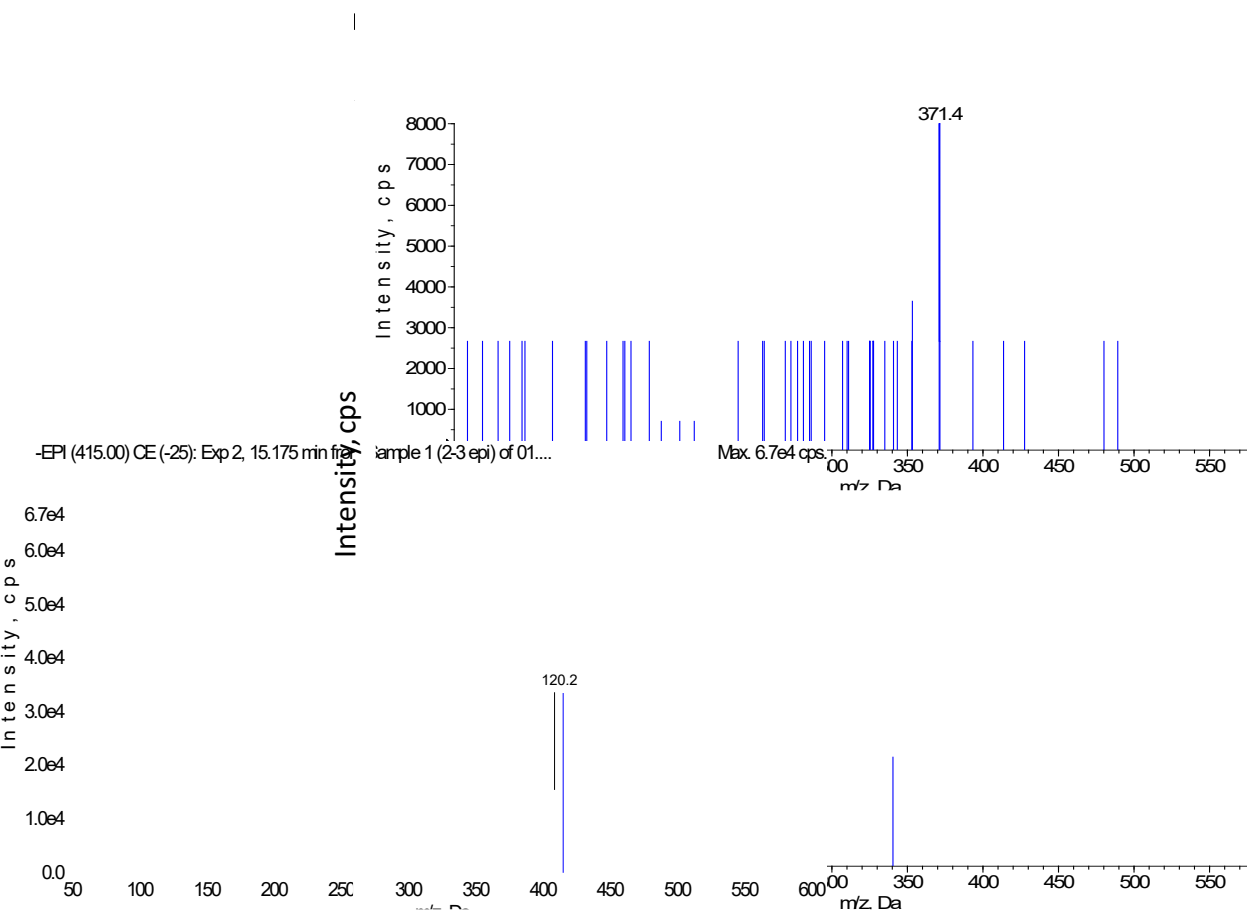
Compound name: DHAT-*O*-MG-*N*-OP (11)

Precursor ion /product ion (*m/z*): 371 /151



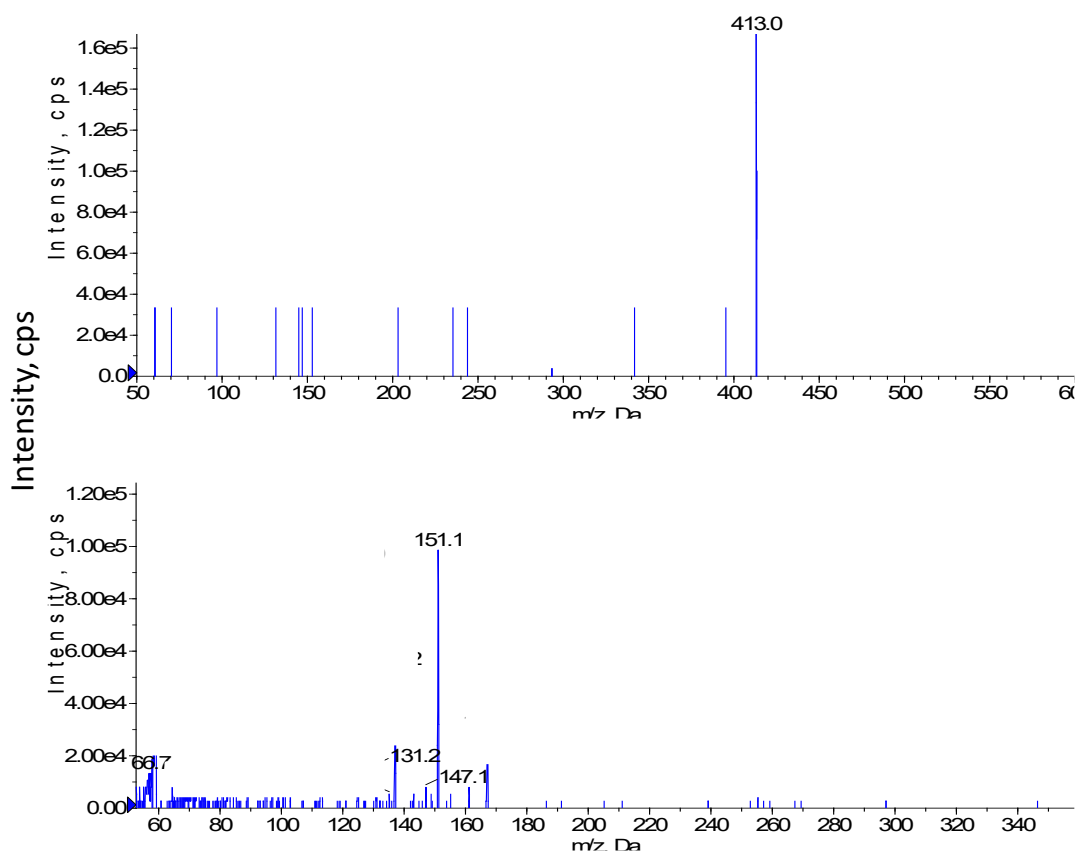
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Precursor ion /product ion (m/z): 371 /195



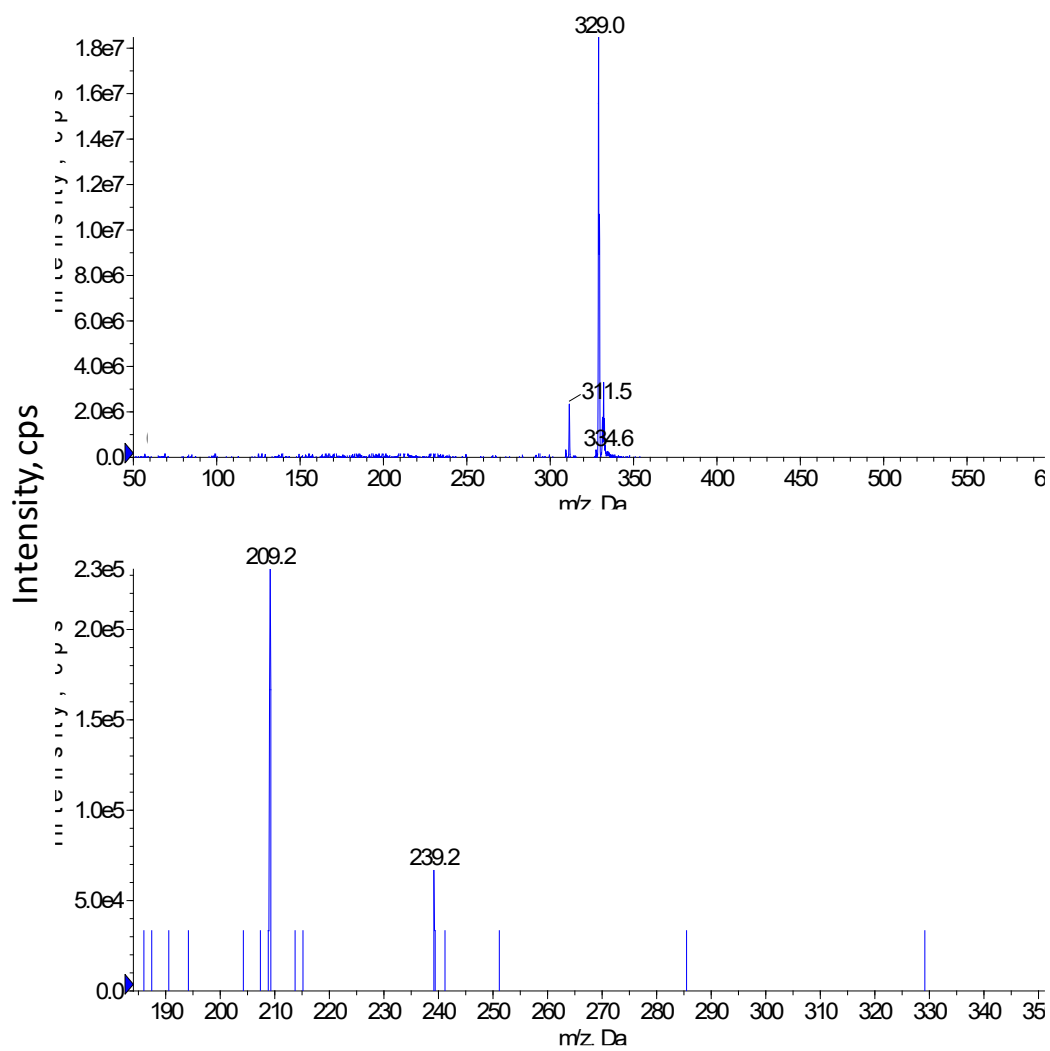
Compound name: HANT-C-MG-N-OB (3)

Precursor ion /product ion (m/z): 413 /151



Compound name: HANT-C-MG (4)

Precursor ion /product ion (m/z): 329 /209



Compound name: HANT-*O*-MG (5)

Precursor ion /product ion (*m/z*): 329 /151

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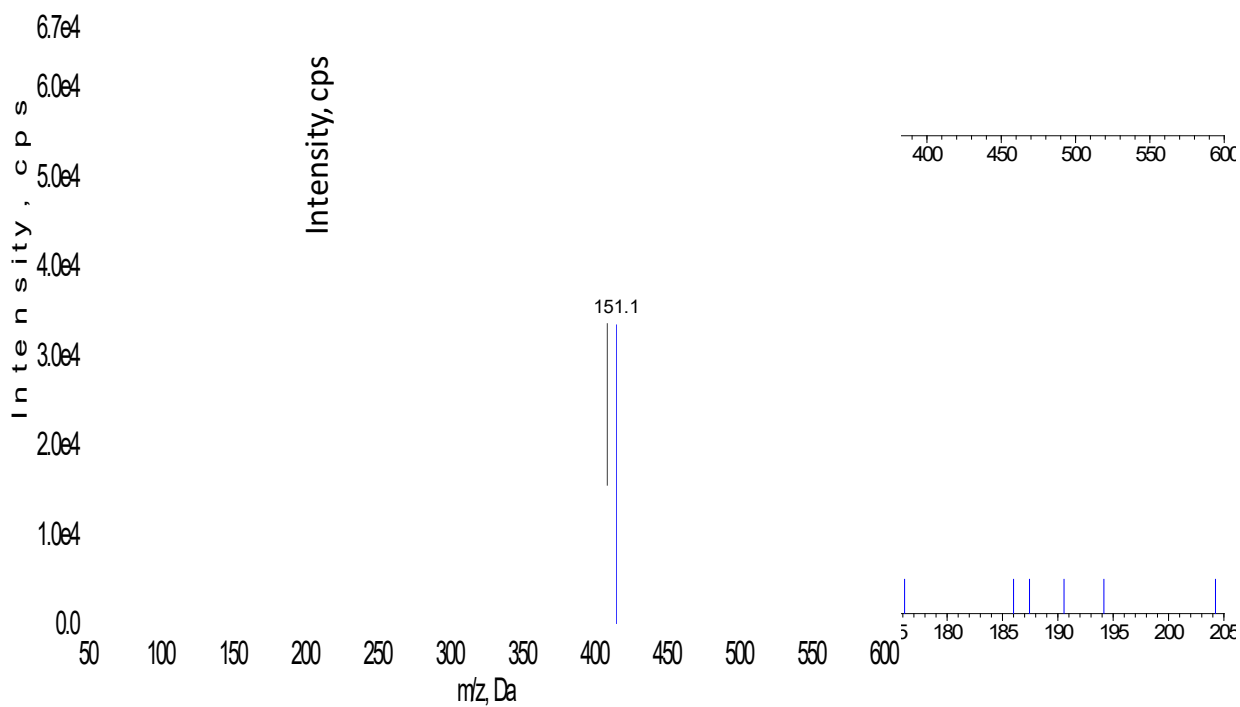
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329.0

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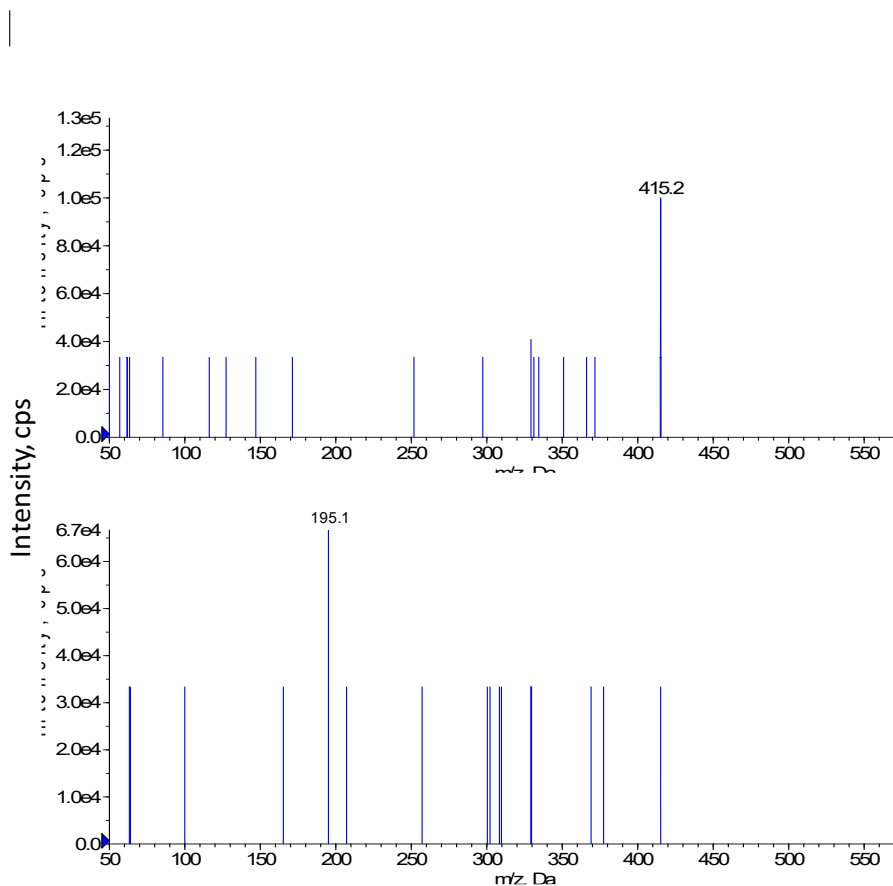
Max. 6.7e4 cps.

Intensity, cps



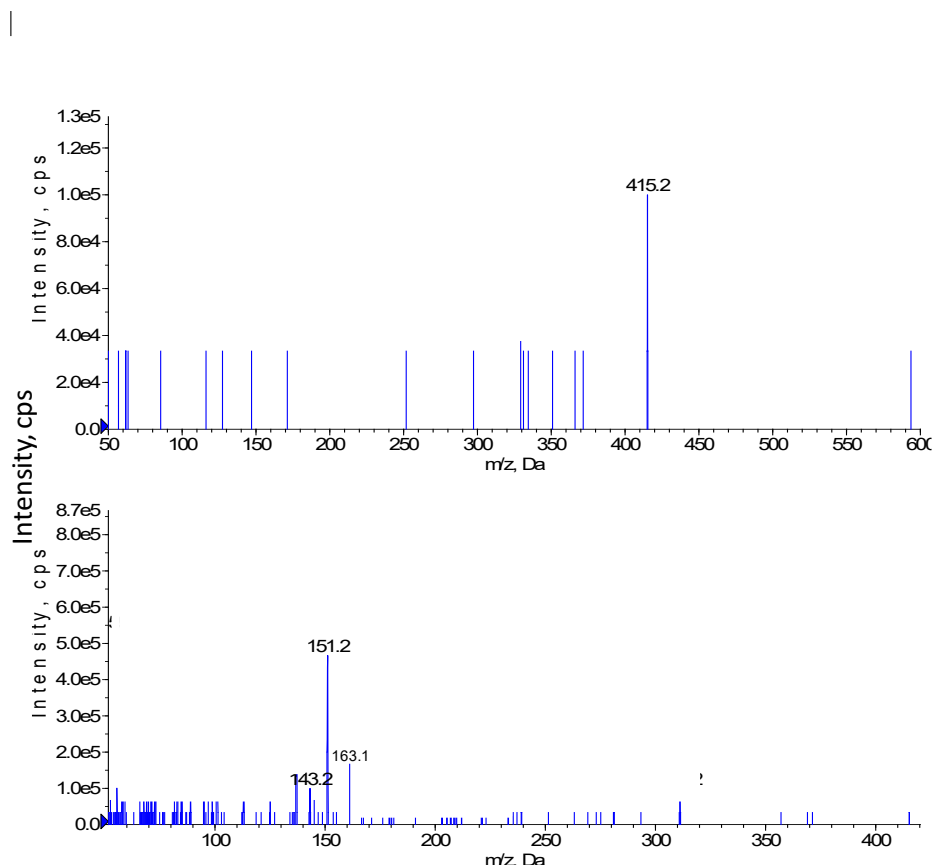
Compound name: HANT-*O*-MG-*O*-M (6)

Precursor ion /product ion (*m/z*): 415 /195



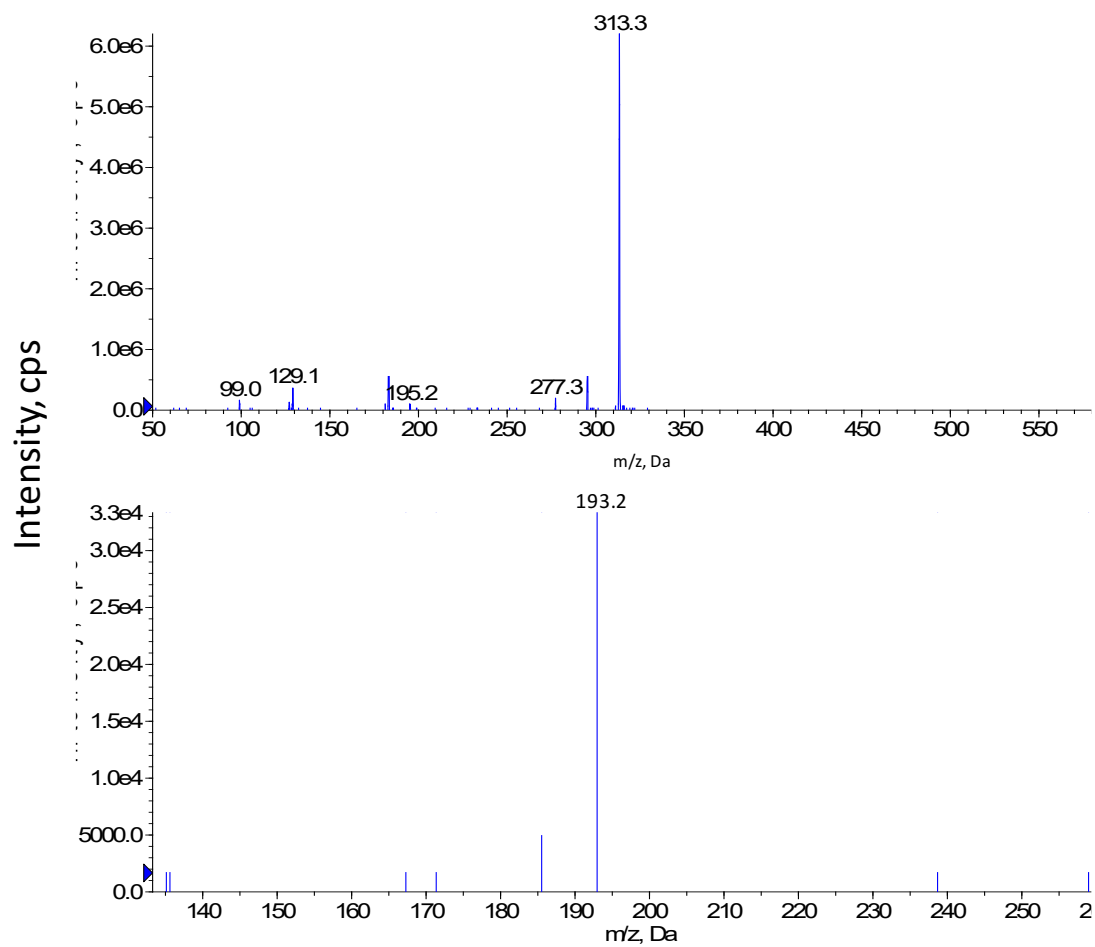
Compound name: HANT-C-MG-O-M (7)

Precursor ion /product ion (m/z): 415 /151



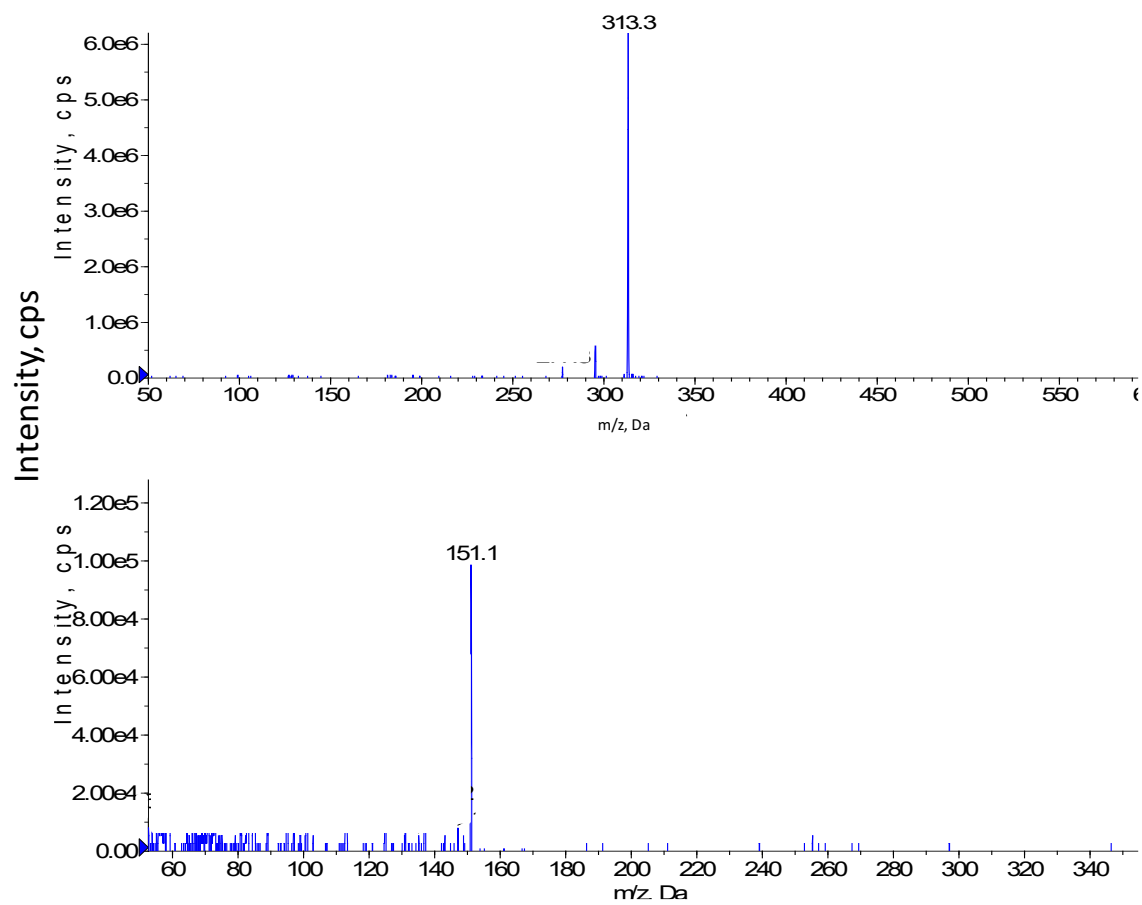
Compound name: ANT-*N*-MG (8)

Precursor ion /product ion (*m/z*): 313/193



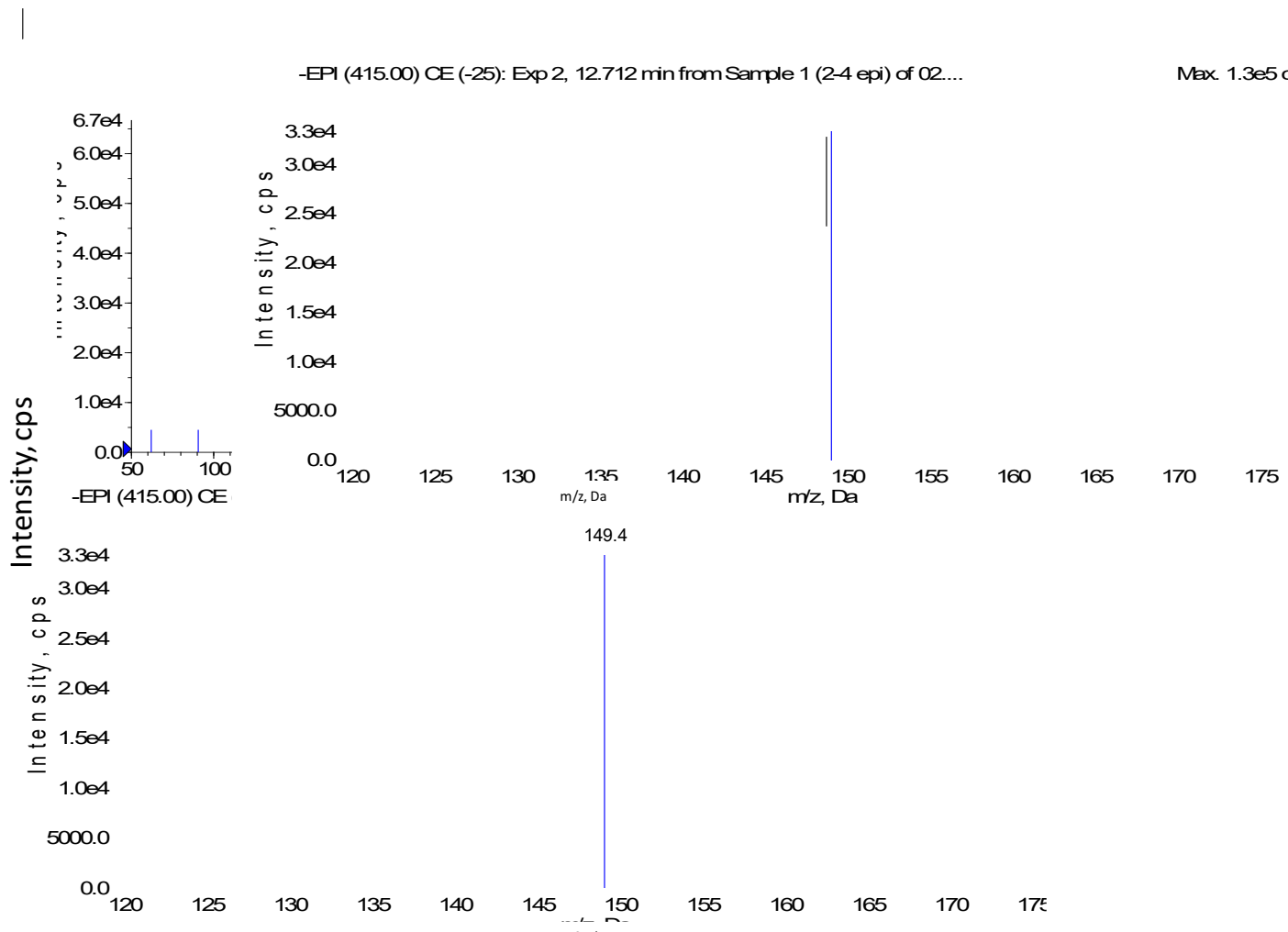
Compound name: ANT-C-MG (9)

Precursor ion /product ion (m/z): 313 /151



Compound name: ANT-C & N- DG (10)

Precursor ion /product ion (m/z): 475 /149



Appendix IV

(Chapter 5 data)

i) Aphids on 2,6-DNT treated *A. thaliana*:

Survival:

Table 1: Survival of *M. persicae* and *B. brassicae* on 2,6-DNT treated *A. thaliana* after 4 days of exposure to 14 day old (post cultivation). Data points are the mean of aphid survival from 15 replicates (7 aphids per treatment).

2,6-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
Control	7 $\pm$ 0.02	7 $\pm$ 0.04
0.25% ethanol contration	7 $\pm$ 0.03	7 $\pm$ 0.03
5	4.3 $\pm$ 1.21	4.4 $\pm$ 0.44
15	3.5 $\pm$ 0.24	2.8 $\pm$ 0.34
25	3 $\pm$ 0.43	1.2 $\pm$ 0.25
35	1.5 $\pm$ 0.44	0.5 $\pm$ 0.25
45	0.46 $\pm$ 0.45	0.14 $\pm$ 0.43
55	0	0

Mean Relative Growth rate:

Table 2: Mean Relative Growth Rate (MRGR) of *M. persicae* and *B. brassicae* on *A. thaliana* treated with 2,6-DNT in Phytigel media. The data is the mean of weight from

ten aphids (on 10 replicates-12 day old plants) at 18⁰C with 16:8 h day:night photoperiod. The negative values show the aphid weight loss. Units stand for mass increase (g) per aboveground mass (g) per day (d).

2,6-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
Control	0.27±0.05	0.245±0.023
0.25% ethanol control	0.28±0.042	0.24±0.05
5	0.19±0.044	0.017±0.043
15	0.17±0.032	-0.05±0.043
25	0.022±0.045	-0.088±0.056
35	-0.129±0.0355	-0.199±0.034
45	-0.16±0.043	-0.46±0.034
55	0	0

Fecundity

Table 3: Fecundity of *M. persicae* and *B. brassicae* on Phytigel grown 2,6-DNT treated *A. thaliana*. The data is mean of no. of nymphs (produced by a single aphid) from 15 replicates (plants) at 18⁰C with 16:8 h day:night photoperiod.

2,6-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
Control	17.37±1.11	14.33±1.02
0.25% ethanol control	17.32±1.64	14.21±1.3
5	12.78±1.13	11.22±1.81
15	11.3±1.01	8.35±1.22
25	9.55±1.08	7.95±1.31

35	7.44±0.56	3.9±0.44
45	3.57±0.19	2.1±0.21
55	0	0

Time course study of weight

Table 4: Weight (mean) of (A) *M. persicae* and (B) *B. brassicae* on the Phytigel media grown *A. thaliana* against different concentrations of 2,6-DNT. The first instar nymphs were analysed after 4 days of infestation on 2,6-DNT treated *A. thaliana* (14 days old plants after 2 days of post germination). The survival data is mean and weight is average of six aphids on 10 replicates (plants) at 18⁰C with 16:8 h day:night photoperiod. The red data point represents mock control (0.25% ethanol control).

A

2,6-DNT concentration	2nd day	4th day	6th day
Control	0.147±0.01	0.28±0.012	0.441±0.02
0.25% ethanol control	0.137±0.02	0.28±0.023	0.443±0.024
5	0.135±0.03	0.26±0.023	0.303±0.03
15	0.083±0.02	0.22±0.02	0.281±0.022
25	0.0386±0.023	0.117±0.022	0.157±0.018
35	0.0253±0.012	0.057±0.025	0.087±0.023
45	0.034±0.011	0.058±0.012	0.078±0.022
55	0.0141±0.22	0±0	0±0

B

2,6-DNT concentration	2 nd day	4 th day	6 th day
Control	0.29±0.09	0.51±0.045	0.813±0.057
0.25% ethanol control	0.287±0.07	0.48±0.045	0.811±0.057
5	0.186±0.08	0.26±0.08	0.371±0.056
15	0.137±0.07	0.18±0.0712	0.25±0.076
25	0.094±0.08	0.121±0.0612	0.22±0.07
35	0.058±0.06	0.098±0.0513	0.138±0.09
45	0.021±0.05	0.03±0	0.0447±0.05
55	0±0	0±0	0±0

v) Aphids on 2,4-DNT treated *A. thaliana*:

Survival:

Table 5: Survival of *M. persicae* and *B. brassicae* on 2,4-DNT treated *A. thaliana* after 4 days of exposure to 14 day old (post cultivation). Data points are the mean of aphid survival from 15 replicates (7 aphids per treatment).

2,4-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
Control	7±0.03	7±0.04
0.25% ethanol contration	7±0.04	7±0.02
0.05	3.9±1.21	4.2±0.44
0.1	2.77±0.24	2.6±0.34

0.25	1.9 $\pm$ 0.93	1.66 $\pm$ 1.03
0.5	1.23 $\pm$ 0.43	1.38 $\pm$ 0.25
1	0.28 $\pm$ 0.44	0.19 $\pm$ 0.25
2.5	0.6 $\pm$ 0.45	0.66 $\pm$ 0.43
5	0	0

Fecundity:

Table 6: Fecundity of *M. persicae* and *B. brassicae* on Phytigel grown 2,4-DNT treated *A. thaliana*. The data is mean of no. of nymphs (produced by a single aphid) from 15 replicates (plants) at 18⁰C with 16:8 h day:night photoperiod.

2,4-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
Control	19.8 $\pm$ 1.22	15.2 $\pm$ 1.67
0.25% ethanol control	19.6 $\pm$ 1.34	15.27 $\pm$ 1.51
0.05	16.47 $\pm$ 1.29	13.73 $\pm$ 1.44
0.1	14.67 $\pm$ 1.21	12.8 $\pm$ 1.34
0.25	12.4 $\pm$ 1.19	10.8 $\pm$ 1.31
0.5	9.73 $\pm$ 1.08	7.8 $\pm$ 1.23
1	4.6 $\pm$ 0.98	4.6 $\pm$ 0.76
2.5	2.33 $\pm$ 0.78	1.8 $\pm$ 0.87
5	0	0

Mean Relative Growth rate:

Table 7: Mean Relative Growth Rate (MRGR) of *M. persicae* and *B. brassicae* on *A. thaliana* treated with 2,4-DNT in Phytigel media. The data is the mean of weight from ten aphids (on 10 replicates-12 day old plants) at 18⁰C with 16:8 h day:night photoperiod. The negative values show the aphid weight loss. Units stand for mass increase (g) per aboveground mass (g) per day (d).

2,4-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
Control	0.27±0.021	0.25±0.023
0.25% ethanol control	0.26±0.031	0.24±0.05
0.05	0.106±0.022	0.16±0.043
0.1	0.044±0.032	0.1.9±0.043
0.25	-0.022±0.023	0.04±0.056
0.5	-0.063±0.0355	-0.017±0.034
1	-0.24±0.043	-0.233±0.034
2.5	-0.315±0.021	-0.34±0.056
5	0±0	0±0

Time course study of weight

Table 8: Weight (mean) of (A) *M. persicae* and (B) *B. brassicae* on the Phytigel media grown *A. thaliana* against different concentrations of 2,4-DNT. The first instar nymphs were analysed after 4 days of infestation on 2,4-DNT treated *A. thaliana* (14 days old plants after 2 days of post germination). The survival data is mean and weight is average of six aphids on 10 replicates (plants) at 18⁰C with 16:8 h day:night photoperiod. The red data point represents mock control (0.25% ethanol control).

A

2,4-DNT concentration	2 nd day	4 th day	6 th day
Control	0.14±0.012	0.24±0.013	0.44±0.023
0.25% ethanol control	0.126±0.012	0.24±0.034	0.40±0.012
0.05	0.071±0.012	0.15±0.023	0.22±0.023
0.1	0.066±0.032	0.106±0.034	0.17±0.032
0.25	0.041±0.032	0.096±0.023	0.132±0.021
0.5	0.031±0.023	0.074±0.034	0.113±0.021
1	0.011±0.023	0.03±0.023	0.057±0.032
2.5	0.0071±0.032	0.013±0.023	0.042±0.032
5	0±0	0±0	0±0

B

2,4-DNT concentration	2 nd	4 th day	6 th day
Control	0.306±0.087	0.52±0.067	0.83±0.087
0.25% ethanol control	0.28±0.067	0.49±0.068	0.811±0.067
0.05	0.184±0.078	0.35±0.078	0.57±0.078
0.1	0.134±0.065	0.31±0.078	0.48±0.065
0.25	0.127±0.068	0.23±0.023	0.37±0.068
0.5	0.08±0.078	0.197±0.098	0.286±0.078
1	0.052±0.098	0.09±0.078	0.118±0.098

2.5	0.024±0.05	0.033±0.04	0.08±0.05
5	0±0	0±0	0±0

vi) **Aphids on 2,3-DNT treated *A. thaliana*:**

Survival:

Table 9: Survival of *M. persicae* and *B. brassicae* on 2,3-DNT treated *A. thaliana* after 4 days of exposure to 14 day old (post cultivation). Data points are the mean of aphid survival from 15 replicates (7 aphids per treatment).

2,3-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
Control	7±0	7±0
0.25% ethanol contration	7±0	7±0
0.05	4.9±1.21	2.8±0.44
0.1	2.11±0.24	1.33±0.34
0.25	1.3±0.43	0.99±0.25
0.5	0.5±0.44	0.43±0.25
1	0.3±0.45	0.26±0.43
2.5	0	0
5	0	0

Mean Relative Growth rate:

Table 10: Mean Relative Growth Rate (MRGR) of *M. persicae* and *B. brassicae* on *A. thaliana* treated with 2,3-DNT in Phytigel media. The data is the mean of weight from ten aphids (on 10 replicates-12 day old plants) at 18⁰C with 16:8 h day:night

photoperiod. The negative values show the aphid weight loss. Units stand for mass increase (g) per aboveground mass (g) per day (d).

2,3-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
Control	0.28±0.034	0.25±0.036
0.25% ethanol control	0.28±0.051	0.23±0.041
0.05	0.26±0.055	0.11±0.041
0.1	0.25±0.054	0.08±0.034
0.25	0.196±0.076	0.0066±0.016
0.5	0.047±0.012	-0.172±0.071
1	-0.22±1.	-0.486±0.018
2.5	0	0
5	0	0

Table 11: Fecundity of *M. persicae* and *B. brassicae* on Phytigel grown 2,3-DNT treated *A. thaliana*. The data is mean of no. of nymphs (produced by a single aphid) from 15 replicates (plants) at 18⁰C with 16:8 h day:night photoperiod.

2,3-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
Control	19.8±1.34	15.2±1.42
0.25% ethanol control	19.6±1.12	15.267±1.72
0.05	17.3±1.23	13.6±1.31
0.1	15.067±1.24	11.4±1.56
0.25	13.54±1.52	9.8±1.81
0.5	11.33±1.33	6.2±1.21

1	9.2±1.23	4.93±0.98
2.5	0	0
5	0	0

Time course study of weight

Table 12: Weight (mean) of (A) *M. persicae* and (B) *B. brassicae* on the Phytigel media grown *A. thaliana* against different concentrations of 2,3-DNT. The first instar nymphs were analysed after 4 days of infestation on 2,3-DNT treated *A. thaliana* (14 days old plants after 2 days of post germination). The survival data is mean and weight is average of six aphids on 10 replicates (plants) at 18⁰C with 16:8 h day:night photoperiod. The red data point represents mock control (0.25% ethanol control).

A

2,3-DNT concentration	2nd day	4th day	6th day
Control	0.15±0.021	0.298±0.032	0.45±0.021
0.25% ethanol control	0.14±0.023	0.297±0.032	0.446±0.023
0.05	0.078±0.032	0.24±0.034	0.406±0.032
0.1	0.053±0.034	0.22±0.035	0.384±0.034
0.25	0.026±0.023	0.133±0.023	0.315±0.023
0.5	0.0228±0.034	0.11±0.028	0.176±0.034
1	0.017±0.034	0.038±0.029	0.06125±0.034
2.5	0.0028±0.01	0.007±0.028	0
5	0	0	0

B

2,3-DNT concentration	2 nd day	4 th day	6 th day
Control	0.306±0.098	0.54±0.098	0.833±0.098
0.25% ethanol control	0.286±0.078	0.52±0.078	0.788±0.078
0.05	0.184±0.067	0.32±0.067	0.474±0.067
0.1	0.126±0.054	0.28±0.054	0.43±0.054
0.25	0.0588±0.076	0.16±0.076	0.321±0.076
0.5	0.05±0.056	0.104±0.056	0.154±0.056
1	0.009±0.023	0.02±0.023	0.043±0.023
2.5	0±0	0±0	0±0
5	0±0	0±0	0±0

vii) Aphids on 3,4-DNT treated *A. thaliana*:

Survival:

Table 13: Survival of *M. persicae* and *B. brassicae* on 3,4-DNT treated *A. thaliana* after 4 days of exposure to 14 day old (post cultivation). Data points are the mean of aphid survival from 15 replicates (7 aphids per treatment).

3,4-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
Control	7±0	7±0
0.25% ethanol contration	7±0	7±0
0.05	4.6±1.21	4.02±0.44

0.1	3.33±0.24	3.33±0.31
0.25	2.6±0.43	1.66±0.25
0.5	1.3±0.92	0.5±0.25
1	0.99±0.45	0.36±0.41
2.5	0.6±0.47	0
5	0	0

Mean Relative Growth rate:

Table 14: Mean Relative Growth Rate (MRGR) of *M. persicae* and *B. brassicae* on *A. thaliana* treated with 3,4-DNT in Phytigel media. The data is the mean of weight from ten aphids (on 10 replicates-12 day old plants) at 18°C with 16:8 h day:night photoperiod. The negative values show the aphid weight loss. Units stand for mass increase (g) per aboveground mass (g) per day (d).

3,4-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
Control	0.28±0.08	0.25±0.054
0.25% ethanol control	0.278±0.092	0.24±0.044
0.05	0.22±0.076	0.182±0.034
0.1	0.18±0.074	0.16±0.065
0.25	0.1040.066	0.079±0.046
0.5	0.068±0.054	0.0366±0.065
1	0.066±0.063	-0.065±0.087
2.5	-0.051±0.067	0
5	0	0

Table 15: Fecundity of *M. persicae* and *B. brassicae* on Phytigel grown 3,4-DNT treated *A. thaliana*. The data is mean of no. of nymphs (produced by a single aphid) from 15 replicates (plants) at 18⁰C with 16:8 h day:night photoperiod.

3,4-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
Control	19.73±0.065	15.2±0.068
0.25% ethanol control	19.6±0.077	15.267±0.027
0.05	17.67±0.065	12.733±0.032
0.1	15.07±0.076	10.933±0.054
0.25	13.6±0.044	6.4±0.043
0.5	9.8±0.076	3.93±0.065
1	5.2±0.034	2.93±0.066
2.5	3.2±0.056	0
5	0	0

Time course study of weight

Table 16: Weight (mean) of (A) *M. persicae* and (B) *B. brassicae* on the Phytigel media grown *A. thaliana* against different concentrations of 3,4-DNT. The first instar nymphs were analysed after 4 days of infestation on 3,4-DNT treated *A. thaliana* (14 days old plants after 2 days of post germination). The survival data is mean and weight is average of six aphids on 10 replicates (plants) at 18⁰C with 16:8 h day:night photoperiod. The red data point represents mock control (0.25% ethanol control).

A

3,4-DNT	2nd day	4th day	6th day
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concentration			
Control	0.17±0.025	0.26±0.025	0.47±0.032
0.25% ethanol control	0.16±0.024	0.25±0.024	0.45±0.034
0.05	0.12±0.024	0.2±0.024	0.34±0.025
0.1	0.11±0.028	0.167±0.028	0.298±0.023
0.25	0.087±0.032	0.146±0.032	0.215±0.019
0.5	0.081±0.034	0.12±0.034	0.19±0.025
1	0.07±0.025	0.12±0.034	0.189±0.024
2.5	0.04±0.023	0.086±0.025	0.137±0.024
5	0±0	0±0	0±0

B

3,4-DNT concentration	2 nd day	4 th day	6 th day
Control	0.306±0.043	0.52±0.048	0.83±0.087
0.25% ethanol control	0.28±0.045	0.503±0.054	0.798±0.078
0.05	0.18±0.054	0.36±0.052	0.63±0.065
0.1	0.16±0.053	0.31±0.049	0.59±0.054
0.25	0.104±0.042	0.23±0.052	0.43±0.056
0.5	0.05±0.056	0.14±0.048	0.35±0.045
1	0.009±0.023	0.09±0.047	0.23±0.056
2.5	0±0	0±0	0±0

5	0±0	0±0	0±0
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Metabolites

Table 17: 2A6NT concentration in *M. persicae* and *B. brassicae* feeding on 2,6-DNT treated *A. thaliana*.

2,6-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
5	0.032±0.012	0.038±0.012
15	0.13±0.011	0.213±0.032
25	0.35±0.016	0.38±0.022
35	0.11±0.014	0.16±0.012
45	0	0
55	0	0

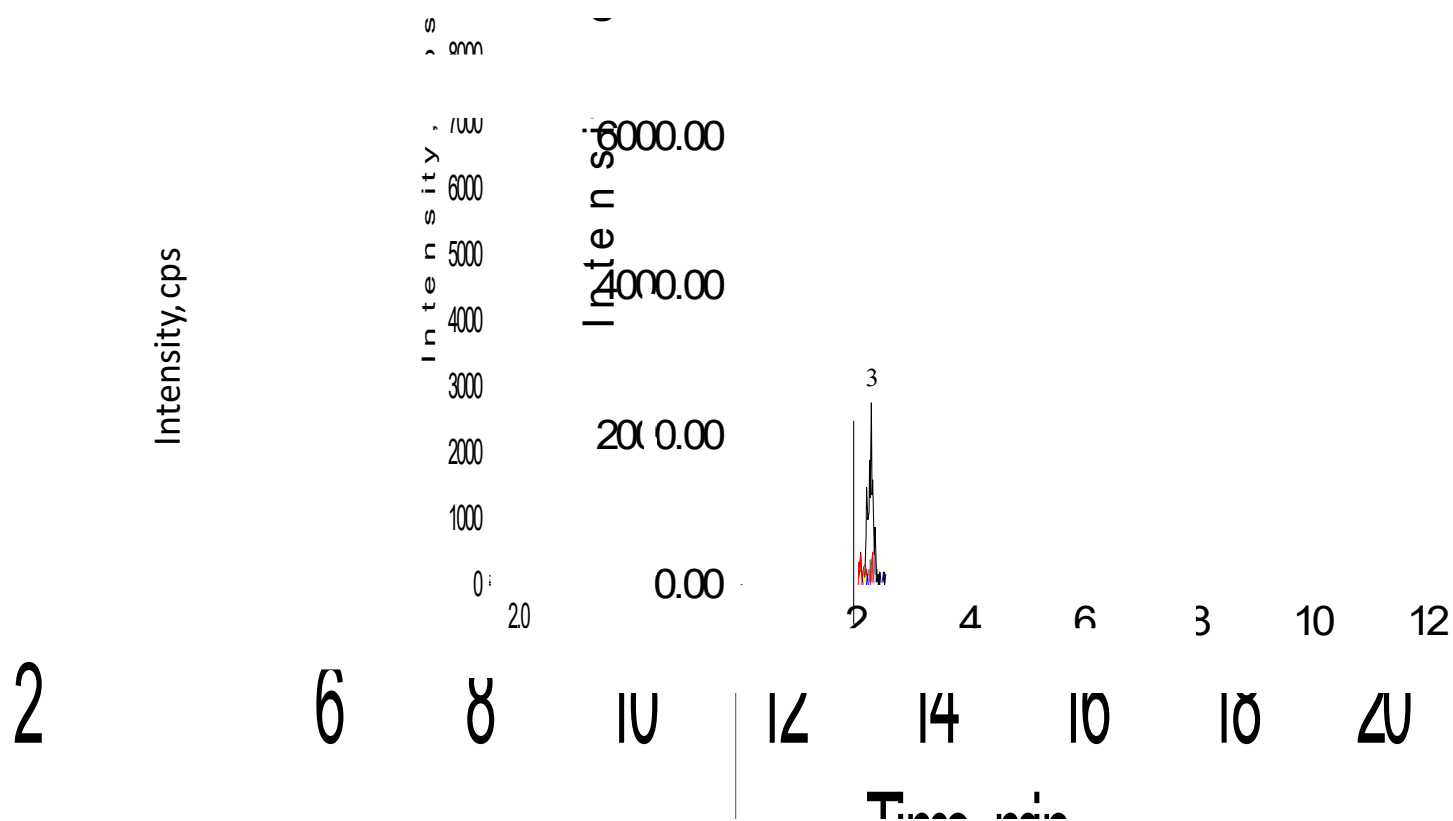


Figure 2: MRM scan (one of three technical replicates) of *M. persicae* fed on 2,6-DNT treated *A. thaliana* with concentration of 2.5 mg /L of Phytigel media (14 days post cultivation) and cultivated under growth temperature of $20\pm3^{\circ}\text{C}$ for 16:8 h day:night period. (1) DHAT-*O*-MG-*N*-OP; (2) DHAT-*C*-MG-*N*-OP; (3) HANT-*C*-MG-*N*-OB (4) HANT-*C*-MG; (7) HANT-*C*-MG-*O*-M; (8) ANT-*N*-MG.

Table 18: Relative concentration of glucosyl conjugates in *M. persicae* feeding on 2,6-DNT treated *A. thaliana*

2,6-DNT concentration	DHAT- <i>O</i> - MG- <i>N</i> -OP (1)	DHAT- C-MG- N-OP (2)	HANT- <i>C</i> - MG (4)	ANT- <i>C</i> - MG (9)	ANT- <i>N</i> - MG (8)	HANT- <i>C</i> - MG- <i>N</i> - OB (3)
5	0.324±0.04	0.21±0.03	0.302±0.02	0.301±0.01	0.299±0.03	0.20±0.01
15	0.59±0.05	0.41±0.01	0.403±0.01	0.303±0.05	0.303±0.04	0.29±0.02

25	0.64±0.06	0.52±0.02	0.506±0.02	0.405±0.04	0.39±0.01	0.301±0.04
35	0.69±0.08	0.64±0.01	0.607±0.03	0.506±0.01	0.504±0.02	0.502±0.02
45	0.76±0.07	0.73±0.03	0.71±0.01	0.709±0.02	0.707±0.01	0.693±0.03
55	0	0	0	0	0	0

Table 19: Relative concentration of glucosyl conjugates in *B. brassicae* feeding on 2,6-DNT treated *A. thaliana*

2,6-DNT concentration	DHAT-O- MG-N-OP (1)	DHAT- C-MG- N-OP (2)	HANT-C- MG (4)	ANT-C- MG (9)	ANT-N- MG (8)	HANT-C- MG-N- OB (3)
5	0.53±0.03	0.43±0.02	0.40±0.04	0.302±0.04	0.302±0.03	0.299±0.04
15	0.74±0.05	0.63±0.01	0.507±0.02	0.303±0.03	0.603±0.04	0.402±0.01
25	0.82±0.06	0.73±0.03	0.607±0.01	0.405±0.01	0.704±0.01	0.604±0.02
35	0.897±0.08	0.84±0.04	0.71±0.05	0.506±0.01	0.806±0.02	0.704±0.01
45	0.95±0.07	0.94±0.01	0.93±0.01	0.709±0.05	0.907±0.01	0.904±0.02
55	0	0	0	0	0	0

Table 20: 2A4NT concentration in *M. persicae* and *B. brassicae* feeding on 2,4-DNT treated *A. thaliana*

2,4-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
0.05	0	0
0.1	13.4±1.12	16.24±1.74
0.25	54.32±2.23	61.02±2.34

0.5	34.45 $\pm$ 2.43	38.71 $\pm$ 2.44
1	14.76 $\pm$ 2.46	16.55 $\pm$ 2.43
2.5	0	0
5	0	0

Table 21: 4A2NT concentration in *M. persicae* and *B. brassicae* feeding on 2,4-DNT treated *A. thaliana*

2,4-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
0.05	6.5 $\pm$ 0.33	6.7 $\pm$ 0.12
0.1	12.34 $\pm$ 0.45	14.45 $\pm$ 2.43
0.25	34.55 $\pm$ 1.33	41.22 $\pm$ 2.19
0.5	65.43 $\pm$ 1.45	68.75 $\pm$ 1.87
1	14.21 $\pm$ 1.31	18.22 $\pm$ 1.78
2.5	0	0
5	0	0

Table 22: Relative concentration of glucosyl conjugates in *M. persicae* feeding on 2,4-DNT treated *A. thaliana*

2,4-DNT concentration	DHAT-C-MG-N-OP (12)	HANT-C-MG-O-M (17)	HANT-O-MG (15)	ANT-C-MG (19)	DHAT-O-MG-N-OP (11)	HANT-C-MG-N-OB (13)
0.5	60.56 $\pm$ 8.23	56.77 $\pm$ 5.4	54.22 $\pm$ 3.2	42.32 $\pm$ 2.1	39.11 $\pm$ 6.78	33.01 $\pm$ 1.3
		3	2	1		3

0.1	66.77±6.78	65.92±5.3 2	61.67±2.1 1	54.55±4.2 2	45.98±8.32	41.12±1.2 1
0.25	72.32±5.66	71.22±4.5 2	65.9±4.2	62.34±3.2	53.45±8.77	49.98±0.9 8
0.5	81.66±9.87	79.31±6.3 3	73.42±6.1	69.44±5.2 3	59.11±12.3 4	51.01±1.4 5
1	86.33±10.9 7	81.56±3.1 2	80.54±7.0 1	71.56±8.4 3	64.98±10.9 8	56.78±1.2 2
2.5	95.33±9.87	93.22±4.2 2	89.12±4.3 4	86.77±5.9 7	75.65±9.34	72.78±1.0 9
5	0	0	0	0	0	0

Table 23: Relative concentration of glucosyl conjugates in *B. brassicae* feeding on 2,4-DNT treated *A. thaliana*

2,4-DNT concentration	DHAT-C- MG-N-OP (12)	HANT-C- MG-O-M (17)	HANT- O-MG (15)	ANT-C- MG (19)	DHAT- O-MG-N- OP (11)	HANT-C- MG-N- OB (13)
0.5	59.43±6.78	57.34±4.32	55.67±3.2 3	54.22±3.2 1	41.22±3.1	31.22±4.2 3
0.1	71.86±8.32	69.12±8.32	67.89±3.1 1	64.23±4.1	51.23±2.5	45.63±5.3 2
0.25	79.23±8.77	76.32±7.45	72.32±4.2 3	68.78±5.1	60.22±4.2 2	51.23±4.2 3
0.5	87.76±12.3	81.56±5.67	76.77±6.5	70.99±3.6	68.12±6.1	61.23±4.2

	4		5		1	2
1	91.65±10.9	90.78±10.9	89.33±4.2	78.12±2.9	71.31±8.3	65.43±4.3
	8	8	2		2	2
2.5	96.34±9.34	94.33±8.32	90.42±3.1	89.34±3.2	82.3±6.22	71.23±3.2
			1	1		2
5	0	0	0	0	0	0

Table 24: 2A3NT concentration in *M. persicae* and *B. brassicae* feeding on 2,3-DNT treated *A. thaliana*

2,3-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
0.05	12.33±1.55	16.53±1.12
0.1	23.21±1.34	29.87±1.32
0.25	34.52±1.54	41.22±1.41
0.5	14.11±1.33	18.11±1.64
1	0	0
2.5	0	0
5	0	0

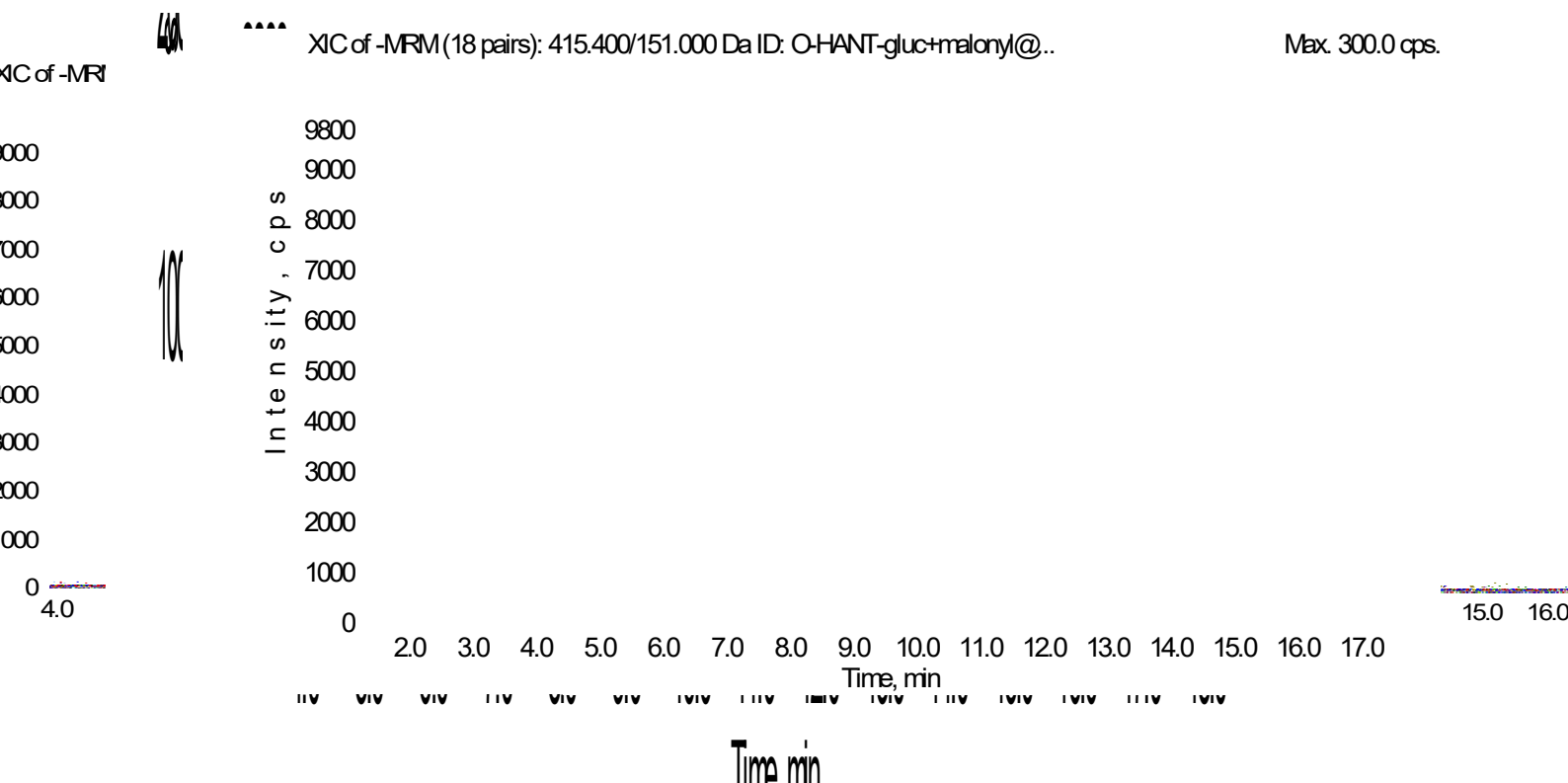


Figure 2: MRM scan (one of three technical replicates) of *M. persicae* fed on 2,4-DNT treated *A. thaliana* with concentration of 2.5 mg /L of Phytigel media (14 days post cultivation) and cultivated under growth temperature of $20\pm3^{\circ}\text{C}$ for 16:8 h day:night period. (11) DHAT-*O*-MG-*N*-OP; (12) DHAT-*C*-MG-*N*-OP; (13) HANT-*C*-MG-*N*-OB; (15) HANT-*O*-MG; (17) HANT-*C*-MG-*O*-M; (19) ANT-*C*-MG.

Table 25: Relative concentration of glucosyl conjugates in *M. persicae* feeding on 2,4-DNT treated *A. thaliana*

2,4-DNT concentration	HANT- <i>O</i> - MG (25)	ANT- <i>N</i> - MG (28)	HANT- <i>C</i> -MG (24)	DHAT- <i>O</i> -MG- <i>N</i> -OP (22)	HANT- <i>O</i> - MG- <i>O</i> -M (26)	ANT- <i>C</i> - MG (29)
0.5	64.56±0.92	57.75±1.11	54.88±2.1	44.56±1.3	35.33±0.98	36.54±0.12

0.1	66.77±1.12	66.22±0.76	58.21±1.2	49.43±0.8	38.98±0.44	47.12±0.8
0.25	80.32±1.02	76.97±1.2	60.21±0.9	51.89±0.4	46.55±1.32	48.99±1.8
0.5	82.66±0.55	79.34±0.55	73.22±0.3	62.33±3.3	47.82±2.35	50.14±1.4
1	87.33±1.02	82.32±0.98	74.32±2.3	64.21±2.4	59.13±3.21	63.56±2.09
2.5	92.33±0.77	89.32±1.06	86.56±1.6	81.62±3.2	72.32±4.22	80.32±2.11
5	0	0	0	0	0	0

Table 26: Relative concentration of glucosyl conjugates in *B. brassicae* feeding on 2,4-DNT treated *A. thaliana*

2,4-DNT concentration	HANT-O- MG (25)	ANT-N- MG (28)	HANT-C- MG (24)	DHAT-O- MG-N-OP (22)	HANT-O- MG-O-M (26)	ANT-C- MG (29)
0.5	65.43±0.8	61.54±1.21	56.77±1.34	49.56±2.13	34.45±0.56	35.67±0.45
0.1	74.86±1.1	67.88±1.23	62.12±1.45	57.88±0.23	35.67±1.98	37.72±0.11
0.25	81.23±0.98	78.32±0.98	68.32±2.31	59.21±0.87	37.88±0.67	43.23±1.87
0.5	87.76±0.43	80.34±1.02	75.32±0.98	70.21±1.67	41.12±2.83	45.44±1.08
1	90.65±1.08	86.56±2.01	78.81±1.36	73.34±2.98	43.11±0.11	52.31±1.98
2.5	95.34±0.53	91.76±1.11	88.22±2.34	85.56±3.22	64.45±2.09	71.55±4.22
5	0	0	0	0	0	0

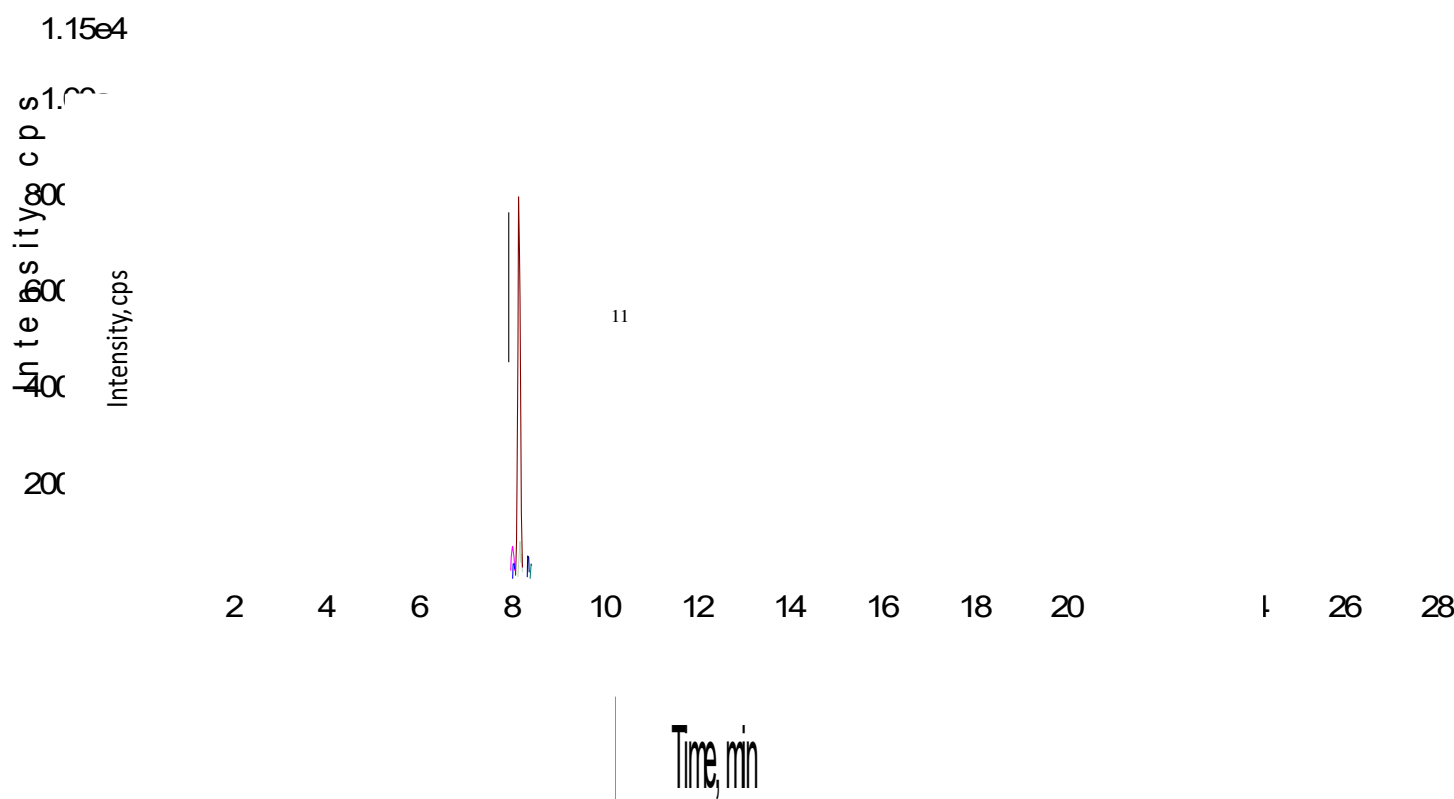


Figure 3: MRM scan (one of three technical replicates) of *M. persicae* fed on 2,3-DNT treated *A. thaliana* with concentration of 2.5 mg /L of Phytigel media (14 days post cultivation) and cultivated under growth temperature of $20\pm3^{\circ}\text{C}$ for 16:8 h day:night period. (11) DHAT-*O*-MG-*N*-OP; (12) DHAT-*C*-MG-*N*-OP; (13) HANT-*C*-MG-*N*-OB; (15) HANT-*O*-MG; (17) HANT-*C*-MG-*O*-M; (19) ANT-*C*-MG.

Table 25: Relative concentration of glucosyl conjugates in *M. persicae* feeding on 2,3-DNT treated *A. thaliana*

2,3-DNT concentration	DHAT- <i>C</i> - MG- <i>N</i> -OP (12)	HANT- <i>C</i> - MG- <i>O</i> -M (17)	HANT- O-MG (15)	ANT- <i>C</i> - MG (19)	DHAT- <i>O</i> - MG- <i>N</i> -OP (11)	HANT- <i>C</i> - MG- <i>N</i> - OB (13)
0.5	60.56 $\pm$ 8.23	56.77 $\pm$ 5.4	54.22 $\pm$ 3.2	42.32 $\pm$ 2.1	39.11 $\pm$ 6.78	33.01 $\pm$ 1.3
		3	2	1		3

0.1	66.77±6.78	65.92±5.3 2	61.67±2.1 1	54.55±4.2 2	45.98±8.32	41.12±1.2 1
0.25	72.32±5.66	71.22±4.5 2	65.9±4.2	62.34±3.2	53.45±8.77	49.98±0.9 8
0.5	81.66±9.87	79.31±6.3 3	73.42±6.1	69.44±5.2 3	59.11±12.3 4	51.01±1.4 5
1	86.33±10.9 7	81.56±3.1 2	80.54±7.0 1	71.56±8.4 3	64.98±10.9 8	56.78±1.2 2
2.5	95.33±9.87	93.22±4.2 2	89.12±4.3 4	86.77±5.9 7	75.65±9.34	72.78±1.0 9
5	0	0	0	0	0	0

Table 26: Relative concentration of glucosyl conjugates in *B. brassicae* feeding on 2,3-DNT treated *A. thaliana*

2,3-DNT concentration	DHAT-C- MG-N-OP (12)	HANT-C- MG-O-M (17)	HANT- O-MG (15)	ANT-C- MG (19)	DHAT- O-MG-N- OP (11)	HANT-C- MG-N- OB (13)
0.5	59.43±6.78	57.34±4.32	55.67±3.2 3	54.22±3.2 1	41.22±3.1	31.22±4.2 3
0.1	71.86±8.32	69.12±8.32	67.89±3.1 1	64.23±4.1	51.23±2.5	45.63±5.3 2
0.25	79.23±8.77	76.32±7.45	72.32±4.2 3	68.78±5.1	60.22±4.2 2	51.23±4.2 3
0.5	87.76±12.3	81.56±5.67	76.77±6.5	70.99±3.6	68.12±6.1	61.23±4.2

	4		5		1	2
1	91.65±10.9	90.78±10.9	89.33±4.2	78.12±2.9	71.31±8.3	65.43±4.3
	8	8	2		2	2
2.5	96.34±9.34	94.33±8.32	90.42±3.1	89.34±3.2	82.3±6.22	71.23±3.2
			1	1		2
5	0	0	0	0	0	0

Table 27: 3A4NT concentration in *M. persicae* and *B. brassicae* feeding on 3,4-DNT treated *A. thaliana*

3,4-DNT concentration	<i>M. persicae</i>	<i>B. brassicae</i>
0.05	0	0
0.1	12.11±1.03	10.41±1.48
0.25	22.31±2.11	24.35±1.41
0.5	8.21±1.34	14.11±1.32
1	0	0
2.5	0	0
5	0	0

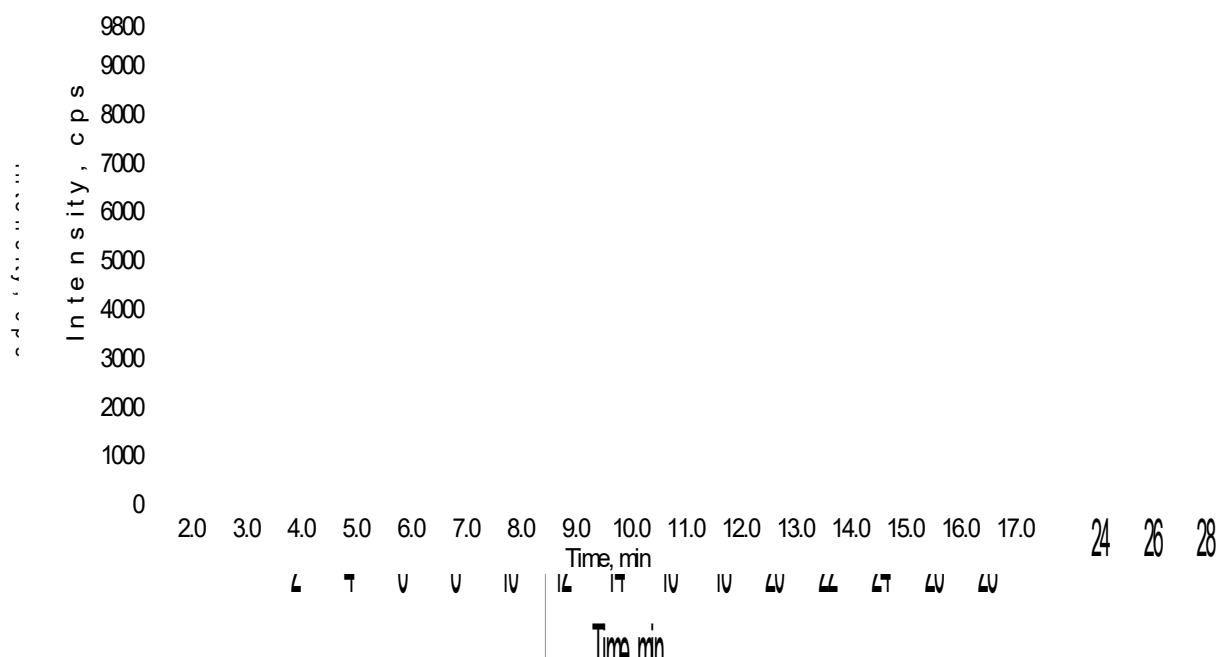


Figure 4: MRM scan (one of three technical replicates) of *M. persicae* fed on 3,4-DNT treated *A. thaliana* with concentration of 2.5 mg /L of Phytigel media (14 days post cultivation) and cultivated under growth temperature of $20\pm3^{\circ}\text{C}$ for 16:8 h day:night period. HANT-C-MG (34); ANT-C & N- DG (40); ANT-N-MG (38); HANT-O-MG-O-M (36); DHAT-O-MG-N-OP (31); HANT-C-MG-N-OB (33).

Table 28: Relative concentration of glucosyl conjugates in *M. persicae* feeding on 3,4-DNT treated *A. thaliana*

3,4-DNT concentration	HANT-C- MG (34)	ANT-C & N-MG (40)	ANT-N- MG (38)	HANT-O- MG-O-M (36)	DHAT-O- MG-N-OP (31)	HANT-C- MG-N-OB (33)
0.5	56.44±0.02	51.77±1.02	49.67±0.32	41.36±0.34	36.01±0.12	34.32±1.09
0.1	67.23±1.27	61.23±0.54	56.55±1.22	43.43±0.54	43.45±0.22	34.56±2.98
0.25	76.04±2.84	71.23±1.23	58.21±1.36	47.85±0.6	45.67±0.32	39.54±5.67

0.5	81.94±1.35	76.44±2.09	60.12±2.44	52.31±0.98	50.77±1.45	47.88±3.45
1	88.22±2.87	85.67±3.98	76.12±3.21	63.01±1.43	59.1±2.89	49.12±5.65
2.5	91.78±3.55	86.02±1.29	84.32±1.22	75.96±1.23	69.91±4.52	59.18±2.89
5	0	0	0	0	0	0

Table 29: Relative concentration of glucosyl conjugates in *B. brassicae* feeding on 3,4-DNT treated *A. thaliana*

3,4-DNT concentration	HANT-C- MG (34)	ANT-C & N-MG (40)	ANT-N- MG (38)	HANT-O- MG-O-M (36)	DHAT-O- MG-N-OP (31)	HANT-C- MG-N-OB (33)
0.5	57.82±1.23	52.88±4.8	50.78±2.34	45.02±1.09	38.54±1.33	35.12±1.32
0.1	69.22±3.34	66.91±2.45	59.85±4.52	49.99±0.98	45.9±3.09	36.67±4.65
0.25	78.23±6.68	75.81±5.6	63.01±2.65	57.03±1.3	49.33±3.77	40.77±3.26
0.5	82.34±6.3	79.31±1.23	68.23±1.34	58.71±2.05	51.02±2.98	49.23±1.56
1	89.67±3.2	87.5±3.2	79.66±6.32	75.98±4.3	61.31±2.1	56.11±2.3
2.5	92.34±2.1	91.21±4.33	85.11±7.54	83.45±3.54	73.41±1.1	65.89±1.23
5	0	0	0	0	0	0