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EFFECTS OF OSMOTIC STRESS INDUCED BY POLYETHYLENE GLYCOL (PEG) 6000 AND MANNITOL ON SEED GERMINATION AND SEEDLING GROWTH OF *DURUM WHEAT*

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ABSTRACT

Seed germination is generally the critical step in seed establishment and thus the determination of successful crop production. This study was focused at examination of the biochemical and germination parameters effected by low water potential which was generated by polyethylene glycol (PEG) 6000 and mannitol, related to drought stress and growth of Waha durum wheat genotype. Two tests were carried out in a growth chamber; the first comprises seed germination into Petri dishes in the presence of different concentrations of the two osmoticums (0, 5, 10, 15 and 20 % of PEG6000 and mannitol). The second test was carried out in nutrient solution BD medium. Our results shows that Both PEG -6000 and mannitol reduced germination. Therefore, a rapid increase was observed in the rate of germination both for the control plants and the plants subjected to a concentration of 5 g/L and 10 g/L and changes in proportion to the time. For the concentration of 15 g/L and 20 g/L, this phase is very short, which explains the reduced germination rate due to the inhibitory effect of the two osmoticums on germination. In this study, PEG-6000 treatments resulted in an increase of some proteins and a decrease of others. Waha displayed 12 bands for control plants, 40 bands for PEG-6000 stressed plants (all treatments) and 35 bands for mannitol treatments.

Keywords: Germination, mannitol, PEG6000, SDS PAGE, wheat.

INTRODUCTION

Wheat yield is significantly subjected to global climate changes in the world and lack of water resources in the environment (Metwali et al., 2011; Mwadzingeni et al., 2016). Drought is one of the most stress able environmental factor which greatly limit the crop production in the most parts of agricultural fields of the world (Nezhadahmadi, 2013) this situation has been worse in the recent global climatic changes (Cheeseman, 2013).

Drought affects morphological, physiological, molecular, and biochemical processes to inhibit its growth (Marcin´ska Izabela et al., 2013). The severity and time period of environmental stresses effects the extent of these changes (Sallam et al., 2019). Researchers suggest that different stages of growth, such as emergence, germination, growth and yield should be considered separately when evaluating genetic material for tolerance against salt. Such evaluations can facilitate the development of cultivars with characteristics such as tolerance against salt throughout the plant ontogeny (Robin et

al., 2014). Murillo et al., (2001) report that selection for all traits is desirable during the germination and early rooting phase.

Germination is considered as one of the most critical stage in the plant life cycle. In addition, under stress conditions, seed germination and the first phase of seedling growth are critical stages for plant establishment (Alaoui et al., 2013). Passing the germination stage is decisive and crucial in all seedling development and growth. During germination, the seed rehydrates as soon as it is placed in the soil, provided that the water content of its environment is sufficient. The imbibition of the seed then generates hormonal changes, which will lead to enzymatic reactivation, allowing the start of reserves mobilization. These processes lead to the radical's breakthrough out of the seed coat and the seed is germinated. However, a good development of the processes leading to germination depends on the environment close to the seed, which is strongly influenced by temperature, water, oxygen content and soil structure.

Germination of seed is generally considered as a critical stage in the establishment of seed and thus, help in the determination of a successful crop production. The late development under the saline stress, favors toxic ions accumulation which can lead towards the death of plants before the development cycle ends. Therefore, earliness in germination can help assess the tolerance against salt (Krichen et al., 2014).

The following variables: the species, its variety, osmotic concentration, its growing conditions and developmental stage of the plant all responsed differently to osmotic stress (Yadav et al., 2019; Lokhande et al., 2010).

Thus, several methods were developed and used in the laboratory to create water stress in plants to assess drought tolerance of wheat plants by the using different chemicals such as mannitol and polyethylene glycol (PEG) etc (Molnar et al., 2004). PEG 6000 was most frequently used chemical to induce water stress to the plant seedlings which were hydroponically grown (Li et al., 2013).

Hence, the present work focuses mainly on these aspects studied under laboratory conditions. The water potential was altered by using polyethylene glycol PEG "6000" and mannitol as external osmotic agents; and aims to assess the impact of osmotic stress on the germination and seedlings of durum wheat genotype by monitoring several parameters related to germination processes in durum wheat genotype.

MATERIAL AND METHODS

Plant Material

The present work focused on the durum wheat (*Triticum durum* Desf) WAHA genotype originating from ICARDA SYRIA. The characteristics of the WAHA variety are presented in Table 1.

Table 1: Characteristics of the studied variety.

Variety	Origin	Agronomic and cultural characteristics	Technological characteristics	Resistance to diseases and different climatic conditions
WAHA	ICARDA SYRIA	High efficiency	- TGW: high Quality - Semolina: very good - Mixing: sensitive - Protein content: 13.95%	- Oidium sheet: resistant - Oidium ear: resistant - Brown rust: very sensitive - *Septorio*sis: moderately sensitive - Tolerant to drought - Tolerant to cold

In this study, two experiments were carried out.

First Experiment

The experiment was carried out in a culture chamber. It consists in studying the effect of two osmoticums, polyethylene glycol (PEG 6000) and mannitol at different concentrations on the growth of durum wheat (*Triticum durum* Desf.) from germination. The chosen seeds were selected according to their size and shape.

For the variety studied, the 90 seeds are disinfected with 10% bleach for 20 min, and then rinsed several times with distilled water. They are then put to germinate in kneaded boxes, these are lined with three layers of filter paper, and each box contains 10 seeds. In one case, we soaked the boxes containing seeds with distilled water (Witness), in the other cases; we soaked the boxes with a solution containing 5%, 10%, 15% and 20% of PEG 6000 and the same concentrations for mannitol. The boxes were put in the dark in a culture chamber at a temperature of 25°C.

Germination Parameters

Final Germination Rate

This parameter constitutes the best means of identification of the saline concentration which presents the physiological limit of germination of the seeds.

It is expressed by the ratio of number of germinated seeds to total number of seeds (ISTA, 2003)

$$G\% = 100\ (XT/N)\ (ISTA, 2003)$$

XT- the total number of germinated seeds

N- the total number of seeds put to germinate - Kinetics of germination: to better understand the physiological significance of the germinative behavior of the studied variety, the numbers of germinated seeds were counted daily until the 7th day of the experiment. Average daily germination (MDG = Mean Daily Germination), MDG is the Percentage of final germination / number of days to final germination.

Reversibility of the Action of Salt

This parameter has the advantage of determining the origin of the depressive effect of salt, if it is of osmotic and / or toxic nature. Thus, the seeds are put to germinate in the presence of different concentrations of PEG 6000 and mannitol for 4 days. On the fourth day, the non-germinated seeds are rinsed three times to remove the unabsorbed salt and then transferred to other Petri dishes containing distilled water for an additional four days.

Coefficient of velocity of germination speed (CVG) according to Kotowski (1926):

$$CVG = 100.\ (N1 + N2 + \ldots + Nx) / (N1T1 + \ldots + NxTx)$$

N- Number of seeds germinated each day (the 1st, the 2nd day, and so on until the last day 'x'.

Germination Index

It allows expressing the germination energy responsible for the exhaustion of seed reserves. The germination index defined by (IG) (germinated seeds / day).

$$IG = (N1)\ x1 + 1/2\ x\ (N2 - N1) + 1/3\ x\ (N3 - N2) + \ldots\ldots + 1 / n\ x\ (Nn - Nn\text{-}1)$$

IG- Number of seeds germinated during the days of the test 1.2.3 …… n-1, n.

N- Number of seeds germinated ...N1, (N1- N2) .....Nn (Haddad and Husein, 2001).

Second Experiment Hydroponics Culture

The term hydroponics comes from the Latin "hydro" (water) and "ponos" (work) consists in emerging the root part of the plant in a nutritive medium, this mode of culture, was chosen because it allows: a better control and a better homogenization of the mineral intake, obtaining healthy roots, free from any disturbance that may interfere with the plants' own response (Dubos, 2001) and also makes it possible to avoid the constraints of the soil, which allows growth rapid and good plant development under fully controlled conditions. In hydroponics, you must first germinate the plants until you get the roots to be able to provide the mineral elements found in the nutrient solution. For all the advantages brought by this culture, we chose the BD medium (Broughton and Dillworth, 1971) which consists of a precise quantity of macroelements, microelements and iron dissolved in a volume of water determined to meet the needs of plants. For the preparation of the culture medium (Table 2), we used stock solutions previously prepared with known concentrations and a precise storage date.

Table 2: Medium composition BD medium (Broughton and Dillworth, 1971).

Stock Solution	For 100 ml	Volume to be with drawn
$CaCl_2\,2H_2O$	50g	5ml/10L
$KH_2\,PO_4$	20g	5ml/10L
$MgSO_4\,7H_2O$	15g	
$K_2\,SO_4$	10g	5ml/10L
$Mn\,SO_4$	0.05g	
Fe EDTA	1g	12.5ml/L
Trace element	For 500ml	
H_3BO_3	0.20g	
$MgSO_4\,7H_2O$	0.20g	5ml/10L
$Cu\,SO_4 5H_2O$	0.10g	
$Co\,SO_4 7H_2O$	0.50g	
$NaMoO_4 2H_2O$	0.50g	

Application of Abiotic Stress

The study of the effect of abiotic stress, was carried out by adding two osmoticums (polyethylene glycol: PEG 6000 and mannitol) to BD medium solution. The PEG 6000 used does not require purification before use, its molar mass is high enough to limit root absorption, while preserving the fluidity of the nutrient solution, effectively it is an osmoticum ideally usable in hydroponic environments. Germinated grains were placed in goblets then in five large plastic tubes with holes each tube contains ten goblets and each goblet contains three sprouted grains, and then emerged in a BD nutrient solution.

We carried out in these latter, the transplanting of our young seedlings in culture medium, at the rate of five seedlings per pot and 10 pots per tube, each tube represents a treatment (1 control and the stressed: 5%, 10%, 15% and 20% of PEG 6000 with the same treatments for Mannitol). The following measurements have been taken:

Total Protein Analysis by SDS-PAGE

Freshly dissected leaves were immediately grinded in pestle mortar with liquid nitrogen and stored at -80°C until extraction. Proteins were precipitated overnight using a solution containing [trichloroacetic acid (TCA) (10 %, v/v), β-mercaptoethanol (0.07%)] / acetone, followed by centrifugation at 10 000 rpm, 4°C for 20 min. Protein pellets were washed

two times with 0.07 % β-mercaptoethanol / acetone, dried and resuspended in the appropriate buffer for gel electrophoresis.

Characterization of protein profiles was carried out using Sodium Dodesyl Sulfate Poly Acryl amide Gel Electrophoresis (SDS-PAGE), according to the method described by Leammeli (1970) in a 12 % resolving gel and a 4% stacking gel. Equal amount of protein was loaded in each lane of the SDS-polyacrylamide gel. Electrophoresis was run at 100 V until the dye front reached the bottom of the gel. The gel was removed from the plates and shaken in staining solution (100 ml ethanol and 1g Coomassie Brilliant Blue R-250) for 2h and then transferred to a distaining solution (50 ml methanol, 70 ml acetic acid and 880 ml distilled water) until protein bands appeared. Electrophoregram for each variety was scored using ECapt, an image processing and analysis program.

Data Analysis

For all concentrations used, each result corresponds to the average of 10 repetitions. The analysis of variance is carried out by the Ficher test at α = 5 % using XLSTAT-Excel 2014.5.

With regard to the analysis of total proteins by SDS-PAGE, the gel obtained was treated by the software “E-Capt” which allows a good visualization of the bands as well as the calculation of their molecular weights as a function of the weight marker. The presence of the bands is coded by "1" and their absence by "0".

RESULTS AND DISCUSSION

Analysis of Germination Parameters

The germination rate is variable depending on the treatments PEG6000 and mannitol, and their different concentrations. The figure 1 shows that, the germinative capacity of the stressed seeds is the same compared to the control for the first and the second concentration of PEG which is the best value of TG (100 %). On the other hand, it is reduced comparatively to the witness and this for the last two concentrations which mark as a minimum value of (80 %). For mannitol, only the first concentration (5 %) which shows the same value as that of the control, then the TG begins to reduce at the level of the second and third concentration which marks a low value of the order of (30 %) and approach zero under severe stress (S4: 20 %) where there is a total absence of germination.

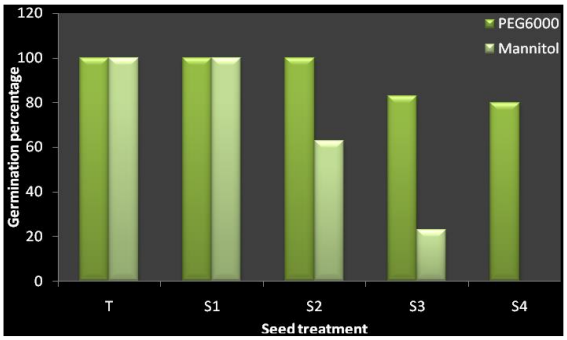

Figure1: Germination percentage.

The figure 2 presents evolution of germination of the studied variety for all the treatments as a function of time. The germination curves make it possible to distinguish 3 phases: a latency phase, necessary for the appearance of the first germinations, and the germination rate was indicated low. The concentration of osmoticums acted as a dependent variable during this phase. It is absent in control plants and those irrigated by a concentration of 5 g/ L and 10 g/L of PEG 6000 and mannitol. However, it becomes more or less long, especially in plants subjected to the treatment of 15 g/L and 20 g/L of the two osmoticums and phase extended to 2 days. A substantially linear phase, corresponding to a rapid increase in the rate of germination which changes in proportion to the time for the control plants and the plants subjected to a concentration of 5 g/L and 10 g/L. For the concentration of 15 g/L and 20 g/L, this phase is very short, explain the inhibition effect of the two osmoticums on germination which reduced germination rate. For the 20 g/L mannitol concentration this phase is absent therefore the germination rate is zero.

The final percentage of the germination was represented by the third phase. Germination capacity decreases with the concentrations applied of the two osmoticums.

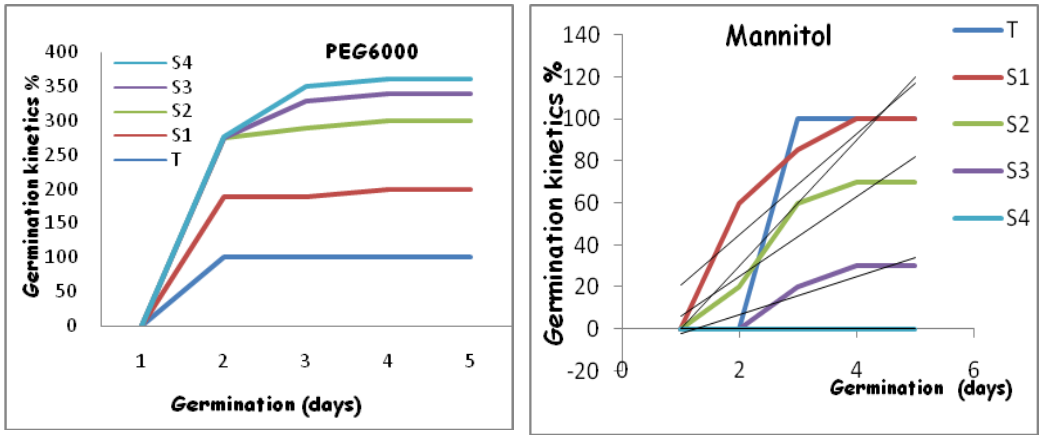

Figure 2: Germination kinetics under PEG6000 and Mannitol seed treatment.

The daily average germination (figure 2) is always higher for PEG6000 than mannitol except for the concentration 5 % which is the same average in the two osmoticums. Our results show a significant reduction of this parameter under stress caused by mannitol to 15 % of 6 germinated seeds/day with a total absence of germination under severe stress (20 %). The germination rate coefficient is affected by PEG6000 and mannitol and varies according to different concentrations (fig. 3). Thus, the CVG (Coefficient of germination velocity) under mannitol treatement decreased significantly compared to PEG6000, wheras no CVG at concentration of 20 % (S4), we marks completely inhibited germination.

The germination index is higher for PEG6000 than for Mannitol (fig. 4). In addition, for PEG6000, the germination index is not too affected at 5 % (S1) and 10 % (S2), unlike the concentrations 15 % (S3) and 20 % (S4) where the GI decreases by report to witness. For mannitol, the GI is reduced at all concentrations until absence to 20 % (S4).

The studied parameters previously have shown that mannitol exerts, in high doses, a depressive effect on the seeds germination of the studied wheat variety. This inhibition can be osmotic and / or toxic. In so far as it is of osmotic origin, we should expect a resumption of germination after lifting this constraint.

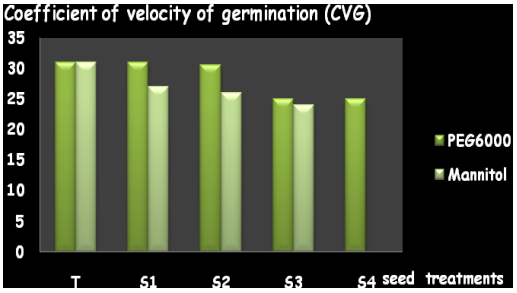

Figure 3: Coefficient of germination velocity under osmotic stress.

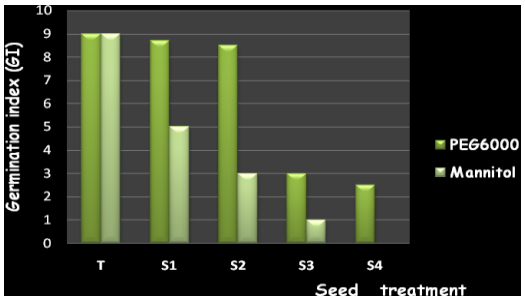

Figure 4: Germination index under different level of osmotic stress.

On the other hand, if ionic toxicity phenomena occur, we can predict the absence of this resumption of germination (Hajlaoui et al., 2007). The transfer of non-germinated seeds of the concentration 10 g /L, 15 g/L and 20 g/L to distilled water is followed by a resumption of germination.

However, the germination capacity remains lower than that obtained in seeds placed directly on the control medium (fig. 5). Regarding this parameter, we note a resumption of germination for seeds germinated and subjected to severe stress (20 %) from the first day until the third day, followed by germinated seeds subjected under stress S3 (15 %), unlike S2 treatment (10 %) there is a slight recovery from the second day. Our results support the work of Prado et al., (2000) in quinoa (*Chenopodium quinoa* Willd) , in which they revealed that, the reduction in the germination rate is due to an osmotic dormancy process developed under these stress conditions, thus representing a strategy of adaptation to environmental constraints.

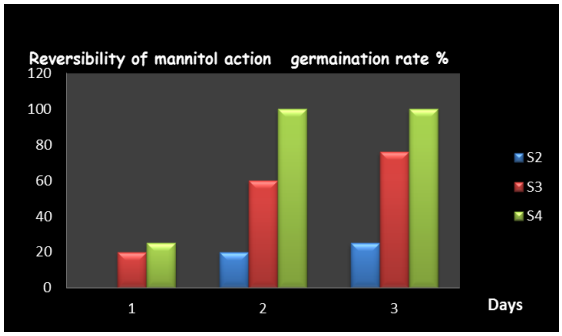

Figure 5: Reversibility of mannitol action.

PEG 6000 not only reduces the germination rate, but also delays germination by slowing down its speed. This delay may be the result of alteration of enzymes and hormones found in the seed. It could also be a problem with hydration of seeds following a high osmotic potential resulting in a certain inhibition of the mechanisms leading to the exit of the radicle out of the integuments and consequently a delay in seed germination (Daroui, 2012).

Effect of PEG-6000 and Mannitol on Protein Patterns using SDS-PAGE

Total leaf proteins of Waha cultivar were extracted from control and both PEG-6000 and mannitol treated plants (5, 10, 15 and 20 %). Protein bands detected ranged from 15 to 96 kDa (fig. 6). In general PEG-6000 treatments resulted in an increase of some proteins and a decrease of others. Waha displayed 12 bands for control plants, 40 bands for PEG-6000 stressed plants (all treatments) and 35 bands for mannitol treatments. The observed differences in bands number between control and treated plants may be attributed to alternation in DNA-nitrogenous bases, in the amino acid sequences or the protein sites or frame shift mutations (Eid, 2019). Therefore, the number of genes is smaller than the number of different polypeptide bands arising from protein synthesis in a genome, but they can still be considered as gene markers. Therefore, it is necessary to study the plant stress responses at the protein level Zhou et al., (2011). Waha displayed five bands with 17, 31, 47, 59 and 68 kD in control and in plants treated with 5 and 20 % PEG-6000. While the following five bands were only observed in plants treated with 10 and 15 %: 16, 19, 39, 45 and 56 kD. Overall, the studied genotype exhibited more new bands with higher intensity in the presence of 20 % of PEG-6000. Our results are similar to the results reported by Bayoumi et al., (2008) and by Hellal et al., (2018). For the osmotic stress experiment using mannitol Waha displayed four bands with 21, 29, 52 and 61kD in plants treated with 10 % and 20 % and tree bands with 23, 26 and 40kD in plants treated with 15 % and 20 %. In contrast to PEG-6000 the lowest concentration of mannitol led to higher intensity. According to Diana et al., (2002) the protein concentration is directly related to band intensity in the wheat seedlings. It is widely known that many genes are commonly expressed under several types of dehydration stresses. Higher plant exposed to such constrains exhibit a characteristic set of cellular and metabolic response, including a decrease or increase in the synthesis of protein. Some proteins were expressed at a lower level under stress. This could be due to the inhibitory effects of stress on transcriptional process. For the newly synthesized ones or for those with an enhanced expression under osmotic and drought stress, it could be because they are

encoded by stress responsive genes. Such proteins could be involved in the tolerance to a cellular dehydration, osmotic adaptation, numerous signaling pathways and the cellular protective enzymes, Phukan et al., (2017). The results also revealed that the expression of those proteins was genetically regulated, depending on the used osmoticum (PEG-6000 or mannitol) as well as the concentration. Therefore is necessary to further investigate the structural and functional roles of these stress responsive polypeptides to enhance our understanding of the osmotic responses in Algerian wheat cultivars.

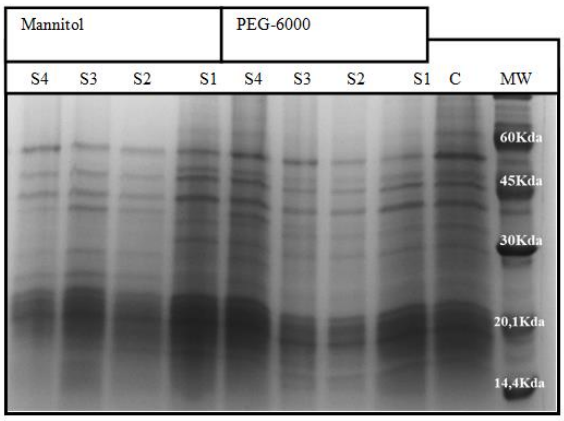

Figure 6: Protein Profile on SDS-PAGE of the genotype Waha under control (C), PEG-6000 stress and Mannitol stress.

CONCLUSION

In conclusion and across our results, we noticed that the diverse germination parameters were affected by the two applied osmoticums and the responses vary according to the, the germination capacity, stress levels, and the germination rate of the tested variety and they decrease with the increase of the concentration of PEG6000 and Mannitol added. Also, the obtained results, demonstrate that the adaptation of our variety is closely dependent and favored by the different physiological and biochemical responses under stress conditions, by comparative analysis of some physiological and biochemical parameters. The most relevant remark, taken from the present study, concerns the effect of stress on total proteins; our results show the appearance of new bands under severe stress S3 and S4 of PEG-6000 which marks the highest number of bands. This confirms that the studied variety controls its metabolic function according to the conditions of the culture medium whose aim is to tolerate the stress.

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