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Effects of putrescine application in culture medium in improving chamomile [*Chamomilla recutita* (L.) Rauschert.] tolerance to osmotic stress under *in vitro* conditions

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Abstract: In order to assess the effect of osmotic stress induced mannitol under *in vitro* conditions on some growth parameters of chamomile [*Chamomilla recutita* (L.) Rauschert], treatments were arranged and compared for the main effect of osmotic stress induced by mannitol in four levels (0, 2, 4, and 6 g/l), and the interaction effect of osmotic stress x putrescine (0, 0.5, and 1 mM). Osmotic stress, especially induced by 4 and 6 g/l of mannitol, were found to significantly reduce shoot height, root length, the number of shoot and root per plant, the fresh weight of shoot, and the fresh weight of root. When plants were grown on 0.5 mM of putrescine, the fresh weight of root, carotenoid, chlorophyll a, and chlorophyll b were increased, compared to plants grown on medium with 0 and 1 mM of putrescine. Plants grown on medium with 0.5 and 1 mM of putrescine had an increased level of flavonoid, phenolic acid, and proline under four levels of mannitol. The amount of 0.5 mM of putrescine significantly improved plant biomass and essential oil content in plants grown on medium containing 0 and 2 g/l of mannitol. The results showed that the use of putrescine could improve chamomile tolerance to osmotic stress.

1. Introduction

Chamomile [*Chamomilla recutita* (L.) Rauschert.] is an important medicinal plant, believed to have many properties. Several studied have indicated the medicinal effects of chamomile on many diseases (reviewed in Singh *et al.*, 2011). With the growing importance of chamomile applications in modern medicine, many studies have focused on the investigation of exogenous factors such as plant growth regulators and environmental stresses on the growth parameters and physiological characteristics. It has been suggested that the medicinal properties of chamomile result from its essential oil and antioxidant content (Edris, 2007; Wei and Shibamoto, 2007; Roby *et al.*, 2013). However, these properties are negatively affected by environmental factors such as osmotic stress (Baghalian *et al.*, 2011; Jeshni *et al.*, 2017). A study on drought effects on physiological and phytochemical traits of chamomile reported that agro-morphological characters, essential oil content and composition are significantly decreased in this condition (Baghalian *et al.*, 2011). Afzali *et al.* (2006) showed that osmotic stress induced by polyethylene glycol and mannitol decreases the growth parameters at early growth stages of chamomile.

Polyamines have been found to involve in plant response to biotic/abiotic stress including osmotic stress, and other types of stress such as drought and salinity which impose osmotic stress on plants rather than their own specific effects (Alcázar *et al.*, 2010; Shabala and Munns, 2017; Shokri-Gharelo and Noparvar, 2018). It has been shown that high levels of polyamines in plants are associated with tolerance to abiotic stress (Alcázar *et al.*, 2010; Mandal *et al.*, 2014; Pál *et al.*, 2018). It has also been shown that exogenous application of polyamines, including putrescine, increases the tolerance of plants to stressful conditions (Talaat *et al.*, 2005; Bibi *et al.*, 2010; Hassanein *et al.*, 2013). Exogenous application of putrescine has been shown to improve morphological parameters (plant height, root length, number of shoots and roots, and plants biomass), physiological characters, and phytochemical properties in wheat (Mostafa *et al.*, 2010), geranium (Ayad *et al.*, 2010), and Egyptian carnation (El-Ghorab *et al.*, 2006). Furthermore, positive effects of exogenous putrescine application have been shown to improve growth characters and tolerance under abiotic stress (Ali *et al.*, 2007; Bibi *et al.*, 2010; Hassanein *et al.*, 2013; Mandal *et al.*, 2014).

There is no published evidence on the application of putrescine *in vitro* culture medium and its effects on morphological, physiological, and essential oil content of chamomile under different levels of osmotic stress induced by mannitol. The objectives of this study were therefore to investigate the effect of osmotic stress induced by mannitol under *in vitro* conditions and to evaluate the effects of putrescine on ameliorating the negative effects of osmotic stress on chamomile.

2. Materials and Methods

Plant materials and experimental conditions

Seeds of German chamomile [*Chamomilla recutita* (L.) Rauschert.] were used in this work. The experiment was carried out under *in vitro* conditions. Test tubes were used as the experimental unit and one plant was cultured in each test tube. All test tubes used in the experiments were sterilized. Plants were grown in a growth room with temperature 25±2°C, relative humidity 50% and 60% during day and night respectively, and 14h photoperiod throughout the experiment.

In order to measure the main effects of osmotic stress on chamomile and the effects of putrescine application in culture medium in reducing the stress effects, two experiments were designed with the same laboratory conditions. The first was based on a completely randomized design with one factor with four levels (osmotic stress). The second was arranged in factorial design based on completely randomized design (4 x 3) with two factors. Four replications were used in both experiments, and 16 units for the first experiment and 48 for the second experiment were analyzed.

Medium culture and experiments

Basic MS (Murashige and Skoog, 1962) was used as the culture medium. Seeds of chamomile were sterilized in a commercial chlorine solution (5%) for 20 minutes and then washed three times using distilled water. Seeds were then gently placed on culture media.

In the first experiment, one treatment including mannitol in four levels (control, 2, 4, and 6 g/l in culture media) was studied. In the second experiments, two treatments were studied; four mannitol levels were used, 0 (control), 2, 4, and 6 g/l in culture media to create osmotic stress combined with three levels of putrescine 0 (control), 0.5, and 1 mM.

Morphological traits

The morphological parameters of chamomile measured in this experiment were following, shoot height (cm), root length (cm), shoot (n/plant), root (n/plant), fresh weight of root (RFW g/plant), and plant biomass (%). Plant height was measured from the crown to the tip of the stem. Fresh root was carefully washed with tap water after harvest and measured from the crown to the tip of the main root. To measure dry weight, plants were dried in an oven with 72°C temperature for 72 hours.

Chlorophyll content (a and b) and carotenoid

The amount of 0.2 g of fresh leaves were ground in 10 mL of 99% methanol, then centrifuged at 3000 rpm for 5 min. The extract was used to measure light absorption at 653, 470, and 666 nm (Lutts *et al.*, 1996). The following equations were used for calculating chlorophyll content:

CHLa=chlorophyll a= 15.65 A666-7.34 A653
CHLb=chlorophyll b= 27.05 A653-11.21 A666
Cx+c=carotenoid=1000 A470 -2.860 CHLa- 129.2CHLb

Measurement of flavonoid and phenolic acid

The semi-dried samples were solved in 0.1 mol/l sodium acetate at 20:1 ratio (liquid: sample) at room temperature. The mixture was homogenized and centrifuged at 20000 g for 30 minutes at 4°C. The supernatant were aspirated and used to determine flavonoid and phenolic acid content. The procedure described by Zhishen *et al.* (1999) was followed to measure flavonoid. The sample mixed with a solution containing aluminium chloride and sodium nitrite was added to 30 µl of sodium nitrite (10%), 60 µl of aluminium chloride hexahydrate (20%), 200 µl of NaOH (1M) and 400 µl of water. The absorbance reading was recorded at 510 nm every 20 s for 1 minute. The absorbance reading was compared to a standard curve drawn from catechin (69-689 µmol/l). The data were expressed as µmol catechin equivalents per gram of fresh or dry matter.

To measure phenolic acid, 2.5 mL of the Folin-Ciocalteu reagent and 2 ml of saturated sodium carbonate (75 g/L) were mixed with 50 µl of sample and homogenized for 10 s and heated for 30 minutes at 45°C. The absorbance reading was recorded at 720 nm and compared to the standard curve made from gallic acid (235-1176 µmol/l). The data were expressed as µmol gallic acid equivalents per gram of fresh or dry matter.

Proline content

To determine proline content of shoot, 0.5 g of the sample were homogenized in 3% (w/v) sulphosalycylic acid and then filtered through filter paper (Bates *et al.*, 1973). Acid ninhydrin and glacial acetic acid were added into the mixture and then heated at 100°C for 1 h in a water bath. Toluene was used to extract the mixture and the absorbance of fraction was read at 520 nm. Proline concentration was determined using calibration curves and expressed as µmol proline g FW.

Essential oil content

Hydrodestillation was used for the extraction of essential oil, where the sample of 25 g of chamomile herb dried in an oven was homogenized and boiled in 600 mL of distilled water in Clevenger for 3 hours. Then, water was gently removed from the tank and the amount of extracted essential oil was measured.

Statistical analysis

Three weeks after culturing, the data were analyzed by one-way and two-way ANOVA using JMP8-Statistics Software. Mean values were separated with Duncan's multiple range test ($P \leq 0.05$).

3. Results

Effect of osmotic stress on growth parameters

The statistical analysis of data from first experiment (16 experimental units) showed that osmotic stress had significant effects on shoot height and fresh weight of shoot at $P<0.01$, and on root length, number of shoots, number of roots, and fresh weight of roots at $P<0.05$. The main effect of osmotic stress on morphological traits is shown in figure 1, with an evident reduction in morphological traits under M2, M4, and M6. Mean comparison of data showed that morphological traits decrease with increasing levels of osmotic stress. Control plant (without stress) showed the highest morphological traits compared to plants grown under M2, M4, and M6. Plants grown on medium with 6 g/l of mannitol showed significant reduction (Fig 1B). The main effect of osmotic stress at M2 and M4 levels was more adverse on shoots than on roots. Root length, number of roots, and fresh weight of root were significantly decreased under M2 and M4 compared to a control group according to Duncan's multiple-range test ($P \leq 0.05$), but no significant difference was observed between plants grown under M2 and M4 conditions (Fig. 1B). The number of shoots showed a significant decrease, and plants which were grown on culture medium containing 2 and 4 g/l of mannitol, had no significant difference ($P \leq 0.05$) (Fig. 1B). Under M6 conditions, all growth parameters especially shoot traits showed sever reduction.

Effect of putrescine application on morphological traits under osmotic stress

The second experiment compared two treatments including osmotic stress and application of putrescine (a total of 48 experimental units). Variance analysis of data revealed that interaction effects of osmotic stress and putrescine (OS x Pu) were significant for shoot length, root length, number of shoots, and fresh weight of shoots (Table 1), while the main effect of putrescine was a significant on the fresh weight of roots (Fig. 2). The Interaction effect and the main effect of treatments were not significant on number of roots per plant (Data not shown).

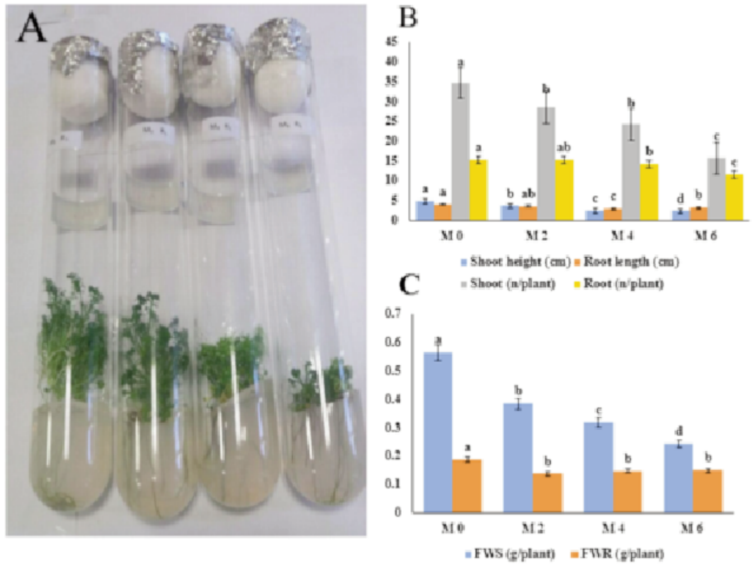

Fig. 1 - Main effects of osmotic stress induced by mannitol (M0= without mannitol, M2= 2 g/l of mannitol, M4= 4 g/l of mannitol, and M6= 6 g/l of mannitol) in culture medium on morphological traits of chamomile. (A) Morphological traits under different levels of osmotic stress (M0= without mannitol, M2= 2 g/l of mannitol, M4= 4 g/l of mannitol, and M6= 6 g/l of mannitol), (B) Mean values of shoot height, root length, number of shoots, and number of roots, (C) Mean values of fresh weight of shoots and roots. Different letters above each bar indicate significant differences according to Duncan's multiple-range test (P≤0.05). FWS= fresh weight of shoot, FWR= fresh weight of root.

Table 1 - Interaction effects of osmotic stress induced by mannitol and putrescine on morphological and chemical traits of chamomile)

Treatment		Morphological traits				Chemical traits		
Mannitol (g/l)	Putrescine (mM)	Shoot length (cm)	Root length (cm)	Shoot (no./plant)	SFW	Flavonoid	Phenolic acid	Proline
M0	Pu0	3.55 b	4.425 b	33.5 a	0.6168 b	33.61 cde	32.87 cde	10.65 d
	Pu0.5	6.025 a	6.325 a	37.5 a	1.615 a	42.02 c	43.24 c	12.86 cd
	Pu1	5.6 a	6.075 a	35.5 a	1.357 a	36.35 cde	34.47 cd	12.26 d
M2	Pu0	2.1 cde	3.275 c	23 b	0.34 bcd	38.38 cd	32.5 cde	16.67 bc
	Pu0.5	2.475 cd	4 bc	24 b	0.5138 bc	22.69 e	17.7 e	15.99 bc
	Pu1	1.6 ef	2.15 e	15 b	0.323 bcd	25.67 de	22.72 de	15.27 bc
M4	Pu0	1.925 def	3.325 c	14.75 cd	0.126 d	70.08 b	62.32 b	18.41 bc
	Pu0.5	2.775 c	3.45 c	19.25 bc	0.4565 bcd	36.77 cde	30.6 cde	19.27 b
	Pu1	1.1 f	1.275 f	8.25 e	0.1505 d	45.11 c	44.76 c	21.46 ab
M6	Pu0	1.125 f	2.3 de	11.5 de	0.2212 cd	71.68 b	67.52 b	24.21 a
	Pu0.5	1.9 def	3.125 cd	17.25 c	0.5102 bc	69.89 b	62.81 b	20.92 ab
	Pu1	1.3 ef	1.175 f	10.75 de	0.193 cd	90.01 a	90.26 a	20.66 ab
Significance								
OS		**	**	**	**	**	**	**
Pu		**	**	**	**	*	*	*
OS x Pu		**	**	*	**	**	**	**

Different letters within each column indicate significant differences according to Duncan's multiple-range test (P≤0.05).
* P<0.05 and **0.01, indicate level of significance.
OS= osmotic stress; M0= without mannitol; M2= 2 g/l of mannitol; M4= 4 g/l of mannitol; M6= 6 g/l of mannitol; Pu0= without putrescine; Pu0.5= 0.5 mM of putrescine; Pu1= 1 mM of putrescine; SFW= fresh weight of shoot.

Putrescine significantly increased the fresh weight of roots at 0.5 and 1 mM compared with plants grown on basic MS medium (without putrescine). In terms of shoot length, root length, number of shoots, and fresh weight of shoots, plants grown on a basic MS medium containing 0.5 and 1 mM of putrescine and without mannitol showed significantly increased traits compared to control plants (without mannitol and putrescine) and other groups (Table 1). Plants grown on medium containing 2 and 4 g/l of mannitol plus 0.5 mM of putrescine (M2Pu0.5 and M4Pu0.5) showed significantly better traits compared to plants grown on medium containing 2 and 4 g/l of mannitol plus 0 and 1 mM of putrescine (M2Pu0, M2Pu1, M4Pu1, and M4Pu1). However, plants grown on medium with 6 g/l of mannitol (severe osmotic stress) and with/without putrescine showed significantly the lowest means compared to other groups (Table 1).

Effect of putrescine application on physiological traits under osmotic stress

Variance analysis of physiological traits showed that main effect of putrescine on carotenoid, chlorophyll a and b was significant (Data not shown), while interaction effect of osmotic stress x putrescine was significant on flavonoid, phenolic acid and proline (Table 1).

The carotenoid, chlorophyll a and b of chamomile were significantly increased in plants grown on medium with 0.5 mM of putrescine (Fig. 2). The flavonoid, phenolic acid, and proline content in plants grown on medium with 1 mM of putrescine plus 6 g/l of mannitol showed the highest content compared to other groups (Table 1). The lowest contents of flavonoid, phenolic acid, and proline in each group (M0, M2, M4, and M6) were observed in plants grown on medium without putrescine (Pu0) and the highest contents in each group were observed in medium with 1 mM of putrescine (M0Pu1, M2Pu1, M4Pu1, and M6Pu1) compared to control groups.

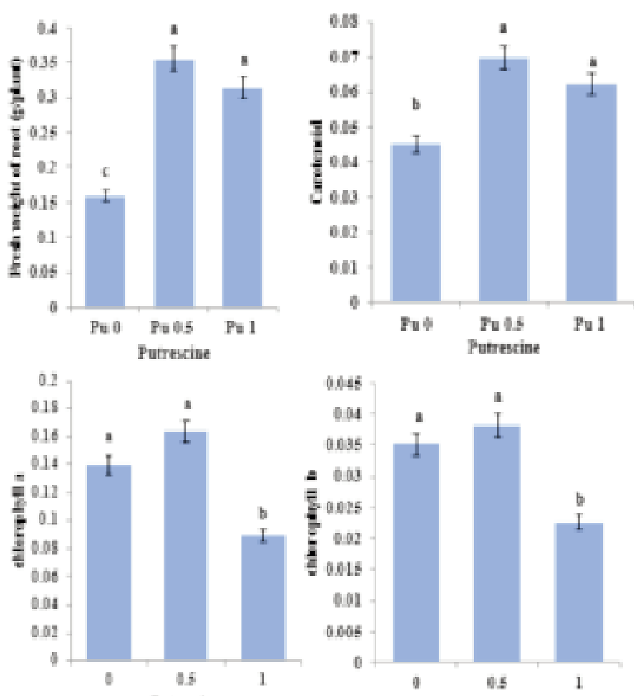

Fig. 2 - The effect of putrescine on morphological and physiological traits of chamomile (fresh weight of root, carotenoid, chlorophyll a and b). Putrescine was added in 0 (control), 0.5, and 1 mM in culture media. Different letters above each bar indicate significant differences according to Duncan's multiple-range test (P≤0.05).

Effect of putrescine application on plant biomass and essential oil under osmotic stress

Variance analysis of data related to plant biomass and essential oil showed that the interaction effect of osmotic stress x putrescine was significant (P<0.05) (Data not shown). Plants grown on medium containing 2, 4, and 6 g/l of mannitol (M2, M4, and M6) plus 0.5 and 1 mM of putrescine (Pu0.5 and Pu1) had the lowest plant biomass and essential oil content compared to the control group (M0Pu0, M0Pu0.5, M0Pu1) (Fig. 3 and 4).

The largest increase in biomass was observed in plants on medium without application of mannitol but treated with 0.5 and 1 mM of putrescine

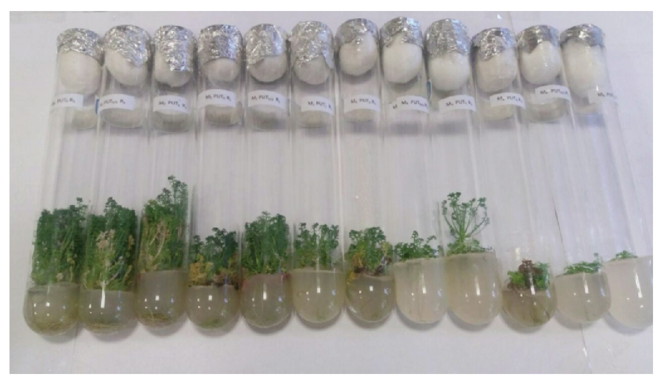

Fig. 3 - Interaction effects of osmotic stress x putrescine on morphological traits of chamomile. Mannitol was used in four levels; 0 (control), 2, 4, and 6 g/l in culture media to create osmotic stress. Putrescine was added in 0 (control), 0.5, and 1 mM in culture media.

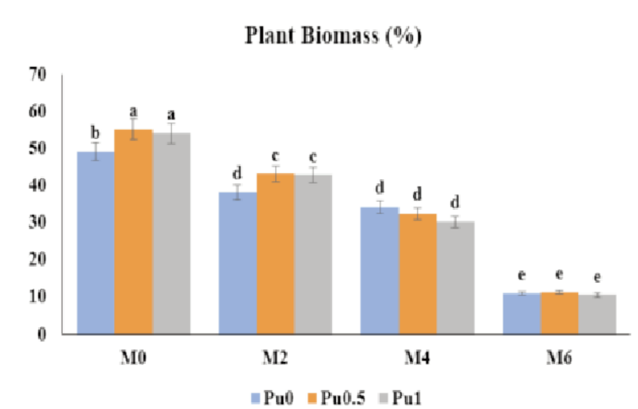

Fig. 4 - Interaction effects of osmotic stress x putrescine on the plant biomass of chamomile. Different letters above each bar indicate significant differences according to Duncan's multiple-range test (P ≤ 0.05).

(M0Pu0.5 and M0Pu1). Plants grown on medium with 2 g/l of mannitol plus 0.5 and 1 mM of putrescine had a larger biomass compared to plants grown on M2Pu0, but significantly lower biomass compared to the control group. In plants grown on medium with 4 and 6 g/l of mannitol, no significant difference was observed between plants placed on medium with 0, 0.5, and 1 mM of putrescine (Fig. 3).

The largest amount of essential oil was in the group of plants grown on medium without osmotic stress (M0;) and with 0.5 and 1 mM of putrescine (Pu0.5 and Pu1). The group of plants grown on medium with 2 g/l of mannitol, plants grown on medium with 0.5 and 1 mM of putrescine showed significantly more essential oil (M2Pu0.5 and M2Pu1) compared to plants grown on medium without putrescine (M2Pu0). Even though plants on medium with 0.5 and 1 mM of putrescine plus 4 and 6 g/l of mannitol did not show significant difference compared to plants grown on medium without its application (Fig. 4).

4. Discussion and Conclusions

The effect of putrescine application in culture medium containing mannitol has not previously been reported so far. The studies on other plant species and also other types of stresses will therefore be used for discussion. In this study, the effect of osmotic stress created by mannitol under *in vitro* conditions, the main effect of putrescine on the growth of chamomile, and putrescine effects in ameliorating effects of osmotic stress on chamomile were investigated.

Osmotic stress is a side effect of some abiotic stresses such as drought and salinity in which water absorption is limited, leading to conditions similar to drought (Shen *et al.*, 1999). Drought and salt stress have been found to decrease the morphological traits in many medicinal plants, in terms of length and number of shoots and roots, fresh weight of shoots, and fresh weight of roots (Afzali *et al.*, 2006; Jaleel *et al.*, 2008; Arazmjo *et al.*, 2010; Anjum *et al.*, 2011). In agreement with the findings of this study), Afzali *et al.* (2006) reported a decrease in fresh weight of shoots and roots in chamomile under polyethylene glycol-induced osmotic stress. In another study, Dadkhah (2010) tested the effects of salinity on the plant height and number of shoots of chamomile in a pot experiment. Dadkhah reported a significant decrease in plant height and number of shoots in early stage of the stress. In respect of drought stress induced by mannitol under *in vitro* conditions, Ghaheri *et al.* (2015) findings in *Steviare baudiana* Bertoni are in accordance with the findings of this study.

Regarding putrescine effects in increasing morphological traits, a number of studies have showed that foliar application of putrescine increases plant height, root length, shoot (number per plant), and root (number per plant) (Talaat *et al.*, 2005; Mostafa *et al.*, 2010; Amin *et al.*, 2011; Hassan and Bano, 2016), and improving physiological traits, in terms of chlorophyll a, chlorophyll b, carotenoid (Talaat *et al.*, 2005; Hassan and Bano, 2016), and proline (Hassan and Bano, 2016). This study provides evidence of improved flavonoid, phenolic acid, and other parameters (Table 1, Fig. 2) in chamomiles grown on medium containing 0.5 and 1 mM of putrescine. Studies that investigate effects of putrescine under *in vitro* conditions have not been found, but in agreement with the findings of this study, several studies have revealed that plants treated with putrescine have increased growth parameters and more tolerance to abiotic stresses such as osmotic stress, drought, salinity, and temperature compared to untreated plants (Jaleel *et al.*, 2008; Alcázar *et al.*, 2010; Hassanein *et al.*, 2013). Investigating the effects of putrescine foliar application on chamomile and sweet marjoram under salinity stress, Ali *et al.* (2007) found that putrescine significantly increased flavonoid content. In addition, the use of putrescine was found to enhance chlorophyll a, chlorophyll b, carotenoid, phenolic acid, and morphological traits under stress conditions compared to untreated plants (Amin *et al.*, 2011; Shallan *et al.*, 2012; Hassanein *et al.*, 2013; Hassan and Bano, 2016).

Other authors have indicated positive effects of putrescine application on plant biomass and essential oil in chamomile and sweet marjoram (Ali *et al.*, 2007), wheat (Hassan and Bano, 2016), onion (Amin *et al.*, 2011), and cotton (Shallan *et al.*, 2012). Findings in this study showed that putrescine application improves the plant biomass and essential oil content under normal growth conditions and in chamomiles grown on medium containing 2 g/l of mannitol (Fig. 4 and Fig. 5), whereas plants grown on medium with 4 and 6 g/l of mannitol showed remarkable decreased levels of biomass and essential oil compared to the control group, and the study did not find the positive effects of putrescine under these levels of osmotic stress (M4 and M6). In line with these findings regarding the positive effects of putrescine under normal growth conditions and 2 g/l of mannitol stress, Ali *et al.* (2007) reported an increase in the plant biomass in *chamomile and sweet marjoram*, Ayad *et al.* (2010) in *geranium* and El-Ghorab *et al.* (2006) in *Egyptian carnation* reported a high level of essential oil in plants treated with putrescine.

Pál *et al.* (2018) in their study on wheat reported that putrescine treatment induces stress-responsive genes that overlap with the genes induced by osmotic stress. They suggested that changes induced by putrescine overlap with changes induced by osmotic stress, and lead to better tolerance in plants treated with putrescine. In another study, Bibi *et al.* (2010) showed that putrescine application significantly increases the endogenous putrescine concentration. They suggested that stress tolerance correlates with an increment of putrescine. The results of the present study do not provide evidence at the molecular level and for the endogenous concentration of putrescine in chamomile but the data showed an increased values of observed traits when putrescine was used in medium culture. As suggested by Mandal *et al.* (2014), polyamines including putrescine alleviate oxidative stress induced by osmotic stress. It is believed that oxidative stress created by being exposed to abiotic stresses such as osmotic stress, drought, and salinity is one of most important reasons for remarkable reduction of morphological traits and plant yield (Shokri-Gharelo and Noparvar, 2018). Other studies have shown that plants with efficient antioxidant systems, including high level of flavonoid, phenolic components (reviewed in Shabala and Munns, 2017), and plants with high level of proline content (Ahmad *et al.*, 2016) show more tolerance and these indices have been regarded as one of tolerance characters in different plants. This study revealed that flavonoid, phenolic acid, and proline are increased in chamomile grown on medium with putrescine (Table 1). This could explain the better morphological and physiological traits as well as essential oil content studied in this work in plants under osmotic stress.

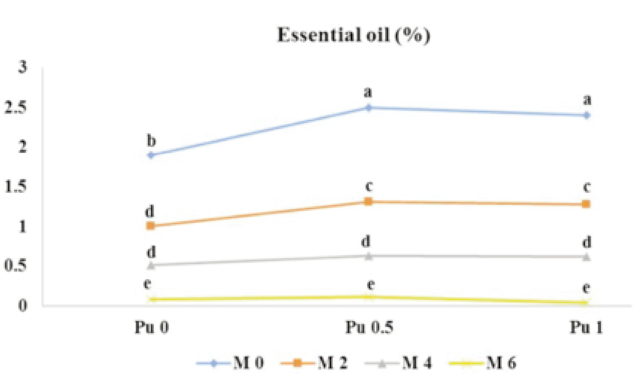

Fig. 5 - Interaction effects of osmotic stress x putrescine on the essential oil of chamomile. Different letters above each bar indicate significant differences according to Duncan's multiple-range test (P≤0.05).

Osmotic stress, especially at M4 and M6 levels (4 and 6 g/l of mannitol) significantly reduce the morphological traits of chamomile under *in vitro* conditions. The main aim of the current study was to use putrescine in culture medium to assess its effects in ameliorating osmotic stress. The values of morphological traits, some physiological traits, and contents of essential oil of chamomile were significantly higher in plants grown on medium containing 0.5 and 1 mM of putrescine compared to plants grown on medium without putrescine application.

Application of putrescine may improve chamomile tolerance to osmotic stress, and may be considered as one of substances that can be used to improve chamomile quality under osmotic stress.

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