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Original Research Article

Effect of shock and elevated levels of mannitol on callus growth, regeneration and proline accumulation in *Ruta graveolens* cultures

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A B S T R A C T

The responses of *Ruta graveolens* callus to different concentrations of mannitol as an induced water stress agent on callus growth, regeneration and proline accumulation in regenerated plantlets were studied. To study callus growth in response to two different treatments of mannitol (direct and gradual), a quantity of 100 mg callus weighting was grown on Murashige and Skoog (MS) medium supplemented with 1 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l kinetin (Kin) then mannitol at the concentration actions 0, 60, 120, 180, 240 or 300 g/l was added. Shoot induction was achieved from callus cultures after transfer to MS medium supplemented with 1.5 mg/l benzyl adenine (BA), and 0.5 mg/l naphthalene acetic acid (NAA). Results revealed that increasing water stress generated by increasing mannitol concentration in direct method caused a progressive reduction in callus fresh weight with a significant increase in response to 240 g/l mannitol after twelve weeks. The mean % of shoot regeneration reached 79.93 and 85.03% at 240 and 300 g/l mannitol for direct and gradual exposure, respectively and a significant increase in proline accumulation in regenerated plantlets was recorded.

Keywords

Ruta graveolens, mannitol on callus growth, regeneration and proline accumulation; Murashige and Skoog

Introduction

Abiotic stress limits crop productivity and plays a major role in determining the distribution of plant species across different types of environments. Abiotic stress and its effects on plants in both natural and agricultural settings is a topic that is receiving increasing attention because of the potential impacts of climate change on rainfall patterns and temperature extremes (Abdel-Raheem *et al.*, 2007). Drought is one of the most common environmental stresses affecting plant growth and productivity particularly in arid and semi arid areas (Rai *et al.*, 2011). However, crop tolerance to drought has not been very well defined and still not clears what aspect of the plant morphology or physiology is the most important factor involved for drought

tolerance. Many efforts should be achieved and still needed in order to define clear target for improving drought tolerance (AL-Taha, 2013). Plants have evolved mechanisms that allow them to adapt and survive periods of water deficit, if not at the whole plant level, at some level or form of plant structure. The plant drought response depends on the species inherent "strategy" but also on the duration and severity of the drought period (Carvalho, 2008).

Plant cell and tissue culture are useful tools for studying stress tolerance mechanisms under *in vitro* conditions, and also used to obtain drought–tolerance plant assuming that there is a correlation between cellular and *in vivo* plant response (AL-Taha, 2013). The technique has also been effectively utilized to induce tolerance which includes the addition of some selective agents to the nutrient medium that permit the preferential survival and growth of desired phenotypes. The selecting agents usually employed for *in vitro* selection including PEG or mannitol for drought-tolerance (Rai *et al.*, 2011).

Ion and osmotic homeostasis are necessary for plant to be salt and drought tolerant. Osmotic homeostasis is accomplished by accumulation of compatible osmolytes in the cytosol for intracellular osmotic homeostasis. These include the accumulation of endogenous free proline which contributes in preventing dehydration and cellular damage via balancing the osmotic potential of the cytoplasm with surrounding environment (AL-Taha, 2013). *Ruta graveolens* (Rutaceae) commonly known as rue, is a strongly scented medicinal and aromatic plant mostly grown in the Mediterranean region as an ornamental because of its yellow-cupped beautiful flowers. It is also used in food stuffs, salads, sandwiches, fish, egg dishes, condiments and pickles (Ahmad *et al.*, 2010; Katzer, 2010).

The herb contains many secondary metabolites such as furocoumarins, furoquinolines and acridone alkaloids, mainly present in the leaves, especially before blooming. The volatile rue oil has a pungent smell and bitter taste, and possesses antibacterial activity against *Micrococcus pyogenes var aureus* and *Escherichia coli*, it is used as insect repellent and antidote for toxins such as snake-bite and scorpion venoms, (Ahmad *et al.*, 2010; Batanouny, 2012). *Ruta* also grown well in direct sun and consumes low water levels and has been reported as a drought tolerant. Thus, it has used been as a ground cover in lands exposed to drought and erosion and could be convenient for other environmental stresses (Hargitt, 2012).

The aim of this study is to examine the response of *Ruta* callus cultures to varying levels of mannitol induced water stress using two types of selection; (a) shock treatment, in which cultures are directly subjected to different concentrations for the stress agent, (b) step wise long term treatment, in which cultures are exposed to stress with gradual increase in the concentrations of the selecting agent. Callus fresh weight, shoot regeneration percentage and proline accumulation in both treatments were determined.

Materials and Methods

Ruta graveolens plants were collected from one of Baghdad nurseries, identified by the herbarium Prof. Ali AL-Musawi, stems of plant were first kept under running tap water for 30 min and then subjected to surface sterilization using 1.5% (v/v) NaOCl (Clorox) for 20 min with vigorous shaking. The stem segments (1.0 cm in length) were

excised aseptically and transferred to sterile agar- solidified MS medium into Petri dishes (20 ml/dish) using forceps. All steps of sterilization were carried out under aseptic conditions (Ahmad *et al.,* 2010). *R. graveolens* explants (Fig. 1A) were inoculated on MS medium (Murashige and Skoog, 1962) supplemented with 1.0 mg/l 2,4-D and 1.0 mg/l Kin. All cultures were incubated at 25 ± 2ºC, 16/8 hrs (light/dark) photoperiod under a light intensity of 1000 lux (Ahmad *et al.,* 2010). Small pieces of calli weighting 100 mg each were recultured onto the maintenance medium containing 1.0 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l Kin and supplemented with different concentrations (0.0, 6, 12, 18, 24 or 30%) of mannitol for direct method. Callus fresh weight/replicate was recorded for all treatments to determine the callus growth inhibition (mannitol vis. callus fresh weight). Callus cultures were re-cultured three times onto the same medium then callus fresh weight was measured after four weeks.

For gradual exposure to mannitol, callus pieces weighting 100 mg were subcultured onto the maintenance medium containing 1.0 mg/l 2,4-D; 0.5 mg/l Kin and subjected to gradual increase in mannitol concentrations (0.0, 6, 12, 18, 24 or 30%). Callus fresh weight/replicate was recorded after four weeks for each concentration before subculture. All stressed callus pieces (Fig. 1B) were transferred into regeneration medium supplemented with 1.5 mg/l BA and 0.5 mg/l NAA, the percentage of callus regeneration and proline content were recorded.

All experiments were designed using a completely Randomized Design (CRD) with 30 replicates except proline experiment was carried out with 3 replicates. Statistical analysis GenStat software was performed using two way analysis of variance (ANOVA), differences between means were determined and least significant differences were compared at $P \leq 0.05$ (Abdul-Jaleel *et al.,* 2008).

Results and Discussion

Results of direct exposure to mannitol revealed that increasing water stress caused a sharp reduction in callus fresh weight (Fig. 2). A significant decrease occurred in the mean of callus fresh weight at 60,120,180, 240 and 300 g/l mannitol (Fig. 3) reached 160, 119, 86.7, 161 and 91.7 mg respectively compared with 0.0 g/l mannitol (206 mg) for shock treatment. While gradual exposure Figure 4 showed no clear decline in callus growth, data shows no significant differences in callus fresh weight between untreated treatment and 60, 120 and 180 g/l mannitol however a significant decrease in callus fresh weight in a mannitol free medium and 240 g/l mannitol reached 415 and 277 mg respectively and in a medium lacking mannitol and 300 g/l mannitol reached 578 and 297 mg respectively for gradual treatment.

The reduction in water content caused a decrease in cell turgor pressure and consequently reduction in callus growth as expressed as callus fresh weight (AL-Taha, 2013). Mohamed and Ibrahim (2012) suggested that mannitol is taken up and metabolized by plants. The *in vitro* growth medium contains a source of carbon, but increasing the carbohydrate concentration in the growth medium up to a limit, increases the water potential, resulting in reduced water uptake and thus caused growth inhibition.

A significant increase in callus growth was recorded in response to 240 g/l mannitol after twelve weeks (Fig. 5) reached 290, 349

and 568 mg respectively after 4, 8 and 12 weeks for 0.0 g/l mannitol, while callus growth under water stress 240 g/l mannitol reached 112, 158 and 188 mg, respectively. It seems that callus growth increased slowly with increasing the time of exposure to mannitol.

Stressed culture medium induced osmotic stress due to reduced cell division, shrinking imbalance due to reduced loss of cell turgor, nutritional imbalance as a result of reduced water uptake, an increase in electrolyte leakage and a decrease in cell water contents with increasing stress (Wani *et al.*, 2010).

Water stress determines cell division and elongation causing growth reduction (Abdul–Qadir and Al-Ka'aby 2011) while Mahmood *et al.* (2012) reported that wheat callus growth and survival significantly decreased after inoculation on a medium supplemented with high concentrations of PEG inducing osmotic stress under belonged stress duration. Mitoi *et al.* (2009) revealed that long term exposure to mannitol at high concentrations, may activate the adaptive mechanisms by which the expression of antioxidant enzymes are correlated. Hadi *et al.* (2014) stated that plants respond to drought stress in very intricate and diverse way, and it is impractical to consider a single gene is responsible for drought tolerance. The gradual exposure of callus to mannitol induces high expression for a number of genes like osmo–proteins (shock proteins), but these proteins do not formed when calli expose to shock treatment with mannitol (Ziegler, 1990). The over expression of these genes has activated the expression of many downstream genes thus plants express improved stress tolerance (Peleg *et al.*, 2011). These results are in line with those reported by AL-Taha (2013) in sour orange who concluded that stepwise treatments are better than shock ones.

Callus cultures (Fig. 6) showed a significant decrease in mean % of shoot regeneration reached 79.93 and 85.03% at 240 and 300 g/l mannitol for direct and gradual exposure respectively compared with 98.33% at 0.0 g/l, after inoculating callus pieces onto a solid MS medium supplemented with 1.5 mg/l of BA and 0.5 mg/l of NAA for 3 months.

Similar results for direct treatment were reported by Wani *et al.* (2010) in rice, who indicated that increasing PEG in the culture medium caused a decrease in callus morphogenic capacity and with Mahmood *et al.* (2012) in wheat, while Matheka *et al.* (2008) reported that plant regeneration from callus under water stress is inversely proportional to PEG concentration in the selection medium. This decrease in the regeneration capacity, resulted under stress, may be explained by the slowdown in all cell physiological processes (Bouiamrine and Diouri, 2012).

Similar results for gradual exposure were reported by Mitoi *et al.* (2009) who indicated that cell cultures exposed to a mild osmotic stress in a medium supplemented with mannitol may not activate the adaptive mechanism. On the other side, raising the exposure time to mannitol allows tolerant cell lines to grow and suppresses the sensitive ones.

The data of % shoot regeneration from callus pieces after employment of high concentrations of mannitol (240 and 300 g/l) for shock and gradual exposure respectively are considered highly optimistic compared with the results of Sakthivelu *et al.* (2008) who reported that a decrease in the total number of viable soybean plantlets regenerated from cotyledonary nodes at 60 g/l PEG. Plants respond to the stresses at both molecular and cellular levels (Peleg *et*

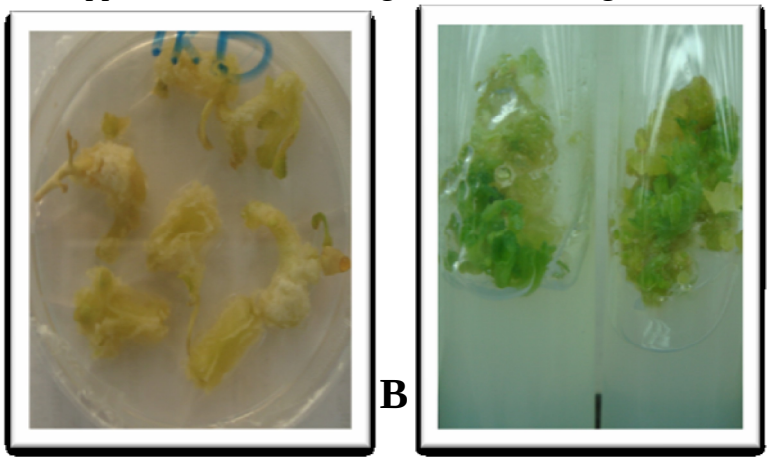
al., 2011). Expression of a variety of genes has been demonstrated to be induced by these stresses; the products of such genes are thought to function not only in stress tolerance but also in regulation of other gene expressions and signal transduction as a stress response. Genes being induced during drought stress conditions are thought to protect plants from water deficit by producing important metabolic proteins and the regulation of genes for signal transduction as stress response (Vazquez and Linacero, 2010).

The accumulation of free proline (Fig. 7) is greatly increased in stressed plantlets in both direct and gradual selection systems compared to control, the mean proline concentration increased at 240 and 300 g/l of mannitol reached 154 and 131 μM/g fwt for the two concentrations respectively compared with 0.0 g/l mannitol (128 μM/g fwt). These results are in line with those of AL-Taha (2013) who reported that proline accumulated in shock treatment is higher than that accumulation gradual one in sour orange callus cultures. Proline has long been known to accumulate in plants experiencing water limitation and both proline synthesis and catabolism are required for optimal growth at low water potential. High concentration of proline in shoots regenerated from callus was recorded. Proline, is a compatible solute playing a predominant role in protecting plants from osmotic stress thus high concentrations accumulate in the cell cytoplasm without interfering with cellular structure or metabolism (Peleg *et al.,* 2011; Rai *et al.,* 2011). Munns and Tester (2008) reported that the biosynthesis and accumulation of compatible solutes in plant tissues is an adaptive response of plants to drought stress and this explains the accumulation of high proline concentrations in shoots regenerated from callus cultures exposed to high concentration of mannitol. These results are in accordance with those of Peleg *et al.* (2011) who reported that increasing compatible solutes synthesis is a strategy to improve tolerance to abiotic stresses. Porline serves as an organic nitrogen reservoir ready to be used after stress relief in order to sustain both amino acid and protein synthesis. Besides, being an osmoprotectant, proline also has a role in, stabilization of proteins and protein complexes and as a signaling/regulatory molecule (Lokhande and Suprasanna, 2012).

Munns and Tester (2008) reported that in many halophytes, proline accumulates at sufficiently high concentrations in leaves at levels above 40 μM. The current work revealed very high levels of proline in regenerated *Ruta* plantlets reached 154, 131 μM/g for direct and gradual treatments respectively. Our results has concluded that *R. graveolens* is a drought tolerant species due to the high accumulation of proline as a compatible solute. Hadi *et al.* (2014) reported that high water content was noted in progressive mild stress than severe stress indicating that plants have the ability to sustain their water content under mild stress, whereas this ability is lost under severe stress, while *Ruta* sustained water content under severe stress and even plantlets were regenerated from stressed cultures.

Figure.1 (A) Callus induction of *Ruta graveolens* on MS medium supplemented with 1.0 mg/l 2,4-D and 0.5 mg/l Kin. **(B)** Regeneration of *Ruta graveolens* on MS medium supplemented with 1.5 mg/l BA and 0.5 mg/l NAA

A



B

Figure.2 Effect of mannitol concentration on mean callus fresh weight after inoculation of callus pieces were inoculated onto a solid MS medium supplemented with 1mg/l of 2,4-D and 0.5 mg/l of Kin after four weeks by using direct method of screening for drought tolerance. Initial weight is 100 mg, n=30

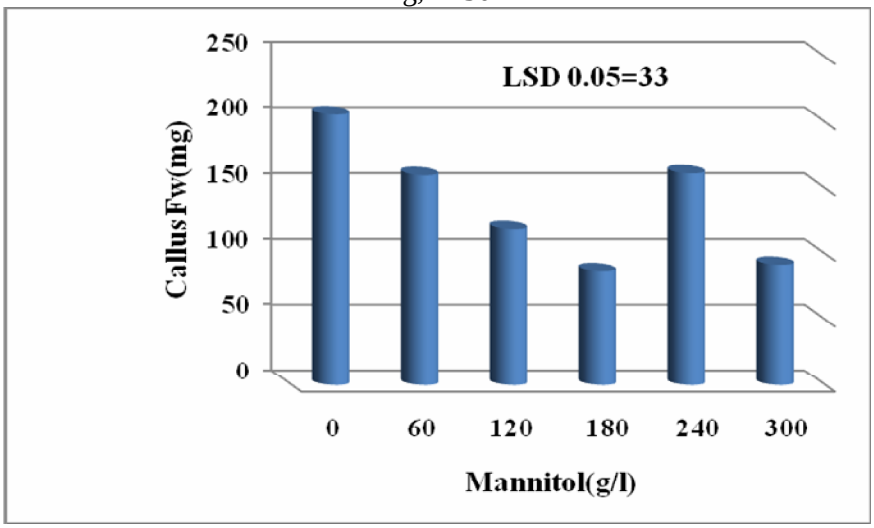


Figure.3 *Ruta graveolens* callus cultures exposed to different concentrations of mannitol showing changes in callus mass grown on MS medium for four weeks

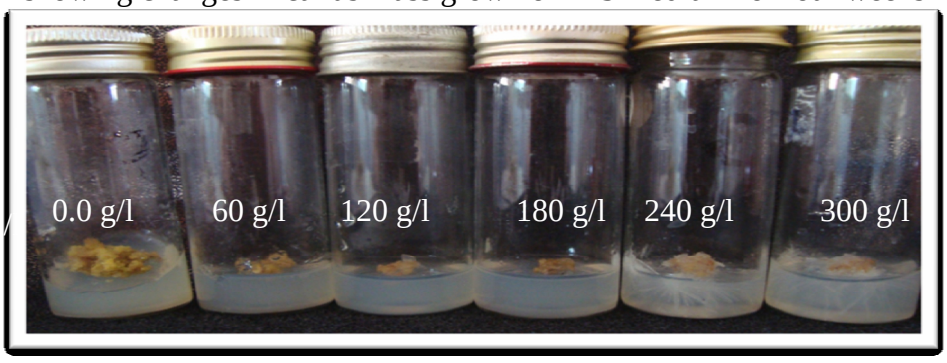


Figure.4 Effect of mannitol concentration on mean callus fresh weight after inoculation of callus pieces onto a solid MS medium supplemented with 1mg/l of 2,4-D and 0.5 mg/l of Kin for twenty weeks using stepwise increments of mannitol concentration at four week intervals. Initial weight is 100 mg, n=30

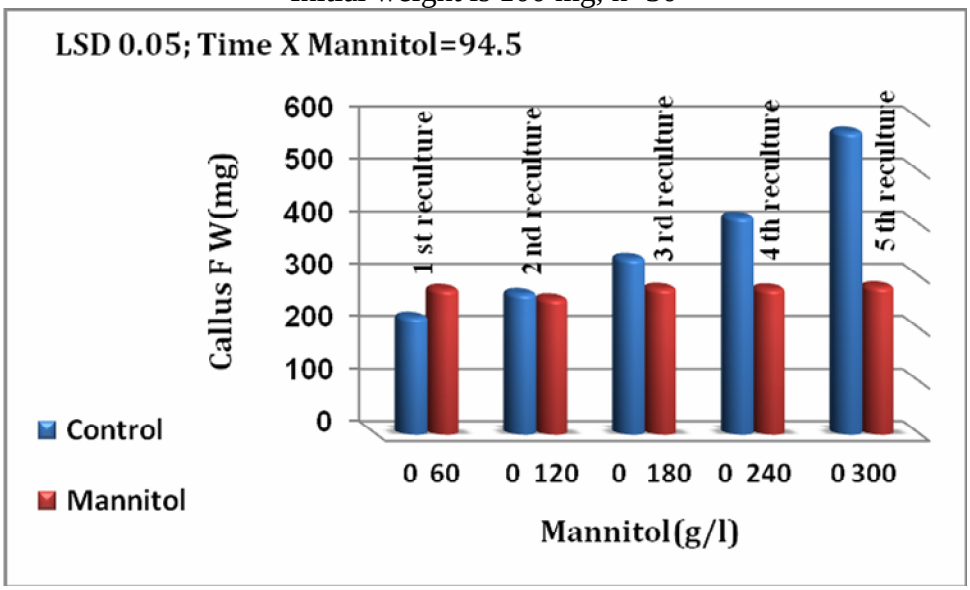


Figure.5 Effect of the time course on *Ruta graveolens* callus fresh weight supplemented with 240 g/l of mannitol, callus pieces inoculated onto a solid MS medium supplemented with 1mg/l of 2,4-D and 0.5 mg/l of Kin for 4, 8 and 12 weeks. Initial weight is 100 mg, n=30

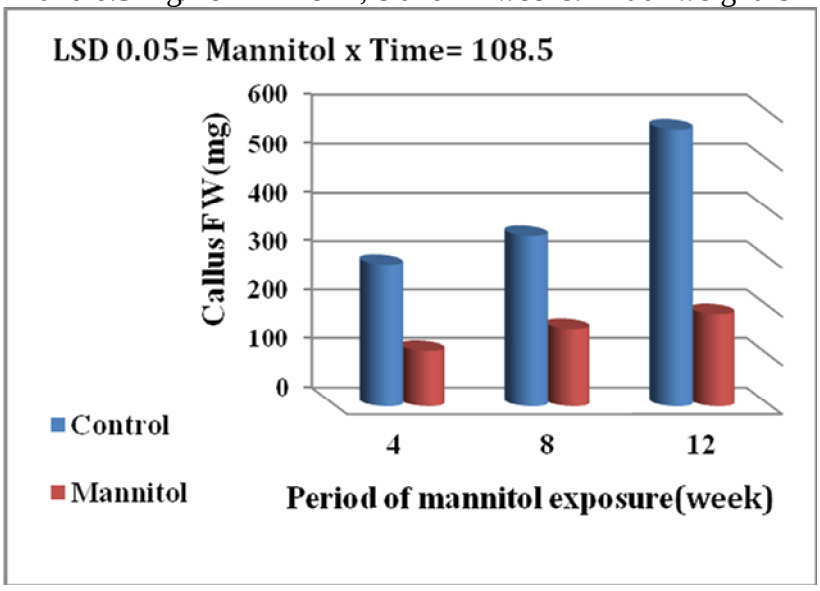


Figure.6 Effect of mannitol (g/l) on the mean no. shoots/callus piece of *Ruta graveolens*, after inoculating callus pieces onto a solid MS medium supplemented with 1.5 mg/l of BA and 0.5 mg/l of NAA for 3 months, n=30

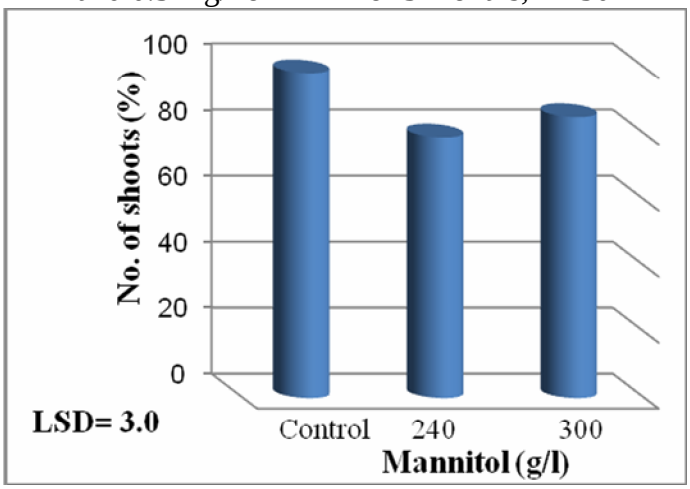
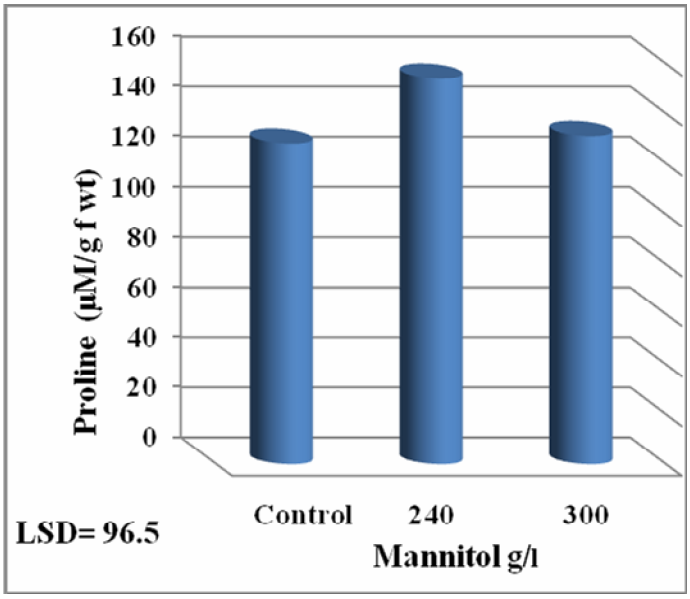


Figure.7 Effect of mannitol (g/l) on the mean proline content in shoots/callus piece of *Ruta graveolens*, after inoculating callus pieces onto a solid MS medium supplemented with 1.5 mg/l of BA and 0.5 mg/l of NAA for 3 months, n=30



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