Indian J. Plant Physiol., Vol. 13, No. 3, (N.S.) pp. 211-216 (July-Sept., 2008)

EFFECTS OF HMX AND TNT CONTAMINATIONS ON BIOCHEMICAL CONSTITUENTS IN *TRITICUM SATIVUM* L. AND *RAPHANUS SATIVUS* L. PLANTS

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Received on 20 Dec., 2007, Revised on 5 May, 2008

SUMMARY

Dose-response experiments formed the basis for evaluating the toxic effects using 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazine (HMX) spiked artificial soils. These stresses to *Raphanus sativus* L. resulted in a reduction in chlorophyll, increase in carotenoid and xanthophylls, reduction in leaf, tuber and root protein, a significant accumulation of free proline in leaves and tuber. HMX decreased proline in root and TNT increased it. HMX stress to *Triticum sativum* L. resulted in an increase in chlorophyll, decrease in carotenoid and xanthophylls, reduction in leaf protein, and increase in tuber and root protein, a reduction in leaf proline, increase in root proline. TNT resulted an increase in leaf and root proline but there are curved behavior to accumulation of free proline in ear. The magnitude of increase in free proline accumulation was higher in the more tolerant plant. Results indicate *Triticum sativum* L. is more tolerant to TNT and HMX than *Raphanus sativus* L.

***Key words*: Chlorophyll, HMX, proline, protein, *Raphanus sativus* L., TNT, *Triticum sativum* L.**

INTRODUCTION

One of the major problems facing the industrialized world today is the contamination of soils, ground water, and air with hazardous and toxic chemicals. A large part of the contamination comes from agricultural, industrial, and military activities. The contamination of soil and water with residues of explosives is a widespread environmental problem in large military complexes (Gorontzy *et al*. 1994). The manufacture, processing, and packaging of 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazine (HMX) at military ammunition plants over several decades have resulted in high concentrations of these contaminants in soils at ammunition production sites (Widrig *et al*. 1997). Disposal of unwanted weapon systems due to demilitarization also adds to the pollution problem at military complexes.

The phytotoxicity of explosives, such as TNT and HMX, on higher plants was reported (Thorne 1999, Krishnan *et al*. 2000, Sunahara *et al*. 2001, Frische and Höper 2003, Elly *et al*. 2006). Some contamination stress results in a wide variety of physiological and biochemical changes in plants. The accumulation of low molecular weight solutes, such as proline and betaine commonly referred to as compatible solutes (Yancy *et al*. 1982) is one example. The accumulation of proline in stressed plants is associated with reduced damage to membranes and proteins (Siripornadulsil *et al*. 2002). In addition to acting as osmoprotectant (Christian 1955, Paleg *et al*. 1984, Delauney and Verma 1993, Taylor 1996), proline

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also serves as a sink for energy to regulate redox potentials (Saradhi and Saradhi 1991), a hydroxy radical scavenger (Smirnoff and Cumbes 1989), a solute that protects macromolecules against denaturation (Schobert and Tschesche 1978), a means of reducing the acidity in the cell (Venkamp *et al*. 1989), a storage compound and nitrogen source for rapid growth after stress (Singh *et al*. 1973), a protein stabilizer (Kuznetsov and Shevyakova 1997, Shah and Dubey 1998) and an inhibitor of lipid peroxidation (Mehta and Gaur 1999). The free proline has also been shown to protect plants against free-radical induced damage by quenching of singlet oxygen (Alia and Matysik 2001, Matysik *et al*. 2002). The higher accumulation of proline could be due to enhanced activities of ornithine aminotransferase (OAT) and pyrroline-5-carboxylate reductase (P-5-CR), the enzymes involved in proline biosynthesis (Kohl *et al*. 1990), as well as due to inhibition of proline oxidase and proline dehydrogenase (PDH) and proline catabolizing enzymes (Kandpal *et al*. 1981). In this study, *Triticum sativum* L. and *Raphanus sativus* L. were exposed for 45 and 75 days respectively, and proline, chlorophyll a+b, carotenoid, xanthopyll and protein content were recorded to evaluate the tolerance of *Triticum sativum* L. and *Raphanus sativus* L. against toxicity using TNT and HMX as the main contaminants.

MATERIALS AND METHODS

Plant TNT and HMX treatments: TNT and HMX were procured from local industrials and other chemicals used were of analytical grade and obtained from E. Merck (Darmstadt, FRG), BDH Poole (England) and Sigma Chemical Co. (USA). Loamy soil from Varamin was spiked (Gholamian and Gholamian 2007) with TNT and HMX each at 0, 50, 200, 350, 500 mg kg^{-1} dw using acetone as a solvent. Solvent-spiked artificial soils served as controls. After spiking, the soils were mixed with a stainless steel spatula and placed in a vented fume hood without illumination for 2 h to allow the acetone evaporation prior to exposure of the test organisms. All units were sprayed with reverse osmosis (RO) water immediately after the test organisms were placed on the soils, and, subsequently, every other day as needed. *Triticum sativum* L. and *Raphanus sativus* L. seeds (from PVR. Seed and Plant Certification Research Institute, Tehran, Iran) were surface-disinfected by immersing in 0.3% sodium hypochlorite for 5 min and rinsing with sterile, deionized distilled water. For the plant test units, 10 seeds were placed in plastic pots containing 1 kg of spiked soil. The pots were watered daily and kept in botanical garden under natural photoperiod of 12–13 h and temperature of 28±4æ%C. All treatments were replicated six times. Care was taken to avoid drainage of soil solution during the treatment by giving water slightly less than the field capacity and draining water, if any, was returned to pots.

Determination of chlorophyll, carotenoid and xanthophyll content: Concentrations of Chl. (a + b) were measured after extraction with 80% acetone. After appropriate intervals of time, 10 segments (~ 0.250 g) were blotted and extracted by boiling in 80% (v/v) ethanol for 10 min repeatedly. The ethanolic extracts were combined and made to 10 ml. Chlorophylls a and b were quantified and expressed as mg g^{-1} fresh weight by transferring 600 µl aliquots of the ethanolic extract into 4.2 ml of 80% (v/v) acetone and determining the absorbance at 663 and 645 nm according to Arnon's method (1949). Chlorophyll a/b ratio was then calculated.

Carotenoid and xanthophyll concentration was measured after extraction with petroleum ether and then 92% methanol from 80% acetone extracts. Xanthophyll extract in methanol and carotenoid remained at petroleum ether layer. Quntification of xanthophyll and carotenoid was performed after saponification of their solution with methanolic potash solution and determining the absorbance at 445 nm (Jensen 1978).

Proline and protein assays: Free proline content was extracted from leaves in 3% aqueous sulphosalicylic acid and estimated using ninhydrin reagent (Bates *et al*. 1973). Protein was extracted in Tris–HCl (pH 6-7) from *Triticum sativum* L. and *Raphanus sativus* L. and then estimated with phenol reagent (Lowry *et al*. 1951).

RESULTS AND DISCUSSION

Plant dose-response of *Raphanus sativus* L. 45 days after sowing (DAS) and *Triticum sativum* L. 75 DAS to TNT and HMX treatment were recorded. Values are mean from five replications. In these analyses, a p-value <0.05 was accepted as significant.

Chlorophyll, carotenoid and xanthophyll content: Chlorophyll content decreased with increase in TNT and HMX concentration as compared to control. Only in *Triticum sativum* L., chlorophyll content increased with increasing concentration of HMX (Fig. 1a). In all cases, with decrease in chlorophyll content, the carotenoid and xanthophyll content increased and with increase in chlorophyll content, the carotenoid and xanthophyll content decreased (Fig. 1b,c). The decrease in chlorophyll concentration could be due to generation of reactive oxygen species (ROS) under TNT and HMX stress leading to the peroxidation of membrane lipids and, thus, the cooxidation of chlorophyll. To effectively eliminate ROS, an antioxidative systems consisting of low-molecular-weight antioxidants (such as ascorbate, carotenoids and xanthophyll) are important quenchers of the singlet state of chlorophyll and singlet oxygen (Candan and Tarhan 2003). The results indicated that during HMX stress to *Triticum sativum* L., HMX possibly did not convert to reactive oxygen species and acted as nutrient source, therefore, its chlorophyll content increased with increasing of HMX concentration.

Free proline and protein content: There was a linear increase in free proline accumulation with increasing severity of stresses in leaf and root of *Triticum sativum* L., leaf and tuber of *Raphanus sativus* L., while a reduction of proline in root of *Raphanus sativus* L. with

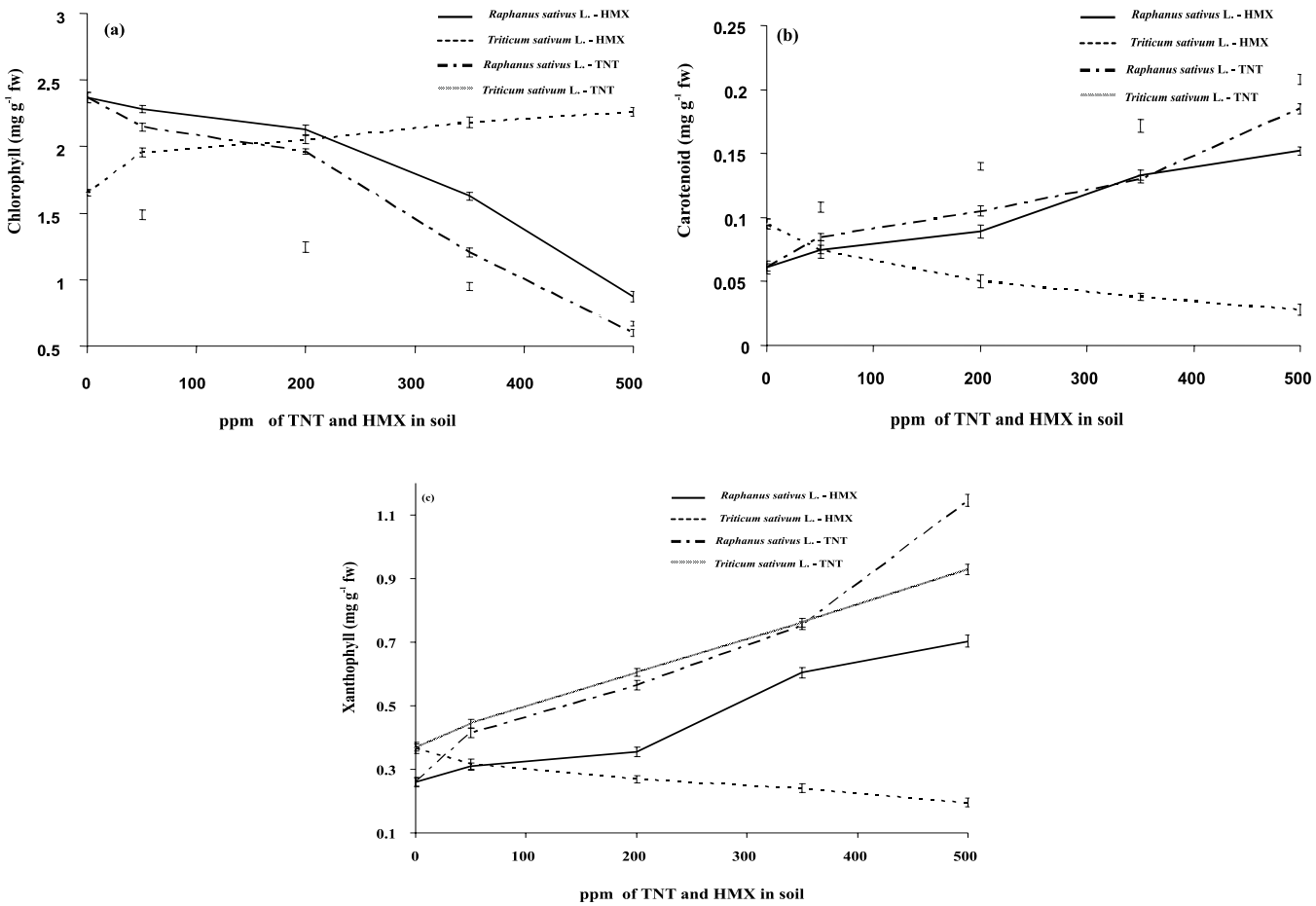

Fig. 1. Chlorophyll, carotenoid and xanthophyll content in leaves of *Triticum sativum* L. (75 DAS) and *Raphanus sativus* L. (45 DAS) as affected by TNT and HMX concentrations

increasing HMX concentration was observed (Fig. 2). More or less similar trends were observed for TNT stress (Fig. 3). Significantly higher proline accumulation in ear of *Triticum sativum* L. at 50 to 200 ppm of HMX and up to 350 ppm of TNT Kg^{-1} soil was observed.

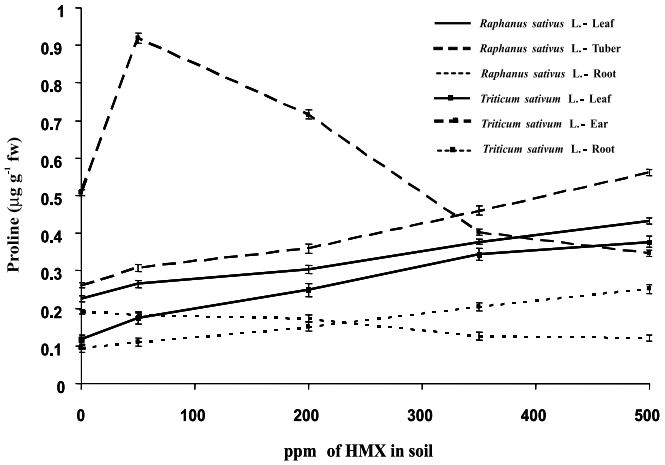

Fig. 2. Free proline content in different plant parts of *Triticum sativum* L. (75 DAS) and *Raphanus sativus* L. (45 DAS) as affected by HMX concentrations

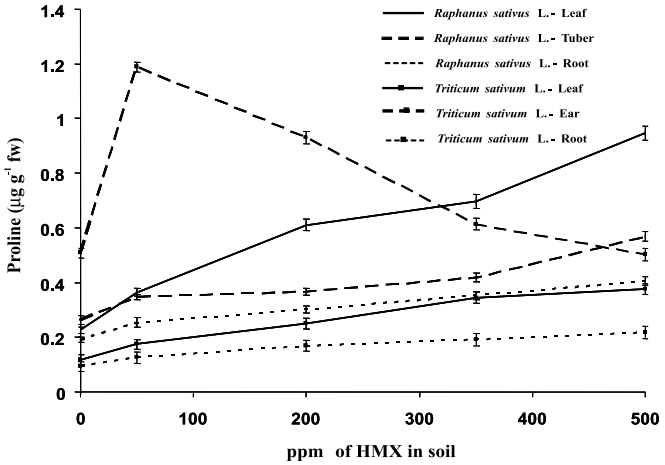

Fig. 3. Free proline content in different plant parts of *Triticum sativum* L. (75 DAS) and *Raphanus sativus* L. (45 DAS) as affected by TNT concentrations

Proline content increased up to 81% and 29% at 50 and 200 ppm of HMX and 133%, 83% and 21% at 50, 200 and 350 ppm of TNT, respectively. At higher concentration of HMX and TNT, the proline content in the ear significantly decreased in both the species. A reduction in protein content with increasing severity of HMX stresses in leaf, tuber, root of *Raphanus sativus* L. and leaf of *Triticum sativum* L. was observed. There was a linear increase in protein content in root and ear of *Triticum sativum* L. with increase in of HMX stress (Fig. 4). The increasing severity of TNT stress resulted in decrease in protein content except during stress up to 200 ppm of TNT that protein content of ear increased (Fig. 5).

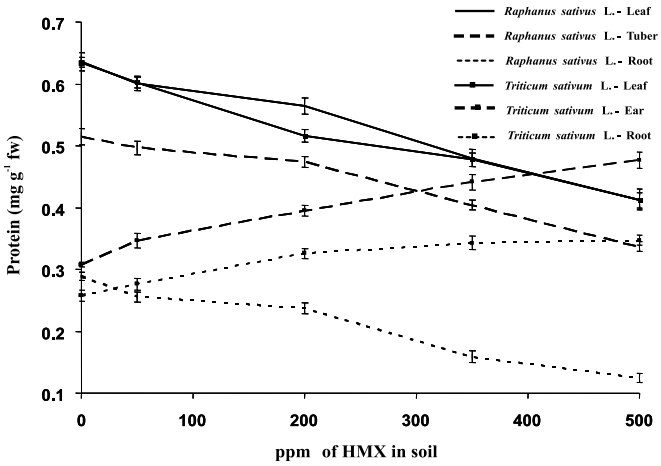

Fig. 4. Protein content in different parts of *Triticum sativum* L. (75 DAS) and *Raphanus sativus* L. (45 DAS) as affected by HMX concentrations

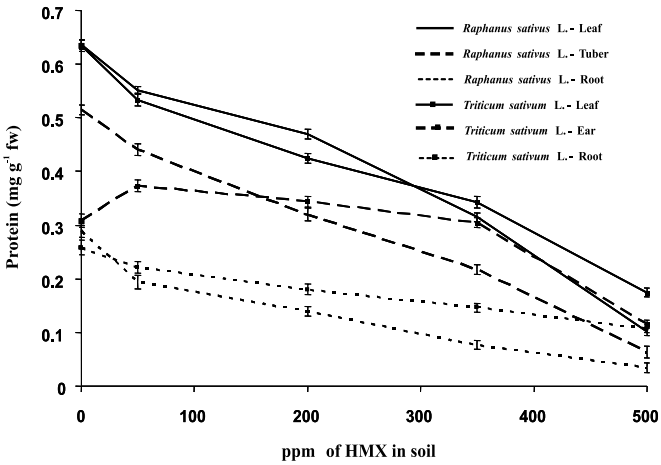

Fig. 5. Protein content in different plant parts of *Triticum sativum* L. (75 DAS) and *Raphanus sativus* L. (45 DAS) as affected by TNT concentrations

Under optimal conditions, the production and destruction in ROS is regulated well in the cell metabolism. Increases in ROS as a consequence of various environmental stresses result in oxidative stress. Environmental stresses, such as heavy metal toxicity, chilling, drought, high light intensity and earth pollutants are directly or indirectly associated with oxidative stress. Plants respond to a variety of stresses by accumulating certain specific metabolites, the most conspicuous being amino acids in general, and proline in particular. Increase in protein in ear and root of *Triticum sativum* L. with HMX treatment might be as a result of protective role of proline (Jimenz *et al*. 1997) and nutrient role of HMX. Proline metabolism was significantly altered and the extent of alteration varied between *Triticum sativum* L. and *Raphanus sativus* L. The maintenance of the turgor by accumulating higher levels of free proline in *Triticum sativum* L., supporting its HMX and TNT tolerance. Furthermore, the HMX and TNT tolerance of *Triticum sativum* L. was indicated by lower amounts of chlorophyll degradation than *Raphanus sativus* L. and higher amounts of protein in ear and root of *Triticum sativum* L. than control samples. In general, results indicate *Triticum sativum* L. is more tolerant of TNT and HMX than *Raphanus sativus* L.

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