

EFFECT OF MANNITOL STRESS ON MORPHOLOGICAL, BIOCHEMICAL AND POLYPHENOL PARAMETERS IN BROCCOLI SPROUTS (*BRASSICA OLERACEA* VAR. *ITALICA*)

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(Received 29th Oct 2017; accepted 13th Mar 2018)

Abstract. To assess the effect of drought stress induced by mannitol at three levels (0, 88 and 176 mM) on biochemical and polyphenolic traits of six F₁ broccoli hybrids, a factorial experiment based on completely randomized design in three replications was implemented at research station of Agricultural Sciences and Natural Resources University, Sari, Mazandaran, Iran in 2016. The results showed that mannitol stress reduced dry weight and shoot length significantly for all varieties but with a different rate. Total phenolic, flavonoid, and anthocyanin contents, the activity of enzymatic antioxidants, and DPPH activity were significantly higher under 176 Mm mannitol application condition than control. In addition, results showed that mannitol stress increased the content of sulforaphane. Marathon genotype showed the highest content of sulforaphane among all varieties under both normal (6.139) and under stress (14.122) conditions. Marathon and Heraklion genotypes could be suggestively used for breeding program to increase content of sulforaphane coupled with other traits. Since the content of sulforaphane along with phenolic compounds and antioxidant activities were higher under both severe and moderate stress conditions, using moderate mannitol stress treatment can be implemented for increasing the content of these suitable compounds in broccoli.

Keywords: *sulforaphane, DPPH, principal component, flavonoid, malondialdehyde*

Abbreviations

ROS	Reactive oxygen species
H ₂ O ₂	Hydrogen peroxide
O ₂	Superoxide radicals
MDA	Malondialdehyde
SOD	Superoxide dismutase
APX	Ascorbic peroxidase
POX	Guaiacul peroxidase
CAT	Catalase
DPPH	2, 2-diphenyl-1-picrylhydrazyl

Introduction

Broccoli (*Brassica oleracea* var. *Italica*) is a member of the Brassicaceae family and its wild form is found throughout the Mediterranean region and is widely cultivated in many countries of Europe and America along with Asian countries (Abou El-Magd et al., 2013). Broccoli is one of the highly valuable vegetable that is rich in nutrients content and it has many benefits for human health. Broccoli sprouts contain a substance called sulforaphane which intensely reduces the number, size, and proliferation of cancerous tumors; and moreover the sprout is rich in fiber, carotenoids, vitamin A, vitamin C and vitamin K (Beecher 1994,

Pereira et al., 2002). This plant is grown as an either spring or autumn crop, but it is sensitive to cold and dry condition especially at early stages. Worldwide, water shortages and drought stress is among the most important abiotic stresses limiting crop production (Lemoine et al., 2010). Due to the uniformity of the soil environment and lack of environmental control in the field, laboratory investigations have a special importance to assess the tolerance of plants against drought stress. To create an artificial environment controlling water potential, substances with high molecular weight are used. Mannitol, as a high molecular weight substance, owing to inducing a condition with osmotic solution identical to natural environment, is often used to control water potential in drought stress studies and controlled environments (Guo et al., 2011).

Drought stress increases the accumulation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-), and hydroxyl ($OH^\cdot$) leading to oxidative stress (Saed-Moucheshi et al., 2014b). Production of reactive oxygen species causes lipid peroxidation along with protein and nucleic acid degradation. Plants are able to reduce the damaging effect of reactive oxygen species by different mechanisms. One of these mechanisms is involved in enzymatic or non-enzymatic antioxidant defense system. Antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POX) are involved in detoxifying oxygen free radicals in plants' cells (Saed-Moucheshi et al., 2014a). In response to increased production of reactive oxygen species, the capacity of antioxidant defense systems and the activity of antioxidant enzymes are increased. The first enzymatic barrier against produced oxidants is SOD, which converts O_2^- to H_2O_2 , a molecule with relatively lower radical activity. H_2O_2 could be detoxified into water and oxygen by the CAT and ascorbate peroxidase (APX) enzymes. Peroxidase has an important role in detoxification of H_2O_2 which can swept away this compound by using ascorbic acid as an electron donor for the reduction of H_2O_2 to H_2O (Saed-Moucheshi et al., 2014b). During this reaction, ascorbic acid is transformed to monodehydroascorbate. POX are glycoproteins that are able to use phenols similar to hydrogen donors to cope with high content of oxygen free radicals. Furthermore, involvement of POX in plant development processes, lignin production, ethylene biosynthesis, plant defenses against stresses, and wound restoration have been proved (Hossain et al., 2015). Changes in the activity of antioxidant enzymes under NaCl stress (Tian et al., 2016) and heat stress (Lin et al., 2010) have been also reported in broccoli sprouts. Additionally, damages to the proteins along with accumulation of some free amino acids such as proline in order to maintain osmotic pressure leading to regulate protein synthesis in cells has been observed under abiotic stresses (Osakabe et al., 2014). Some researchers claimed that the reduction in synthesis of some types of proteins is attributed to decrease in their polysome numbers (Han and Wagner, 2014, Suzuki et al., 2014). In addition to changes in the content of proteins, lipids are affected by induced abiotic stresses. Membrane lipid peroxidation normally results in production of some aldehyde namely malondialdehyde (MDA) which is a quick responsive compound to oxidative stress (Berger et al., 2016). Furthermore, ethylene and salicylic acid are two phytohormones composed of polyphenols which are important in stress responses of the plants which have direct, indirect, and cascading effects on some vital activities of the plants such as photosynthesis and plant developments under stress conditions (Gupta and Huang, 2014).

The current study tends to find out about the responses of the different genotypes of the broccoli to water shortage stress caused by mannitol under laboratory and controlled environment through considering different morphological, biochemical, and polyphenols parameters. In addition, to screen the most useful F₁ hybrids for being used in plant breeding and human nutritional programs. In addition, evaluate the relationship between measured parameters by using advanced and multivariate statistical techniques in order to help breeders to use them as screening criteria for indirect selection of genotypes with higher nutritional qualities.

Materials and methods

Experimental procedure

Seeds of the six F₁ broccoli genotypes (Castle Dome, Green Magic, Heraklion, Marathon, Matsuri and Sacora) prepared from PS America, Inc and TOKITA SEED CO. LTD (Japan) companies were used in a factorial experiment with two factors (Mannitol levels and genotypes) on the bases of completely randomized design (CRD) with three replication and in each replication with 10 petri dishes. The seeds were soaked in ethanol 70% for 2 minutes and then were in undated with Bleach (sodium hypochlorite) 20% for 10 minutes; after that, the seeds were washed 4 times with deionized water. Five seeds were transferred to each sterile petri dish containing wet filter papers. The periods of 16 hours of light and 8 hours of darkness in a growth chamber at 23 °C (day)/ 20 °C (night) temperature with average humidity of 70% were applied and continued during the experiment. The five-day small sprouts of broccoli were treated with 88 and 176 mM mannitol for 48 hours (two days). Therefore, the samples were taken from the seven days old sprout to assess the biochemical and growth-related traits.

Measurements of the traits

Shoot length, root length, and dry weight were measured as growth related traits. Shoot and root lengths were measured using a precise measure on the bases of centimeter. For measurement dry weight, fresh shoots were placed in aluminum foils and kept in an oven for 48 h under 70 °C, and then were weighed with a precise scale on the bases of gram. The mean of the five used sprout in each petri dish was used for each replication.

Total protein content was measured by the method of Bradford (1976) at 595 nm spectrophotometric wave length. MDA and H₂O₂ contents were estimated by the methods of Heath and Packer (1968) and Velikova et al. (2000), respectively. Enzymatic antioxidants consist of SOD, APX, POX, and CAT were measured using the methods of Giannopolitis and Ries (1977), Nakano and Asada (1981), Dazy et al., (2008), and Aebi (1984), respectively. The methods described by Ainsworth and Gillespie (2007), Oomah and Mazza (1996), and Yuan et al. (2009) were used for the measurement of phenol, flavonoid, and anthocyanin contents in broccoli sprouts, respectively. DPPH as the total activities of the sprout to scavenge reactive oxygen species were estimated using Brand-Williams et al. (1995) method. Using HPLC standard, sulforaphane content in the broccoli sprout was measured for each variety under each treatment after determining the peaks based on the method of Brader et al. (2006).

Statistical analyses

Data were analysed by one-way ANOVA in SAS 9.3, followed by Duncan's multiple range test (DMRT) comparison test. PROC CORR of SAS 9.3 was used for assess Pearson's correlation coefficient between all pairs of measured traits. The graphs for mean comparison were drawn by Excel 2016 software. Multivariate analyses namely principal component analysis (PCA) and biplot graph were assessed by Minitab v. 16.

Results

Analysis of variance and mean comparison

Table 1 is presenting the results of analysis of variance for all measured traits. The main effect of genotype was significant ($p < 0.01$) for all traits. In addition, the effect of mannitol showed significant effect on all measured traits ($p < 0.01$) except for root length. The interaction between genotypes and mannitol was significant for all parameters. Since the interaction effect between genotypes and mannitol showed high significant in the ANOVA table.

The results of multiple mean comparison based on Duncan's multiple range test subjected to growth related traits, shoot length and root length, along with some polyphenols consist of total phenol content, flavonoid content, and anthocyanin content are prepared in Table 2. In all genotypes, control treatment showed higher shoot length than two other levels of mannitol application. In Heraklion, Marathon, Green Magic, and Matsuri genotypes, application of 176 mM mannitol resulted in the lowest shoot length, but in other genotypes application of 88 mM mannitol obtained the lowest shoot length with no significant difference from 176 mM. Castle Dome and Matsuri genotypes showed higher shoot length in comparison with other ones. The highest shoot length was achieved in Matsuri genotype under no application of mannitol (6.87 cm), while the lowest shoot length was obtained in Sacora under the application of 88 mM mannitol (3.33 cm). Similar to the shoot length, the response of different genotypes to different levels of mannitol were different for root length. In Sacora, Heraklion and Matsuri genotypes, the control treatment showed higher root length than the two other levels of mannitol application. On the contrary, in Marathon, Green magic, and Castle Dome genotypes no application of mannitol resulted in lower root length in compare to other levels. The highest and the lowest root length were obtained in Marathon genotype under the application of 176 mM mannitol (13.53 cm) and Sacora genotypes under 176 mM mannitol (6.83 cm), respectively. Response of dry weight of different broccoli sprouts to different levels of mannitol application is depicted in Table 2. Dry weight of all F_1 hybrids were reduced by application of mannitol in compare to control. Excluding the Castle Dome genotype, higher level of mannitol application (176 mM) resulted in lower dry weight. Matsuri and Castle Dome genotypes showed higher dry weigh than the other genotypes.

Highest total phenol content was obtained in Marathon genotype under 176 mM ($112.29 \text{ mg g}^{-1} \text{ FW}$) while the lowest content was obtained in Sacora genotype under no application of mannitol ($39.52 \text{ mg g}^{-1} \text{ FW}$). Flavonoid of all genotypes except for Marathon reached a higher content with application of higher mannitol concentration. The highest flavonoid content ($123.4 \text{ mg g}^{-1} \text{ FW}$) was achieved in Heraklion under highest concentration of mannitol (176 mM), while the lowest

content achieved in Matsuri under no application of mannitol (28.73 mg g⁻¹ FW). Anthocyanin content of genotype Matsuri decreased in response to higher application of mannitol. However, the response of all other genotypes to higher concentration of mannitol was to increase the anthocyanin content. Marathon under application of 176 mM mannitol obtained the highest anthocyanin content (0.623 mg g⁻¹ FW) among all treated plots, while castle Dome genotype under no application of mannitol showed the lowest anthocyanin content (0.14 mg g⁻¹ FW).

MDA and H₂O₂ as two important markers indicating the severity of the stresses in plant were measured in all genotypes under all mannitol treatments, which their results are presented in *Table 2*. In all genotypes, higher mannitol content resulted in higher content of both MDA and H₂O₂. Heraklion and Marathon showed highest ratio for increase in the content of MDA in response to mannitol application. Similarly, Marathon and Green Magic genotypes showed the highest difference between control and mannitol application among all genotypes regarding H₂O₂. Green Magic (3.96 μmol mg⁻¹ FW) and Sacora genotype (0.77 μmol mg⁻¹ FW) both under 176 mM mannitol application resulted in the highest content of MDA and H₂O₂, respectively. In contrast, the lowest content of both MDA and H₂O₂ were observed in Castle Dome genotype under no application of mannitol.

SOD, CAT, POX, and APX as the enzymatic ROS scavengers were measured using spectrophotometric tool. The response of all enzymatic antioxidants to higher concentration of mannitol were to increase their activities in all genotypes. The rate of increase in Matsuri genotype regarding SOD was higher than other genotypes (104 and 166% increase in 88 and 176 mM mannitol in relation to control). Similar results regarding other measured enzymatic antioxidant were observed for this variety (*Table 2*). Excluding CAT, Marathon genotype showed the highest enzymatic activities among all genotypes under 176 mM mannitol application. Heraklion genotype under highest application mannitol showed the highest activity for CAT. The lowest activity of SOD, CAT, POX, and APX were observed in Sacora, Matsuri, Green Magic and Sacora under control treatment, respectively. The activity of DPPH which is an abbreviation for 2, 2-diphenyl-1-picrylhydrazyl was also measured as a free radical scavenger (*Table 2*). In genotype Marathon, the antioxidant activity by DPPH under no application of mannitol showed a high significant difference with application of 88 mM mannitol, but application of 176 mM showed a lower significant activity in compare to control. Similarly, in all other genotypes, the higher concentration of mannitol resulted in higher activity of DPPH. The highest activity of DPPH achieved in Heraklion genotype under the application of 176 mM mannitol (85.46 mmol kg⁻¹), but the lowest activity was resulted from control treatment in var. Matsuri (64.71 mmol kg⁻¹).

Sulforaphane as an important compound in broccoli was also measured in all genotypes under all treatments, which its results are presented in *Fig. 1*. The response of all genotypes to application of mannitol were to increase the content of sulforaphane but the rate of increase in some genotypes consist of Sacora and Castle Dome were low. The rate of increase in the content of sulforaphane in Masuri and Heraklion was higher in response to increase the concentration of mannitol with a high positive trend. Marathon and Green Magic showed a great increase from no application to 88 mM application of mannitol, but the difference between 88 and 176 mM mannitol remained insignificant. Under all mannitol levels and in total, the content of sulforaphane was highest in Marathonin comparison to other genotypes.

Table 1. Analysis of variance for measured traits in six F1 hybrids broccoli under mannitol treatment

Source	DF	Mean Square													
		DW	Shoot length	Root length	Sulfo-raphane	MDA	H ₂ O ₂	Phenol	Flavonoid	Antho-cyanin	SOD	CAT	POX	APX	DPPH
Variety	5	0.17**	5.64**	40.3**	46.58**	4.18**	0.32**	3465.78**	1314.91**	0.24**	5910.37**	1220.73**	764.16**	4187.53**	57.45**
Mannitol	2	0.06**	11.42**	1.05ns	139.3**	0.3**	0.07**	1338.42**	1985.89**	0.05**	5891.76**	2493.38**	999.84**	11596.67**	169.84**
Interaction	10	0.02**	1.56**	2.97**	15.17**	0.6**	0.14**	106.31**	999.98**	0.005**	446.94**	135.99**	89.54**	363.52**	58.15**
Error	36	0.001	0.05	0.33	0.27	0.001	0.0004	4.68	2.03	0.0003	2.46	1.04	0.47	0.86	0.57
Coefficient of Variation		3.51	4.59	5.71	6.47	1.23	6.46	3.7	2.06	4.16	2.39	2.98	3.61	1.96	0.92

**, *, and ns: significant at 0.01, 0.05 level, and non-significant at 0.05 level.

DW: Dry weight, MDA: Malondialdehyde, H₂O₂: hydrogen peroxide, SOD: Super Oxide Dismutase, CAT: Catalase, POX: Guaiacul Peroxidase, APX: Ascorbic Peroxidase, DPPH: 1,1-diphenyl-2-picrylhydrazyl

Table 2. Multiple mean comparison of measured traits for interaction between broccoli varieties and mannitol treatment

Variety	Mannitol (mM)	Dry weight (g)	Shoot length (cm)	Root length (cm)	Phenol (mg/g FW)	Flavonoid (mg/g FW)	Antho-cyanin (mg/g FW)	MDA (μmol mg ⁻¹ FW)	H ₂ O ₂ (μmol mg ⁻¹ FW)	SOD (μmol mm ⁻¹ mg ⁻¹ p)	CAT (μmol mm ⁻¹ mg ⁻¹ p)	POX (μmol mm ⁻¹ mg ⁻¹ p)	APX (μmol mm ⁻¹ mg ⁻¹ p)	DPPH (mmol kg ⁻¹)
Sacora	0	0.36H	6.367B	7.833I	39.524L	63.667G	0.451E	2.551H	0.23J1	24.011L	26.472I	10.939J	14.976N	82.323F
	88	0.32I	3.333K	7.667IJ	47.476HIJ	83.067C	0.505D	2.722G	0.35G	37.553J	32.672FG	11.222J	40.422I	84.091BCDE
	176	0.314I	4.467FG	6.833J	66.333D	83.933C	0.582B	2.935F	0.77A	31.146K	35.637E	13.043I	55.255G	84.495ABC
Heraklion	0	0.41G	5.567CD	13.033AB	45.238JK	51.733J	0.487D	2.373I	0.2H	66.522F	33.413F	26.287E	27.013K	82.02FG
	88	0.344H	4.7F	11.1D	52.317EFG	72E	0.534C	3.223D	0.44F	76.005E	48.789C	30.866D	71.515E	85.152AB
	176	0.35H	3.567JK	12.5BC	51.619EFG	123.4A	0.543C	3.869B	0.52E	82.162D	64.764A	37.062B	95.819B	85.455A

Marathon	0	0.355H	4.3GH	12.067C	85.143C	58.333HI	0.423E	2.049K	0.16G	82.394D	32.415FG	15.002H	45.985H	75.96H
	88	0.314I	4.033HI	13.167AB	97.952B	72.133E	0.543C	2.223J	0.3J	101.629B	45.854D	26.929E	84.076C	85.404A
	176	0.293IJ	3.8IJ	13.533A	112.286A	91.133B	0.623A	3.055E	0.53D	126.464A	55.502B	42.997A	110.646A	83.737CDE
Green Magic	0	0.46F	5.1E	8.933GH	45.667IJK	57.086I	0.369F	3.21D	0.18HI	55.538H	21.558J	5.477M	16.437N	82.273FG
	88	0.39G	4.4FGH	8.5GHI	50.571FGH	71.467E	0.482D	3.619C	0.64C	76.06E	31.465G	21.666F	23.257L	83.232DEF
	176	0.35H	3.267K	10.667DE	54.952E	75.533D	0.535C	3.958A	0.71B	96.427C	44.827D	33.826C	34.731J	84.394ABCD
Matsuri	0	0.89A	6.867A	9.933FE	40.952L	28.733K	0.214G	1.695M	0.09JK	18.845M	11.419L	5.334M	18.729M	64.717I
	88	0.83B	5.7C	8.1HI	48.952GHI	53.133J	0.195GH	1.957L	0.06KL	40.68I	29.054H	11.405J	44.655H	82.929EF
	176	0.65E	4.333FGH	7.667IJ	67.238D	60.333H	0.199GH	2.051K	0.16J	74.025E	55.548B	16.032GH	80.11D	84.293ABCD
Castle Dome	0	0.9A	6.6AB	9.333FG	42.667KL	53J	0.14I	1.701M	0.01M	35.627J	10.782L	6.883L	9.382O	81.061G
	88	0.72D	5.233DE	10.667DE	53.524EF	81.267C	0.179H	1.938L	0.05L	59.043G	15.741K	9.733K	18.207M	83.586CDE
	176	0.79C	5.933B	10.833DE	50.238FGH	67.067F	0.219G	2.05K	0.15j	95.877C	20.97J	16.338G	60.532F	84.04BCDE

Means with the same letter(s) in each column are not significantly different (Duncan 1%).

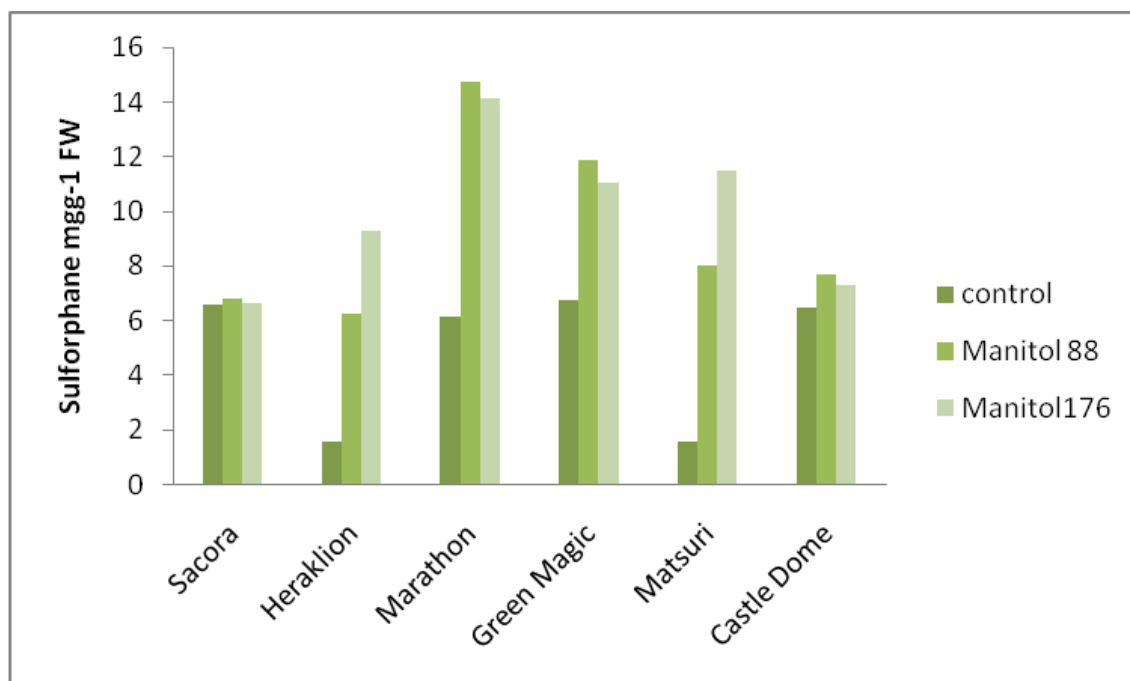


Figure 1. Response of sulfonphan content of different broccoli genotypes to mannitol treatment

Correlation between measured traits

The pairwise correlations between all pairs of measured traits based on Pearson method is presented in Table 3. Except for shoot length (0.74), dry weight showed negative correlations with all other traits. Similar to dry weight, all pairwise correlations of shoot length, excluding dry weight, were negative. The only insignificant correlation of shoot length was related to root length (-0.19). Root length showed negative correlation with shoot length and dry weight but these correlations were not statistically significant. Excluding dry weight and shoot length, all other correlations of root length were positive, but its correlation only with phenol content (0.5), SOD (0.7), and POX (0.67) were significant. Sulfuraphane showed significant correlations with shoot length (-0.61), phenol content (0.65), SOD (0.66), CAT (0.58), POX (0.52), APX (0.58), and DPPH (0.47). The content of sulfuraphane showed no significant correlations with flavonoid (0.29) and anthocyanin (0.31). MDA revealed negative significant correlations with dry weight (-0.67) and shoot length (-0.57). Also, the correlations of MDA with H_2O_2 (0.79), flavonoid (0.61), anthocyanin (0.61), CAT (-0.54), and POX (-0.51) were significant. Similar to MDA, the correlations of H_2O_2 with dry weight (-0.66) and shoot length (-0.63) were negatively significant. Furthermore, H_2O_2 showed significant correlations with MDA (0.79), flavonoid (0.59), anthocyanin (0.63), SOD (-0.43), CAT (-0.64), POX (-0.47). Phenol content showed significant positive correlations with all enzymatic antioxidant. The correlation of flavonoid with CAT was positively significant (0.51), but its correlations with other enzymatic antioxidant were not significant. Anthocyanin showed positive significant correlations with CAT (0.63) and POX (0.69). All internal correlations of enzymatic antioxidants were positively significant. DPPH as a stable compound with antioxidant ability showed positive significant correlation with SOD (0.48), Cat (0.53) and POX (0.49) but its correlations with APX were not significant.

Table 3. Pearson correlation coefficients for all pairs of the traits measured in different varieties of broccoli under mannitol treatment

	DW	SL	RL	DPPH	MDA	H ₂ O ₂	Phenol	Flavonoid	Antho- cyanin	SOD	CAT	POX	APX	Sulfo- raphane
DW	1													
SL	0.74**	1												
RL	-	-	1											
DPPH	0.23ns	0.19ns	0.41ns	1										
MDA	-0.52*	-0.53*	0.04ns	0.22ns	1									
H ₂ O ₂	-	-	0.06ns	-	0.79**	1								
Phenol	0.66**	0.63**	0.46ns	0.16ns	0.12ns	0.18ns	1							
Flavonoid	-	-0.5*	0.5*	0.35ns	0.61**	0.59**	0.16ns	1						
Anthocyanin	-0.53*	0.65**	0.17ns	0.34ns	0.61**	0.63**	0.42ns	0.44ns	1					
SOD	-	-	0.31ns	0.48*	-	-0.43*	0.69**	0.26ns	0.39ns	1				
CAT	0.38ns	-0.52*	0.7**	0.53*	0.22ns	-	0.64**	0.52*	0.63**	0.6**	1			
POX	-	-	0.34ns	-0.54*	-	0.64**	0.52*	0.51*	0.63**	0.6**	1			
APX	-0.58*	-0.6**	0.67**	0.49*	-0.51*	-0.47*	0.51*	0.38ns	0.69**	0.79**	0.82**	1		
Sulforaphane	-	-0.54*	0.44ns	0.3ns	-	-	0.7**	0.38ns	0.44ns	0.67**	0.84**	0.72**	1	
	0.38ns	-	0.16ns	0.47*	0.18ns	-	0.65**	0.29ns	0.31ns	0.66**	0.58*	0.52*	0.58*	1
	0.34ns	0.61**	0.16ns	0.47*	0.18ns	0.19ns	0.65**	0.29ns	0.31ns	0.66**	0.58*	0.52*	0.58*	1

**, *, and ns: significant at 0.01, 0.05 level, and not-significant at 0.05.

To achieve more sophisticated results without the effect of other correlated variables on sulforaphane, the direct effect of all variables by holding all the effect of all other variable fixed using path coefficient analysis were implemented (*Table 4*). The path analysis showed that dry weight, H₂O₂ content, and then phenol content were the most influential variables positively affecting the content of sulforaphane, respectively. On the other hand, shoot length showed a negative coefficient in the path analysis. The coefficient of all other variables in the path analysis were negligible.

Principal component

Principal component analysis was carried out to assess the inter relationship among 14 measured traits in this study. The results showed that the first two PCs accounted for about 81 percent of total variability. Therefore, the biplot for the first two PCs in which both genotypes and parameters can be depicted in a same plot was prepared (*Table 5*). Dry weight and shoot length showed negative association with all other traits because they placed in a revers proximity to all other traits in the two dimensional graph of first two components. Matsuri and Castle Dome as two close genotypes were placed in the area near to the dry weigh and shoot length. Root length, sulforaphane content, phenol content, APX, SOD, POX, and CAT showed acute angles one another and also had positive high coefficients with both first and second components. Marathon was fixed solely in adjacent to these traits nearer to sulforaphane than all other genotypes. H₂O₂, MDA, DPPH, flavonoid content, and anthocyanin content showed positive high coefficients with first component but negative high coefficient regarding the second component. Genotypes Heraklion and Green Magic placed in near to these parameters, while Green Magic showed closest distance with DPPH and Heraklion with flavonoid content. Genotype Sacora showed a revers trend to all other genotypes considered in this study. Sacora obtained negative high coefficient with both components and was solely placed in a separate group (*Fig. 2*).

Table 4. Direct effect of each variable through sulforaphane content

Variable	Coefficient
DW	0.850992
Shoot length	-0.52174
Root length	0.041577
MDA	0.266981
H ₂ O ₂	0.848199
Phenol	0.771737
Flavonoeid	-0.04394
antocyanin	-0.16695
SOD	-0.03846
CAT	-0.32892
POD	-0.37341
APX	0.407253
DPPH	0.458507

Table 5. Proportion of each principal component and the related score of each trait

	PC1	PC2
Eigenvalue	7.9454	3.3633
Proportion	0.567529	0.240236
Cumulative	0.567529	0.807764
DW	-0.323	-0.186
Shoot length	-0.333	-0.062
Root length	0.228	-0.322
sulforaphane	0.172	-0.266
MDA	0.242	0.351
H ₂ O ₂	0.311	0.212
Phenol	0.204	-0.404
Flavonoid	0.262	0.23
Aantocyanin	0.328	0.173
SOD	0.247	-0.305
CAT	0.291	-0.085
POD	0.322	-0.109
APX	0.215	-0.364
DPPH	0.186	0.358

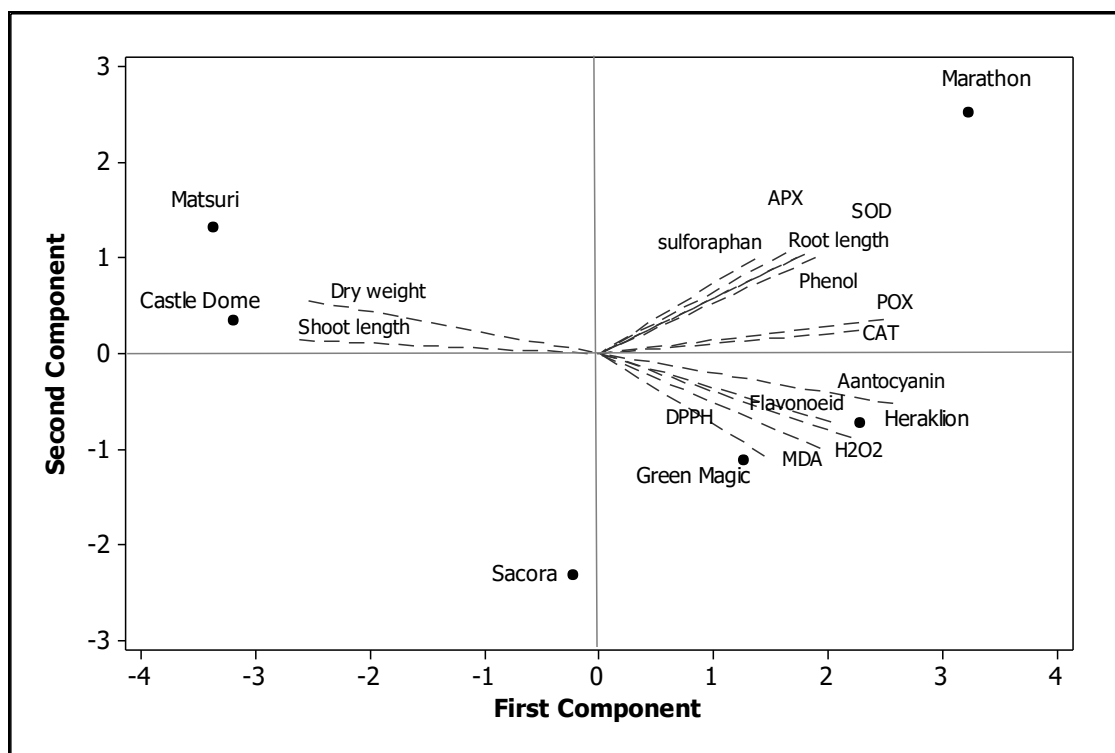


Figure 2. Biplot genotypes and traits using first two components of principal component analysis

Discussion

The results of the current study indicated that application of mannitol significantly decreased shoot dry weight and shoot length in all genotypes. Some genotypes had lower decrease rate in comparison with other genotypes regarding shoot length (Marathon genotype) and shoot dry weight (Marathon and Heraklion genotypes), however they might not show the highest values for these traits. Root length response to mannitol application in different genotypes was varied. Most of the genotypes showed increase in root length in response to mannitol application but some genotypes (Sacora and Heraklion) showed decrease. However, the rate of decrease in Heraklion was not significant. The reduction of shoot length and dry weight might be due to the falling off in the rate of photosynthesis under osmotic stress caused by mannitol application as a representative of drought stress. Chaves et al. (2009) reported that under drought stress the closure of stomata occurs to maintain the water content in the plant cells, which directly affects the growth rate of the plant's shoot. Also, the decline in the leaf area and other photosynthetic part of the plant is doubled with the stomatal closure and make photosynthesis rate to be even more reduced (Berger et al., 2016). On the other hand, the reaction of the root to drought stress might be different from what is predictable in shoot. It is well established that the root of the plants is seeking for water storage by increase its length and number of lateral roots and hairy roots (Chaves et al., 2009). The subject of root system architecture (RSA) under drought stress is revealing that under drought stress, the growth of the root can be increased, maintained at determined level, or even decreased; but the rate of growth in the shoot would normally decrease, and so that the overall growth of the root under drought stress would be always higher than the shoot (Uga et al., 2013). Furthermore, it has been stated by some researchers that tolerant genotypes are normally responding to drought in different ways which is one of the most effective way is to enlarge the root growth, while the susceptible genotypes are not able to manage this growth as properly as the tolerant genotypes (De Dorlodot et al., 2007).

In all genotypes, the content of total phenols, flavonoid, and anthocyanin increased. Marathon and Heraklion genotype showed relatively higher content of these three traits than other genotypes, while the Marathon genotype could be stand out as the highest responsive variety. Based on the study of Gawlik-Dziki (2008) phenolic compounds in vegetables, herb, and other edible plants can make them act as reducing agents and respond to oxidant compounds. In this situation, phenolic compounds can be used as hydrogen donators leading to quench the free radicals. Phenolic acids and flavonoids reportedly have shown strong antioxidant properties. They are able to react to radicals and catalyze oxidative reactions resulted in scavenging free radicals and stabilizing them in plants (Saed-Moucheshi et al., 2014b). Under drought stress, the content of free radicals or ROS in plant's cells is arisen which normally causes damages to cell apparatus; therefore, higher content of phenolic compounds indicates higher tolerance of genotype and plants to drought stress. Moreover, it has been reported that antioxidant activity of edible parts of the plants is linked with anti-mutagenic and anticancer genic properties. Subsequently, genotypes with higher content of natural antioxidants as phenolic compounds can be suggested for being used in nutritional programs and food content of human. In addition to broccoli, the extracts of varies fruits and vegetables have showed higher phenolic compounds in response to drought stress (Gawlik-Dziki, 2008).

MDA and H₂O₂ content are two important markers of oxidative stress induced in plants (Vosough et al., 2015). The lower the content of these two compounds under stress condition, the lower the damages to the plants under this situation (Saed-Moucheshi et al., 2014b). Genotypes and cultivars with lower content of MDA and H₂O₂ under stress condition would reveal higher tolerant to stress. In this study the content of both MDA and H₂O₂ increased under drought stress; however, the response rates of genotypes were varied. Castle Dome showed lower content of these compounds than other genotypes under all mannitol levels. In addition, the difference between mannitol levels in Marathon and Heraklion were relatively lower in compare to other genotypes. Under drought stress, the generation of ROS is increased from the steady level in the plant result in oxidative stress. H₂O₂ and other high reactive oxygen compounds can directly react with lipids and other apparatuses of cell membrane leading to lipid peroxidation and higher content of MDA (Hossain et al., 2015).

The activity of all assessed enzymatic antioxidant in this study increased in all genotypes in response to the increase of mannitol levels. Among all genotypes, Marathon showed relatively higher activity for this antioxidant under mannitol applications. In addition, Heraklion showed higher activity of this antioxidant under drought stress. Additionally, DPPH antioxidant activity indicated that the antioxidant activity of broccoli sprout is higher under drought stress condition. Marathon variety showed a high increase in the DPPH antioxidant activity in response to mannitol levels. The induced response of drought stress is to heighten the generation of ROS, which in turn induces the activity of antioxidant compounds and specially the enzymatic ones. In various studies, higher activity for enzymatic antioxidant under stress condition indicates higher ability of the plants in response to stress condition (Gupta and Huang, 2014; Hossain et al., 2015; Nakano and Asada, 1981). Based on this viewpoint, genotypes with higher activity of enzymatic antioxidant can be considered as higher tolerant genotypes to drought stress. Changes in the activity of antioxidant enzymes under various environmental stresses have been also reported (Baxter et al., 2014). In addition to phenolic compounds and enzymatic antioxidant, DPPH which itself is a radical compound normally acts as a scavenger for other dangerous radicals and therefore its content is a marker for higher antioxidant activity in plants.

The response of all genotypes to application of mannitol were to increase the content of sulforaphane but the rate of increase in some genotypes consist of Sacora and Castle Dome genotypes were low. Marathon and Green magic showed a great increase from zero to 88mM application of mannitol, but the difference between 88 and 176 mM mannitol remained non-significant. Under all mannitol levels and in total, the content of sulforaphane was highest in Marathon in comparison to other genotypes. Owing to the anticarcinogenic and anticancer functions, sulforaphane is an important compound in broccoli sprout. Since sulforaphane is a naturally available inducer of phase II enzymes in human and animal bodies that is able to detoxify cancer-causing chemicals, it would be appropriate to use such foods and plants part as broccoli, which are containing this compound to use its anti-cancerous properties. Additionally, sulforaphane has a cytoprotective effect against oxidative stress (Gu et al., 2011).

Considering the relationship among measured parameters, it is turned out that the shoot dry weight along with shoot length showed negative correlation with other measured traits. There were positive correlations among sulforaphane content, root length, total phenol content, SOD, APX, CAT, and POX. In addition, there were significant correlations among flavonoid content, anthocyanin content, MDA, H₂O₂. Gu et al.

(2011) investigated the effect of sucrose on the sulforaphane content in broccoli sprouts and stated that there was a significant correlation between glucosinolate, sulforaphane and antioxidant activity with phenol contents (137). Baczek-Kwinta et al. (2006) stated that SOD activity was different in different genotypes of cabbage under drought treatment. Similar results were reported by Joon-Ho and Sang (2015) and Dominguez-Perles et al. (2010) and indicated that there was no significant correlation between the sulforaphane content and the antioxidant activity of DPPH in Broccoli and other plants in the *Brassicaceae* family.

Furthermore, grouping of the varieties according to the measured parameters resulted in cluster Marathon genotype as separate group. In this study, Marathon showed relatively high values for the most of the measured traits, while the content of MDA and H₂O₂ were roughly lower than the other varieties.

Conclusion

The overall results of this study showed that the application of mannitol as a compound that decreases the osmotic potential of cells and simulates the drought stress in the *in vitro* condition, is a suitable compound for being used to induce the simulated drought stress in laboratory experiment. In addition, induced stress changed the content of measured traits and activities of enzymatic antioxidant. Mannitol stress reduced shoot dry weight and shoot length significantly for all genotypes but with the different rate. Marathon and Heraklion genotypes showed higher growth related traits under relatively all conditions. The response of marathon to mannitol application related to total phenolic content, flavonoid content, anthocyanin content, the activity of all enzymatic antioxidant, and the activity of DPPH was higher than the other genotypes. H₂O₂ and MDA content as the negative markers of oxidative stress were higher under mannitol stress condition. Results showed that sulforaphane content of the different genotypes under different stress levels was different. Mannitol stress increased the content of sulforaphane. Marathon showed the highest content of sulforaphane among all genotypes under both normal and stress conditions. Considering all these results together put us in touch with a suggestion that Marathon is the most proper genotypes for both cultivation and breeding aims among all studied genotypes. Heraklion genotype showed to be in second order regarding the content of measured traits after marathon with a high significant content of sulforaphane comparing other genotypes. Therefore, Heraklion might be a useful genotype being considered in line with marathon in breeding programs. Furthermore, both severe and moderate stresses increased sulforaphane amount along with phenolic compounds and antioxidant actives; therefore, it could be elucidated that moderate drought stress condition (88 mM concentration of Mannitol) which led to relatively low decreases in growth parameters comparing control condition, might be applicable for inducing higher content of sulforaphane and phenolic compounds in broccoli. More studies are needed to assess whether bioactive molecular accumulation really improve the biological / functional properties of these bioactive molecule enriched-sprouts.

Acknowledgements. The authors are grateful to the Agricultural and Natural Resources University, Sari, Mazandaran, Iran for providing the facilities for the investigations.

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