Bulgarian Journal of Agricultural Science, 25 (No 1) 2019, 55–61

Lead toxicity affects growth and biochemical content in various genotypes of barley (*Hordeum vulgare* L.)

Abdul Latief A. Al-Ghzawi[1*], Wesam Al Khateeb[2], Abdoul Rjoub[3], Abdel Rahman M. Al-Tawaha[4], Iyad Musallam[5], Khaldoun O. Al Sane[5]

[1]*Department of Biology and Biotechnology, Faculty of Science, Hashemite University, P.O. Box 330127, Zarqa 13133, Jordan*
[2]*Department of Biological Sciences, Yarmouk University, P.O Box 566***,** *Irbid 21163, Jordan*
[3]*Department of Computer Engineering, Jordan University of Science and Technology, P.O. Box 3030, Irbid 22110, Jordan*
[4]*Department of Biological Sciences, Al Hussein Bin Talal University, P.O. Box 20, Ma'an, Jordan*
[5]*National Agricultural Research Center (NARC), P.O. Box 639, Amman 19381, Jordan*
[*]*Corresponding author:* ghzawi@hu.edu.jo

Abstract

Al-Ghzawi, A. L. A., Al Khateeb, W., Rjoub, A., Al-Tawaha, A. R. M., Musallam, I., & Al Sane, K. O. (2019). Lead toxicity affects growth and biochemical content in various genotypes of barley (*Hordeum vulgare* L.). *Bulgarian Journal of Agricultural Science, 25*(1), 55–61

Lead is a widely spread environmental pollutant and its concentration is dramatically increasing in our ecosystems. Jordan is facing this significant problem because of the dramatic increase in population size, especially due to the crisis of Syrian refugees. In this study, nine barleys (*Hordeum vulgare* L.) genotypes (two cultivars and seven landraces) were grown under controlled growth conditions, and checked for their tolerance responses against two levels of lead nitrate (0 and 7 mM) in terms of growth (germination, shoot and root length) and biochemical (proline content and lipid peroxidation) responses. Genetic variation between barley landraces was also assessed using ISSR-PCR and results indicated genetic variability among the studied barley landraces. A toxic effect of lead on seed germination, root and shoot systems for all tested genotypes coupled with alterations in biochemical content were observed. LR4 and LR5 showed high growth rates, high proline content and low MDA level compared to the rest of genotypes. Therefore, LR4 and LR5 might be the most appropriate genotypes to be used in future breeding programs to produce lead-tolerant barley cultivars.

Keywords: barley; *Hordeum vulgare* L.; lead toxicity; proline; lipid peroxidation

Introduction

Heavy metals have been recognized as major environmental pollutants that adversely affect biological, evolutionary, nutritional and environmental aspects (Gill, 2014). Those adverse effects were thoroughly investigated on plants in terms of seed germination (Nagati et al., 2015), chlorophyll content (Arshad et al., 2017), plant growth and yield (Ashraf et al., 2017) and metal accumulation in several plant species (Hussain et al., 2013). Lead is one of the most abundant and widely spread heavy metals, and is environmental pollutant and highly phytotoxic (Yadav, 2010; Pourrut et al., 2011). Actually, lead deserves the increasing attention and current public concern all over the world (Sengar et al., 2008). It has been confirmed that lead can

cause morphological, physiological, and biochemical disorders in plants (Hussain et al., 2013; Shahid et al., 2014). However, plant roots are able to absorb some lead from soil, which may then accumulate in plant tissues (Taiz et al., 2015). Lead affect photosynthesis by inhibiting activity of carboxylation enzymes (Stiborova et al., 1987), induces oxidative stress by enhancing the production of reactive oxygen stress (ROS) in plants (Srivastava et al., 2015; Alves et al., 2016) and alter membrane permeability and disturb the mineral nutrition of the cells (Sharma and Dubey, 2005). Lead concentration is increasing rapidly in the environment due to the dramatic increase of world population, and human irresponsible ecological behavior (Singh et al., 1997; Hadi and Aziz, 2015; Kennedy et al., 2016). Jordan, in particular, is suffering from this problem. The inflow of the Syrian refugees to Jordan who are rapidly increasing and making an obvious demographic change and increased social and economic impacts increased the pressure on Jordan environment. Their toxic emissions increase environmental pollution which results in severe deterioration of natural resources, and disturbance of ecosystems.

Large quantities of lead have been discharged into the environment via solid and liquid (sludge) wastes, atmospheric emissions (e.g., car exhausts) and human activities such as mining, fossil fuel combustion, and industrial management practices (Naja and Volesky, 2009). This has resulted in urban pollution with no phase out activities followed (Hashisho and El-Fadel, 2004). Barley (*Hordeum vulgare* L.) was particularly selected for this study since it has been widely cultivated in the arid regions of Jordan. These regions were clean from pollution caused by heavy metals (and lead in particular) for several decades since they were far from urbanization. Nowadays, they became urban regions characterized by dense population which is dramatically increasing since Syrian refugees began to live in camps there. Consequently, lead pollution is increasing and the consumption of barley is increasing as well. In the present work, we are showing the physiological impacts and biochemical changes induced by lead toxicity in barley among two cultivars and seven selected landraces collected from different regions of Jordan. The objective of this study was to study the effects of lead nitrate ($Pb(NO_3)_2$) on seed germination, shoot and root growth and biochemical content in nine barley genotypes during early stage of ontogenesis looking for lead-tolerant genotypes that might be used in future barley breeding programs.

Materials and Methods

Plant material, growth conditions and lead nitrate treatments

Barley (*Hordeum vulgare* L.) landraces used in this study were provided by the gene bank at the National Agricultural Research Center (NARC) in Amman, Jordan, and have been collected from different regions in Jordan. Two cultivars: Rum and Mouta were used in this study. Rum is 6-row barley, originated from Harbinger-Arivat X Attiki in CIMMYT (Mexico) and certified in NARC. Mouta is 2-row barley, Roho/Arabi Abiad/6250 developed in ICARDA and certified in NARC. In addition, seeds of seven collected landraces (LR1-LR7) were also used. LR 1, LR 2 and LR 7 were 2-row barley and the other landraces were 6-row. Seeds of all genotypes were sterilized by immersing in sterilization solution (10% sodium hypochlorite containing 0.3% v/v Tween 20) for 10 minutes, and rinsed with sterile distilled water under a laminar air flow hood for three times (1 min each). Seeds were, then, allowed to germinate on filter paper in Petri dishes (9 cm diameter), moistened with distilled water (control) or 7 mM lead nitrate ($Pb(NO_3)_2$). Plates were incubated in the growth chamber at 23°C (±1) with a 16-h-light/8-h-dark photoperiod.

Petri dishes were arranged in a completely randomized design (CRD) with four replicates for each treatment. The relative germination percentage of barley seeds was recorded for each genotype on the 4th day of planting. Seed showing radical extrusion by ≥ 2 mm long were considered to be germinated seed as described by Montana et al. (2014). Relative germination percent was calculated according to the following equation: number of germinated seeds in the stress media/number of germinated seeds in the control media x 100 (Smith and Dobrenz, 1987). Growth parameters including relative root length, and relative shoot length were recorded on 7th day of planting using a ruler.

Proline content assay

Proline content was measured following the procedure of Bates et al. (1973) with some modifications described by Al Khateeb et al. (2017). Plant material (0.5 g) was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate was filtered. 2 ml of filtrate was transferred to a test tube containing acid ninhydrin (2 ml) and glacial acetic acid (2 ml). The mixture was then incubated in boiling water for 1 h. To stop the reaction, test tubes were transferred to an ice bath. In order to extract the chromophore, 4 ml of toluene was added and mixed vigorously for 15-20 s. The test tubes were, then, incubated at room temperature until the two phases separated. The chromophore, which contains toluene, was transferred to another test tube and measured at 520 nm. Proline concentration was determined by using a standard curve in the range of 20-100 µg/ml and it was calculated on a fresh weight basis of 7-day old seedlings according to the formula:

µmole proline (g^{-1} fresh weight) = [(µg proline ml^{-1} x ml toluene) /115:5 µg $µmole^{-1}$)]/g sample 5^{-1}.

Lipid peroxidation level assay

Lipid peroxidation (LPO) level was measured in terms of a product of lipid peroxidation, namely malondialdehyde (MDA) according to Carmak andHorst's (1991) method using 7-day old seedlings. A weight of 0.5 g of fresh tissue was homogenized in 10 ml of 0.1% trichloroacetic acid (TCA), and the resulting homogenate was centrifuged at 15 g for 10 min. Aliquot of the supernatant (2 ml) was mixed with 20% TCA (4 ml) containing 0.5% thiobarbituric acid (TBA). The mixture was then transferred to a 95°C water bath for 30 min and left to boil. The mixture was then cooled in an ice bath, and then centrifuged at 10 g for 10 min. Finally, the supernatant was measured at 532 nm wavelength. An extinction coefficient of 155/mM per cm was used to calculate MDA content.

Inter Simple Sequence Repeat - Polymerase Chain Reaction (ISSR-PCR) and gel electrophoresis

Genetic variation between barley landraces was assessed using 7 ISSR primers (Table 1). Genomic DNA was extracted from 7-day old seedlings using DNA extraction kit (Promega, Madison Wisconsin, USA) following the manufacturer's instructions. 5 µl of DNA were mixed with 2.5 µl of bromophenol blue and loaded on a 1% agarose gel to check the quality of isolated DNA. The gel was, then, run at 90 V for 60 min in 1x Tris Borate EDTA buffer (TBE). NanoDrop (NanoDrop 2000, Thermo Scientific) was used to measure DNA quantity and quality. DNA amplification was carried out in a thermal cycler in a total volume of 25 containing 12.5 µl of master mix (1x Amplicon), 1.5 µl primer, 2 µl DNA, and 9 µl DNase-free water. Then, PCR amplification according to the following cycling profile: an initial denaturation at 94°C for 5 min, followed by 40 cycles of 50 s at 94°C, 50 s at 49°C, and 1 min at 72°C, with a final extension for 5 min at 72°C. Amplified products were detected by mixing 7 µl of PCR product with 3 µl of loading dye, and then loaded on a 1% agarose gel in 1X TBE buffer at 90 V for 120 min. DNA fragments were visualized under ultraviolet light to detect presence or absence of bands.

Table 1. ISSR primers used to examine genetic variation in barley genotypes from Jordan

Primer name	Primer sequence (5′- 3′)	Annealing temperature, ℃
807	AGAGAGAGAGAGAGT	52
812	GAGAGAGAGAGAGAGAA	52
823	TCTCTCTCTCTCTCTCC	54
840	GAGAGAGAGAGAGAGAYT	58
879	CTTCACTTCACTTCA	40
886	VDVCTCTCTCTCTCTCT	41

Statistical analysis

In all experiments, a completely randomized design with four replications was used. Each experiment was repeated twice. Mean and standard error (SE) values were calculated for each treatment. Data for each experiment was subjected to analysis of variance (ANOVA) using the Statistical Product and Service Solution (SPSS Inc., version 19, 2010). Mean comparison was done using tukey's post hoc analysis. In all statistical analysis, a significance level of 5% was used.

Results

ISSR-PCR and gel electrophoresis

In this study, ISSR was used to assess the genetic variations among seven selected genotypes of Barley (*Hordeum vulgare* L.). To check for the presence of polymorphisms, the banding pattern resulting from DNA amplification was screened. All primers have shown the presence of genetic variation between the seven barley landraces selected (Fig. 1). The presence of genetic variation enabled us to perform our subsequent analyses.

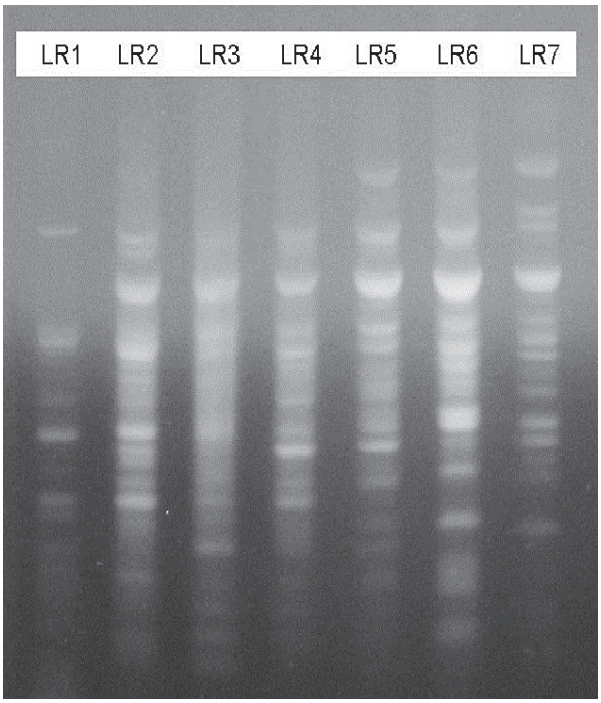

Fig. 1. ISSR analysis used to assess the genetic variations existing among the selected seven landraces (LR1-LR7) of barley (*Hordeum vulgare* L.). Amplified DNA fragments with different primers tested are shown. Different banding patterns resulted which are associated with polymorphisms between the landraces studied

Effect of lead toxicity on germination, root and shoot length

Lead had inhibitory effect on germination %. All seeds treated with lead nitrate had lower germination compared to the control treatment (Fig. 2a). The seven genotypes differ significantly in their germination percentages with LR5 and LR4 being the highest while LR6 and LR7 being the lowest (Fig. 2a). On the other hand, no significant variation was reported between LR1 and LR3, and the same observation was shown between LR2 and both of Rum and Mouta. The reduction of roots and shoot length was observed as the principal symptom of phytotoxicity. Current results showed that all seeds treated with lead nitrate had lower relative root and shoot length compared to the control treatment (Fig. 2a, 2b). Genotypic differences were also observed where; LR1, LR4 and LR5 had the highest relative root length while LR3, LR6 and LR7 were shown to have the lowest relative root length among all genotypes tested (Fig. 2b). Rum and Mouta have shown similar response to lead stress. LR4, LR5 have the highest relative shoot length followed by the two cultivars Rum and Mouta while LR3, LR6 and LR7 were the lowest (Fig. 2c).

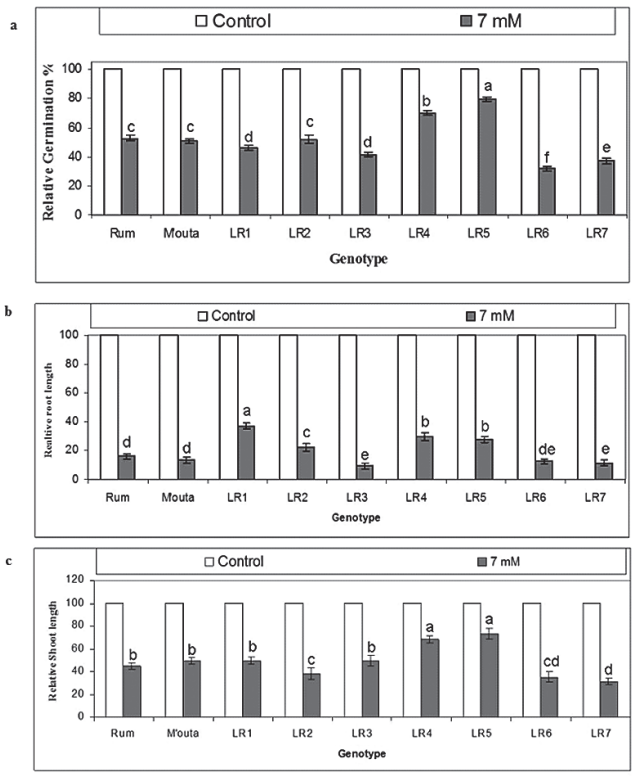

Fig. 2. Effect of lead toxicity on seed germination (a), root length (b), and shoot length (c) of barley (*Hordeum vulgare* L.). Data shown are means of four replicates ± SE. Values with different superscripts (a–e) are significantly different at P < 0.05.

Effect of lead toxicity on proline content and lipid peroxidation level

As expected, all lead-treated genotypes resulted in higher proline content compared to the control (Fig. 3a). It was shown that LR5, LR4, and LR3 have the highest proline content while LR6, LR7, Rum and Mouta produced the lowest values (Fig. 3a). All lead treated genotypes have shown higher LPO levels (MDA) compared to the control treatment (Fig. 3b). LR6 and LR7 have shown the highest LPO level compared to all other genotypes tested while LR3, LR4, LR5 and LR1 were the lowest. Rum and Mouta have shown a moderate response with regards to LPO level (c) (Fig. 3b).

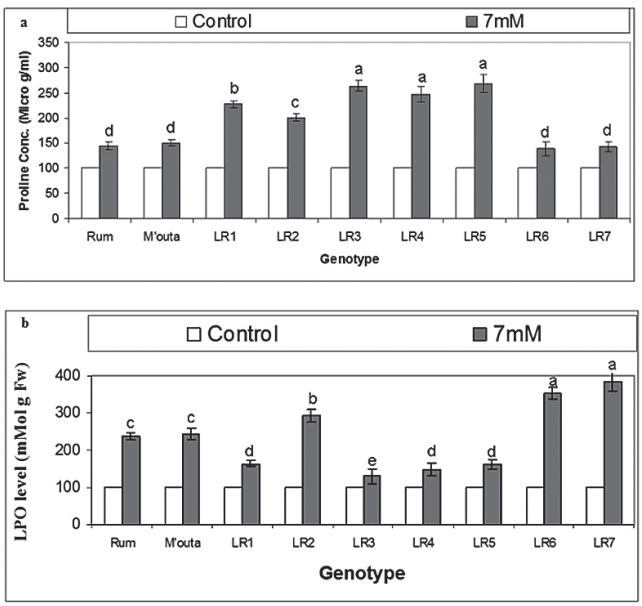

Fig. 3. Effect of lead toxicity on proline content (a) and lipid peroxidation (b) of barley (*Hordeum vulgare* L.). Data shown are means of four replicates ± SE. Values with different superscripts (a–d) are significantly different at P < 0.05.

Discussion

ISSR-PCR and gel electrophoresis

Our study has revealed the presence of genetic variations among the seven barley genotypes analyzed. In agreement with our results, Guasmi et al. (2012) and Mahfouz and Ray-

an (2016) showed that ISSR primers resulted in variation in the percentage of polymorphism among barley genotypes. In spite of being not as high in yield, the stability of genotypes in adverse environmental conditions is typically high. Even though, genetic diversity is expected to decrease over time as a consequence of genetic drift.

Seed germination, root and shoot length

The present study showed that lead was negatively affected germination percentage, root and shoot length. The inhibitory effect of lead on barley germination was also reported by Dal Corso et al. (2010). The decrease in seed germination might be due to the interference of lead with metabolic processes, which cause a decrease of energy generation for the seed embryo which might happen due to the inhibiting effect of lead on ATP synthetase/ATPase enzymes (Tu Shu and Brouillette, 1986; Gill, 2014; Sedzik et al., 2015) and result in inhibition effect on germination of seeds and hence retards growth of seedlings.

The application of lead had a toxic effect on root and shoots elongation and overall development of root and shoots system (Fig. 2). High sensitivity to tested doses of lead was predominantly observed on LR3 and LR7 while LR1, LR4 and LR5 were the most tolerant genotypes. The measured lower relative root length in this study was consistent with the findings of Juknys et al. (2009) and Sedzik et al. (2015), who have studied multiple plant species and revealed that exogenous application of lead nitrate had a significant reduction on root length. In addition, a greater decrease in dry matter accumulation had occurred in the roots of barley compared to the shoots (data not shown); this might be happened because roots of barley accumulated more lead ions than did the leaves (Singh et al., 2003). It also seems that the transport of lead from root to shoot systems is limited as was shown in the current work and confirmed by Sedzik et al. (2015).

As mentioned earlier, all seeds treated with lead nitrate have shown lower relative shoot length in each cultivar and landrace examined compared to the control. Rum, Mouta and LR1 have shown the same response to lead stress which was significantly lower than LR4 and LR5, but higher than all other genotypes tested. Early seedling growth was also inhibited by lead in several plant species including barley (Sedzik et al., 2015). Lead reduced the contents of chlorophyll, Ca and Mg in barley (Wozny et al., 1995; Pourrut et al., 2011). These minerals have a structural role in the biosynthesis of chlorophyll molecule, and triggering several enzymes involved in energy metabolism (Taiz et al., 2015). Hence, reductions in these contents (due to lead effect) result in a severe inhibition of photosynthesis, and consequently negatively affect the plant shoot and root growth (Cenkci et al., 2010). This could be justified from diverse points of view; the stressed plants might have to expend more energy for their survival, growth and development in the undesirable environmental conditions which otherwise would be available for their other growth processes (Gill, 2014). In general, a larger decline in growth of root and shoot was observed when higher amount of lead was applied. It is in agreement with findings of other authors (Moosavi et al., 2012; Piršelová et al., 2015). It is crucial to declare that concentrations of tested lead are relatively high compared to the mean values found in soils. Applying higher concentrations to plant roots in laboratory conditions allows researchers to quickly identify the variations in plant susceptibility to certain contaminant. Thus, this is an extremely significant factor in research of plant tolerance system.

Proline content and lipid peroxidation level

Generation of free radical is one of the preliminary reactions of plants to stress. Free radical's generation and ROS were stimulated in the incidence of heavy metals (Halliwell and Gutteridge, 1993), and this can critically disturb typical metabolism via oxidative damage to cellular components. To diminish and restore the harmful effect caused by active oxygen, plants have built up a multifaceted antioxidant mechanism. These antioxidants had a significant job in the cellular protection strategy against oxidative stress, inducing resistance to heavy metals through protecting labile macromolecules (Galli et al., 1996). It is confirmed that detoxification of metal ions inside plant tissues typically depends on chelation by suitable ligands. Antioxidants such as proline occupy an essential role in detoxification of toxic metal ions (Singh and Sinha, 2005).

All lead-treated genotypes in the current work resulted in higher proline content compared to the control. In agreement with our findings, proline content and accumulation has been reported as a common and well known metabolic response of higher plants to various stress conditions including drought, salinity and heavy metals (Signorelli, 2016). Proline is a part of non-specific defense systems that are activated when barley is subjected to lead stress (Sharma and Dubey, 2005). In this study, accumulation of free proline in response to lead was determined in nine barley genotypes to detect non-tolerant and Pb-tolerant genotypes. Results showed that proline concentration was significantly higher in the Pb-tolerant genotypes (LR5, LR4, LR3 and LR1) than in the non-tolerant genotypes (LR6, LR7, Rum and Mouta). Present results regarding the accumulation of free proline in response to heavy metal exposure were consistent with other findings among different plant species (e.g. Piršelová et al., 2015). Exposure to lead is known to disturb the plant water

balance (Saradhi, 1991; Signorelli, 2016). Proline build up in plants under lead stress is stimulated by a Pb-enforced decline of the plant water potential, and the useful importance of this buildup would lie in its participation to water balance maintenance (osmoregulation) which contributes to lead tolerance (Saradhi, 1991; Piršelová et al., 2015; Signorelli, 2016). Proline can also raise the stress tolerance of plants via preventing enzymes denaturation, and stabilization of protein production (Kuznetsov and Shevyakova, 1997).

Measurements the level of MDA is regularly employed as an index of lipid peroxidation (LPO) under stress situations. In the current study, the content of MDA was significantly increased in all lead treated genotypes, where LR6 and LR7 have shown the highest MDA level amongst genotypes while LR5, LR4 and LR3 were the lowest. This implies that LR5, LR4 and LR3 were better protected themselves from oxidative damage, and be able to rapidly up-regulate the antioxidative mechanism. We suppose that the decline of MDA concentration was attributed to increased antioxidative enzyme activities, which reduced the levels of hydrogen peroxide (H_2O_2) and membrane damage. Our results are in agreement with those of Zhang et al. (2007). On the other hand, it is hard to give explanation whether the synchronized boost in the activity of antioxidant enzymes in barley genotypes exposed to lead toxicity is because of improved expression of the genes controlling the biosynthesis of these enzymes and/or boost the activation of presented enzyme pools. For that reason, additional information is required at the sub-cellular and molecular levels so as to gain more detailed indications about the mechanisms of lead toxicity.

Conclusion

In this study, we have screened nine barley genotypes selected from different regions of Jordan, and tried to identify the most lead-tolerant landraces. Results showed that lead affected the growth of the nine genotypes and this was combined with biochemical changes. LR4 and LR5 were the best performing genotypes in terms of germination shoot and root growth. This was supported the higher level of proline accumulation in LR4 and LR5 compared to the rest of the genotypes. Again, LR4 and LR5 have the lowest MDA concentrations amongst the genotypes. Therefore, it can be concluded that LR4 and LR5 can be selected in future breeding programs to produce cultivars with higher resistance to lead toxicity.

Acknowledgments

Authors would like to thank the Hashemite University (Zarqa, Jordan) for supporting this research work. Thanks are also extended to NARC (Amman, Jordan) for providing the seeds.

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Received: 17.03.2018; ***Accepted:*** 21.05.2018