Original Article

Genotype-Dependent Differential Response of Cereal Germinating Seeds Against Trinitrotoluene

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Abstract

Land mines are one of the tragic problems to human security in many countries. Among many techniques suggested detecting landmines, plant-based detectors can be used as a reliable tool for unexploded ordnance detection. This study was conducted to determine different concentrations effect of trinitrotoluene (0, 5, 10, 15, 20, 30, 45 and 60 ppm) on germination and early seedling growth of 6 species of cereals including Barley, Wheat, Durum wheat, Oat, Rye and Triticale. In contrast to oat and durum, barley and wheat genotypes had the best germination and germination index against different trinitrotoluene (TNT) concentrations. TNT showed no impact on the alpha amylase activity of the tolerant (*Barley var.* Bahman) and susceptible (Durum wheat) genotypes. Under higher TNT concentration, barley had the highest root length and root number and also was the best in dry matter allocation towards root tissues. We measured the different parameters for roots of 6 species of cereals, that oat was sensitive plant when exposed to TNT than others. With emphasis on the wide-range adaptation of cereals to climate and soil characteristics and their extensive and fibrous root systems, it is suggested that barley genotypes especially *Bahman* variety can be a confident plant to carry the essential genetic structure for explosive detection.

Keywords: Landmine Detectors, Trinitrotoluene , Cereals, Germination, Seedling Growth

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Introduction

Landmines are one of the tragic problems to human security in many countries throughout the world. Many techniques have been suggested to detect landmines [1]. Biological and bio-inspired detection methods introduced a promising technique which allows highly sensitive and efficient [2]. Approximately 80% of all kinds of landmines were charged with trinitrotoluene (TNT) [3]. The TNT is a recalcitrant to degradation and has high toxic effects on aquatic and terrestrial organisms [4].

Plant-based detectors which are genetically modified for sensing of explosives and its degradation products can be used as a reliable tool for landmine and unexploded ordnance detection [5]. A key factor of creation and development of the phytosensor is the tolerance of plant species to these compounds for explosive. Frequent studies have demonstrated the impact of TNT on plant cell culture and mature plant behaviors [6]. In the all plant species, phytotoxicity symptoms of TNT were the same and described by chlorosis and growth suppression [7]. TNT is a phytotoxicant because of its detrimental effects on plant growth and development [8]. This compound is metabolized by plants to 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-4,6- dinitrotoluene (4-ADNT), 2,4-dinitrotoluene (2,4-DNT), and 2,6- dinitrotoluene (2,6-DNT) [9, 10]. It seems that the response of plants at the germination stage will play a crucial role for plant-based landmine detectors. Monocotyledonous plants (*Avena sativa* L., and *Triticum aestivum* L.) indicated a high efficiency of remediation than dicotyledons (*Lepidium sativum* L., and *Brassica rapa* Metzg.) on TNT contaminated soils [11]. Grasses with extensive and fibrous root systems and rapid establishment [12, 13], seem to be more sensitive to a component amount of explosive in the contaminated soils. Germination of two forage grasses, *Poa compressa* L. and *Pinus palustris* L. have assessed in TNT contaminated soil [14]. The results showed that TNT contamination strongly declines germination of the species. The highest amount of TNT concentration (60 ppm in nutrient-free agar) did not influence switchgrass germination while increasing TNT concentration from 15 to 60 ppm decreased smooth bromegrass germination capacity [15]. The grasses indicated a different response of tolerances to TNT in agar medium, so that shoot and root of switchgrass grew more against TNT than smooth bromegrass. Both species including switchgrass and smooth bromegrass could not be established on soil containing more than 50 mg/kg of extractable TNT. Based on Krishnan et al. (2000) [16] experiments tall fescue had a better performance in soils with concentrations less than 31 mg/L TNT in comparison with smooth bromegrass which was inhibited by 24 mg/L TNT. Oat plants were capable of tolerating 1600 mg/L TNT and might be useful in the bioremediation of TNT contaminated soils [11]. The studies might consider the plant behavior through edaphic factors in TNT contaminated soils. A wide-range adaptation to climate and soil characteristics is more important element for a candidate plant to be nominated for genetic engineering.

Iran is the second most landmine infested country with an estimated 16 million landmines [17] and generally has an arid and semi-arid climate with the most of the relatively scant annual precipitation falls from October through April. In the most of the country, yearly precipitation averages 250 mm or less. The grazing lands and the pasture areas of Iran are generally enriched with cereal landraces. Cereals may present the best candidates for creating plant-based landmine detectors which are originally evolved in the different region of climatic. With the basis, the main objective of the present work was screening of some compatible cereals against different TNT concentrations to find the best plant for the subsequent genetic engineering.

Materials and Methods

Plant materials and growing conditions

Seeds of barley (*Hordeum vulgare* L. vars. Bahman, Valfajr, Yousef and Kavir), wheat (*Triticum aestivum* L. vars. Azar, Shahpasand, Omid and Sardari), Durum wheat (*Triticum durum* L. var. Yavaros), Oat (*Avena sativa* L. var. Random), Rye (*Secale cereal* L. var. Danko) and Triticale (*Triticosecale* Witt. var. Jovanilo) were obtained from Cereal Research Department of Seed and Plant Improvement Institute, Karaj, Iran. Seeds were soaked in 70% ethanol solution for 30 seconds and surface-sterilized by commercial bleach supplemented with few drops of Tween 20 for 15 min with continuous shaking. For each replication 30 seeds were sown on half strength MS medium [18] containing 0.5% agar and different concentrations of TNT including 0, 5, 10, 15, 20, 30, 45 and 60 ppm under sterile conditions. Petri dishes were incubated at 25˚C for 10 days under dark condition to prevent photodegradation of TNT [19]. The experiment was performed in completely randomized design with 5 replications.

Germination parameters

Seed germination was recorded daily for 7 days and germination was considered to have occurred when the radicle was approximately half of the seed length. Percentage of germination was calculated as follow: Gp= (Ng/Nt)*100 where Ng is the final number of germinated seeds and Nt is the total number of planted seeds. Germination index was calculated using the following formula: GI= ∑(Gt/Tt) where Gt is the number of seeds germinated on t^{th} day and Tt is the number of days up to t^{th} day [20].

Growth parameters

Plants were harvested in the 10^{th} day and growth parameters include shoot and root lengths, shoot and root fresh weight and root numbers were recorded immediately. Dry weight measured after the root and shoot were dried at 70˚C for 3 days. Ratio of root to shoot was calculated on the basis of dry matter. Absolute water content was determined by subtracting dry weight from fresh weight of samples and expressed as percent of fresh weight.

Protein extraction and alpha amylase activity

Enzyme assay was carried out just for two genotypes with the highest tolerance (barley var. Bahman) and susceptibility (durum var. Yavaros) to TNT treatments. Alpha amylase was extracted and assayed as described by Warner et al. (1991) [21] and Bernfeld (1955) [22] with some modifications. In brief, seed samples were ground using a mortar and pestle in 2 ml of 20 mM sodium acetate buffer pH 4.5, containing 1 mM $CaCl_2$. After centrifugation at 20,000 × g for 30 min, the pellet was washed with 2 ml of the extraction buffer and re-centrifuged. The combined supernatants were treated with two volumes of cold acetone on ice to precipitate proteins and centrifuged at 11,000 × g for 10 min. The pellet was re-dissolved in 2 ml of acetate buffer and centrifuged at 11,000 × g for 10 min to sediment insoluble material. The protein amount was determined by Bradford assay kit with BSA as a standard. The enzymatic reaction was prepared by adding 20 µg enzyme solution to 100 µl of starch solution equilibrated at 20˚C. The mixture was incubated for 5 minutes at 20˚C and then was added to 100 µl color reagent solution containing 1.06 M sodium potassium tartrate, 0.4 M NaOH, 48 mM 3,5-dinitrosalicylic acid and immediately placed in a boiling water for 15 min. After cooling on ice to room temperature, 900 µl deionized water was added to the reaction. Absorbance of the final reaction mixture was recorded at the 540 nm. A concentration series of maltose was used to plot a standard curve.

Statistical analysis

The variables were analyzed in completely randomized design ANOVAs with five replications in SPSS software. Multiple comparison tests were used to identify differences among treatment means when the ANOVAs indicated significant differences ($p<0.05$).

Results

Plant response analysis

Among cereals, barley had the best performance to germinate in all TNT concentrations. Germination percentage of wheat varieties (*Triticum aestivum* L. vars. Azar, Shahpasand, Omid and Sardari) had a big variation in comparison to barley genotypes (*Hordeum vulgare* L. vars. Bahman, Valfajr, Yousef and Kavir). Sardari genotype among wheat varieties showed the highest sensitivity to TNT exposure, while Omid and Shahpasand were influenced by no TNT levels. In contrast, other cereals especially at the highest TNT content demonstrated a significant weak germination. As shown in Figure 1, the rate of germination strongly decreased in TNT-containing media for oat and durum wheat. The 5 ppm concentration of TNT had the highest effect on oat germination while rye and triticale were suffered from the highest TNT level. On the basis of germination index, barley and wheat genotypes gained the highest amount of germination rate under different concentrations of TNT exposure, while oat and durum wheat presented the lowest germination rate at the same conditions (Fig. 1). Assimilate allocation in different plant organs (shoot and root) suggested that root tissues in all genotypes had similar response to TNT such as control condition (Fig. 2). It suggests that oat plants have translocated no materials to the root tissues in exposure to the 5 ppm level of TNT treatments. On the other hand, TNT treatments generally decreased dry matter accumulation in shoot tissues. Bahman variety hit the highest point of dry matter allocation in the shoot among Barley genotypes. Wheat genotypes followed the same dry matter allocation in shoot and root in different TNT concentrations, that it may not use all of the seed storage materials for seedling growth. Based on the presented data, Bahman had the highest growth rate in comparison with others.

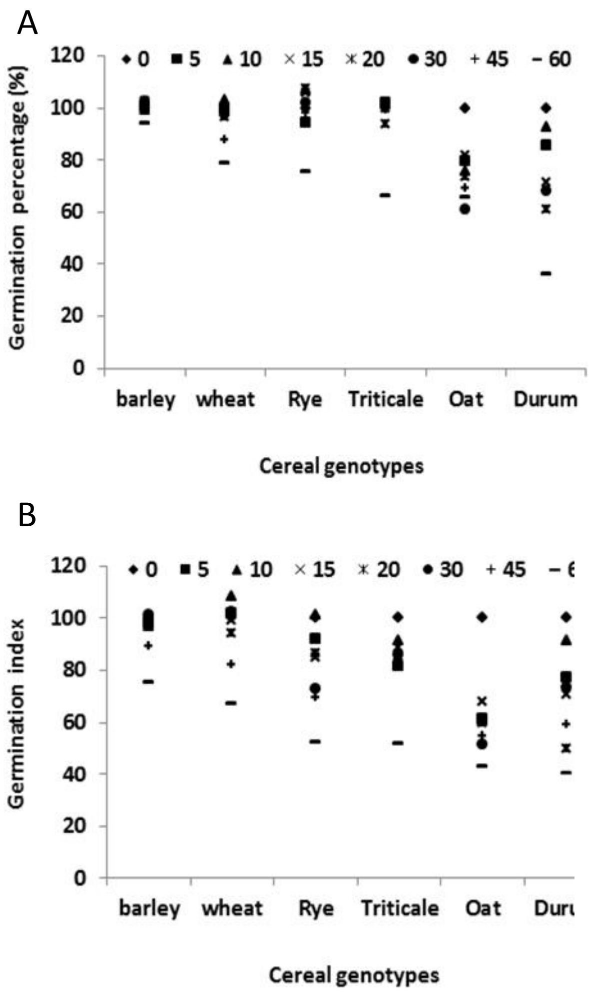

Figure 1. Germination percentage (A) and germination index (B) of the different genotypes affected by different concentrations of TNT contaminations.

At the higher levels of TNT, barley genotypes had the highest root length in contrast to rye and durum which had the highest root length in the lower TNT concentrations. Almost at the all conditions, barley in comparison with wheat genotypes had the best root length. Number of root emergence was different among genotypes. In addition, barely indicated the best efficiency for root generation than wheat with increasing the concentration of TNT.

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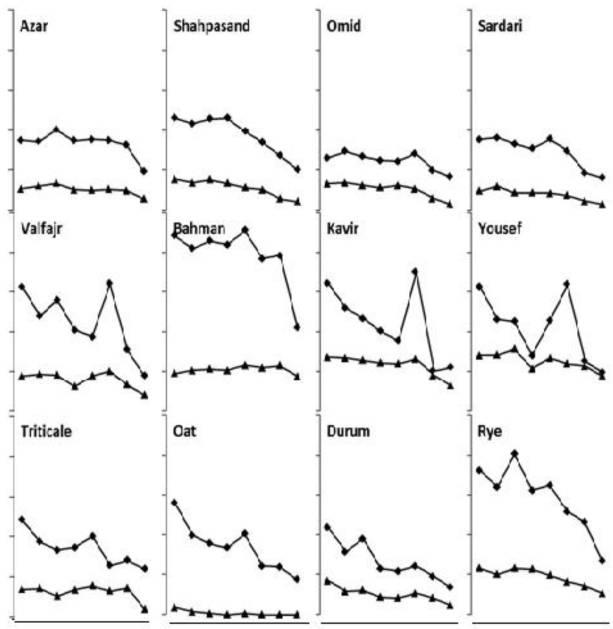

Figure 2. Dry weight allocation among genotypes of cereals. Horizontal axis presents different TNT treatments (0, 5, 10, 15, 20, 30, 45 and 60 ppm, respectively) and vertical axis is percent of dry matter allocation. Square (■) and triangle (▲) are representative of shoot and root, respectively.

Triticale and rye, whereas, rye seedlings generate the most root number at the lower TNT contaminations. For all of the root parameters oat was the weak plant when expose to TNT. As seen, root dry weight was more tolerant character than root length. Root length and root number may be important because of their involvement in TNT uptake under natural soil condition. It seems that plants with a high root:shoot ratio can establish a proper root system to detect a component amount of TNT in soil condition. Barely increased root:shoot ratio along with increase in TNT concentration. Genotypes of Kavir and Yousef experienced up to two folds increase in root:shoot ratio. Durum followed nearly stable ratio in response to TNT. But, all wheat genotypes indicated a decrease in root: shoot ratio in different concentrations of TNT.

Measurement of water content was a weak index for evaluation of growth responses of genotypes against TNT treatments. In addition, water content of all plant organs did not show any understandable response of growth among the genotypes. Plant dry weight seems to be one of the most important factors which can be responsible for packaging TNT or its toxic derivatives. Barley gained the highest amount of whole dry weight equal to 109.8 mg per plant as a mean value of all growth media. After barely, plants of rye, triticale, wheat, durum, and oat produced higher whole plant dry weight, respectively. Barely also was the best in the dry matter allocated towards root tissues.

Alpha-amylase activity

One of the key factors in seed germination process is alpha amylase activity which assimilates translocation to embryo axis. Kinetic activity of alpha amylase enzyme extracted from dry seed of barley (Bahman) as tolerant plant and durum as a susceptible one showed that the enzyme catalyzed starch through the same pattern in both genotypes. Direct effect of TNT contamination on the alpha amylase activity was studied using direct addition of 60 mg/L TNT to enzyme reactions. As shown in figure 3, Durum wheat showed the higher amount in alpha amylase activity in the samples taken after germination while barley of Bahman made the highest activity in enzymes prepared from dry seeds. Enzyme activity in geminating seeds showed the highest activity in comparison with dry seeds. TNT did not make any impact on the enzyme activity even after 48 hours.

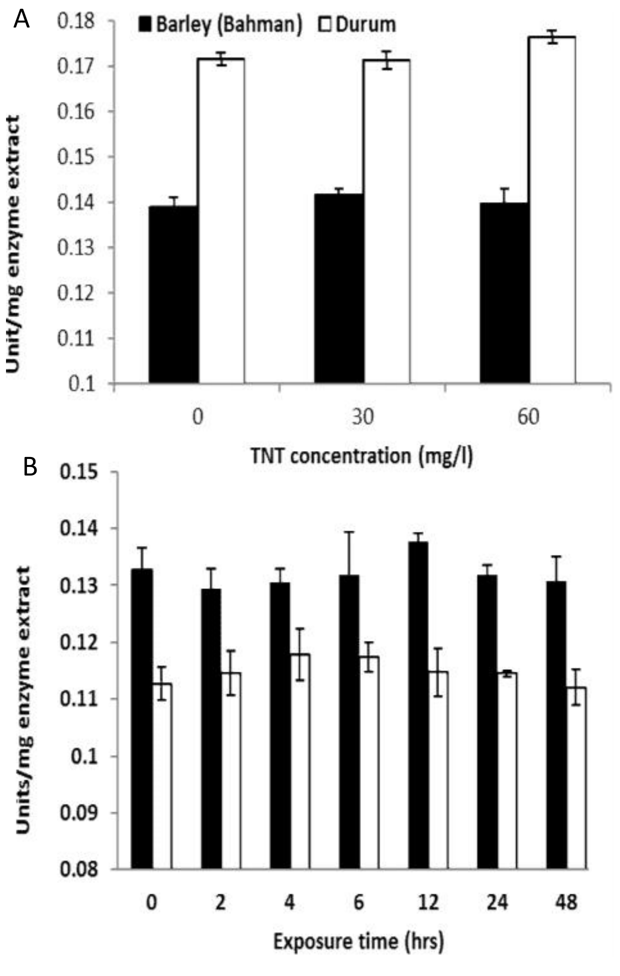

Figure 3. A: Alpha amylase activity in different concentrations of TNT including 0, 30, and 60 mg/L for germinating seeds. B: Alpha amylase activity at times 0, 2, 4, 6, 12, 24, and 48 hours for dry seeds treated with 60 mg/L TNT.

The enzyme prepared from barely in all exposure time had the higher activity in comparison with durum wheat. To assess the results from the direct effect of TNT on the alpha amylase activity, barley and durum seeds were germinated on the MS media containing 30 and 60 mg/L TNT concentrations. The results revealed that TNT did not have a significant effect on the enzyme activity (Fig. 3).

Discussion

Morphological and physiological characteristics of monocot plants in the context of the ecological adaptabilities make them to be the best candidate for TNT survey in the field condition. The polluted soils may exert the highest xenobiotic detrimental effects on the first stages of seedling establishment. Older monocot plants are capable of tolerating higher TNT concentration in the soil condition [16]. In spite of the results, Gong et al. (1999) [11] suggested that energy reserves in seed tissues make emergence less sensitive to environmental pollutants. Our data showed that the plant response to explosive contaminations may depend on genetic potentials. Barley germination was not affected by TNT while germination of oat and durum was severely reduced with increasing in TNT concentration.

In general, performance of plant genotypes at the germination stage was better than seedling growth especially at the higher level of TNT concentrations. Plant organs were followed a differential response to TNT treatments so that root tissues showed the most tolerance than shoot parts. Peterson et al. (1998) [15] reported the same experience on switchgrass and smooth bromegrass in response to TNT. Under TNT contaminations, it appears that assimilate allocation to root parts strikingly conserved in comparison to shoot (Fig. 2). Root tissues are the first to sense and to uptake TNT but the final destination of TNT or its derivatives may be related to the plant ability in transformation and translocation of TNT. With emphasis to unknown mechanism of TNT uptake and its intracellular accumulation site [23], up to 78% of radioactivity of C_{14}-TNT taken up by hybrid poplars was found in the roots after 2 days [24]. But, soybean and maize localized the higher part of radioactivity in plant shoots [25]. Differential genotypic responses at the germination and seedling establishment stages may be addressed using plant ability in TNT management at the biochemical and physiological levels.

Totally, water imbibition causes the activation of catalyzing enzymes and subsequently increases in seed respiration and ATP synthesis. It seems that the translocation of assimilates to the embryonic axis and finally radical protrusion can be first step in these biological processes. TNT concentration and exposure time had no significant effect on alpha amylase activity. It means that the alpha amylase activity as a candidate involved in germination was not arrested in the different treatments of TNT, whereas germination index data revealed that germination rate was affected by TNT levels. In the experiment, shoot dry weight in all genotypes showed the most susceptibility to TNT treatments where a major part of seed assimilates were regularly translocated to the shoot organs. Therefore, it appears that TNT pollution inhibited translocation of seed reserves to the shoot in comparison with enzymatic reactions involved in solubilizing seed reserves. On the other word, TNT tolerant plants have been probably have solved the negative impact of the pollution on the assimilate translocation. As suggested by Adamia et al. (2006) [25], the main pathway of TNT transformation in plant cells is nitro group reduction. The higher total radioactivity in plant shoots and much higher content of low-molecular-weight derivatives in plant roots demonstrated that root organs could effectively metabolize and translocate TNT [26]. It consequently seems that in concert with detoxification mechanisms, extensive biotransformation is part of a strategy for coping with the potentially negative impacts of TNT on plant growth and development [26], so that enhancing TNT transformation directly increased TNT tolerance. Based on our data, increasing of root:shoot ratio and stable root numbers under TNT exposure in tolerant genotypes may highlight critical role of root in TNT transformation and/or translocation.

Conclusion

In conclusion, our data introduced barley and wheat genotypes as TNT tolerance plants that can germinate in contaminated soils. Activity of alpha amylase enzyme as one of the most important factors involved in germination did not changed in genotypes of the tolerant (barley var. Bahman) and susceptible (durum wheat). Barley had the highest root length and root number and also was the best in dry matter allocation towards root tissues. With emphasis on the wide-range adaptation of cereals to climate and soil characteristics and their extensive and fibrous root systems, it is suggested that barley genotypes especially Bahman variety can be a confident plant to carry the essential genetic structure for explosive detection.

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