

In Vitro Effect of Salt Stress on *Prunus Microcarpa* (Potential of Being a Rootstock for All Stone Fruits) and Some Stone Fruit Rootstocks

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Abstract

In this study, in vitro salinity tolerance of three *Prunus microcarpa* genotypes (G1, G2 and G3) selected from South East Anatolia was investigated in comparison with some of standard rootstocks (MaxMa 14, Gisela 5, Pixy, SL-64, and Garnem). To determine salinity tolerance of rootstocks, explants were cultured in vitro on Nas and Read (2004) Medium (NRM) containing 0, 50, 100 or 150 mM NaCl. Four weeks after the application of salinity treatments shoot growth, chlorophyll a, chlorophyll b, Proline, Lipid Peroxidation (MDA), Hydrogen Peroxide (H₂O₂) were analyzed.

Salinity stress inhibited the growth and development of all genotypes in vitro and depending on genotypes the response to salinity stress varied. Under salinity stress particularly at 100 and 150 mM NaCl level Proline and H₂O₂ increased in all genotypes. *Prunus microcarpa*, especially G2 genotypes, seemed to be more tolerant to salinity stress compared to the genotypes of the other species tested.

Prunus microcarpa genotypes of Turkey origin tested in this study could be used as potential rootstocks in soils suitable for fruit culture, in lightly saline soils and when irrigation water is brackish, at least as safe as the other standard rootstocks tested ..

Keywords: *Prunus microcarpa*, salinity, rootstocks, Proline, H₂O₂, Lipid Peroxidation.

1. Introduction

The stone fruits attract great attention in the world in terms of economic value. In 2014, world annual production of stone fruit (apricot, cherry, nectarine, almond, peach and plums) exceeded 42.5 million metric tons (FAO). Demand for stone fruit production is increasing with population growth. Breeding in fruit rootstocks are working around the world as the resulting clonal rootstock that allows replicated in fruit breeding. Fruit cultivation has developed thanks to clonally propagated and dwarfed rootstocks obtained from fruit rooting studies in the world. Researchers apply rootstock for development paths to meet the increasing demand and to provide sufficient products to the market that can provide varieties that are better quality, dwarf, early maturing, and resistant to environmental conditions, pests, and diseases mentioned that *Prunus microcarpa* may have commercial potential for rehabilitation works and farming of types of stone fruits as especially dwarfing rootstock (Nas et al., 2012). Again it is stated that *Prunus microcarpa* may have potential to be a dwarfing rootstock for kinds of *Prunus* like cherry, plum, apricot and almond because of its creation of small crown (being dwarf) (Nas and Bölek., 2012). In this study, the tolerance of three genotypes (G1, G2 and G3) of important stone fruit rootstocks like MaxMa 14, Gisela 5, SL-64, Pixy and Garnem with Turkey originated *Prunus microcarpa* which is hopeful about being rootstock for kinds of stone fruits and still being worked on in present under conditions of *in vitro* against the stress of saltiness are researched.

2. Materials And Methods

2. 1. Plant Material and Culture Condition

The research is conducted on clone plants that are reproduced in tissue culture. Plant breedings of clone rootstocks are raised in flowerpots in greenhouse. The plants in flowerpots are used as explant source for *in vitro* works. In the result of the prolongation, shoots are subjected to superficial sterilization (disinfestation) after the leaves of new shoots belong to rootstocks were cut. Green steels are rinsed

three times by sterile purified water after being waited 15 minutes by occasionally mixed in 20% commercial bleach solution that contains 10 drops Tween-20. The green steels are cut from internodes after superficial sterilization process. Explants which are formed by Individual shoot-tip and nodal explants are cultured on the environment that gelled with $5.5 \text{ g}\cdot\text{L}^{-1}$ agar (Sigma, A-1296) and contains $1.0 \text{ mg}\cdot\text{L}^{-1}$ Benzyladenine (BA) + $0.01 \text{ mg}\cdot\text{L}^{-1}$ Indole-3- butyric acid (IBA) + $30 \text{ g}\cdot\text{L}^{-1}$ sucrose in 25 x 150 mm glass tubes containing 15 mL medium were subjected to 16 h light [under cool-white fluorescent light at $80 \text{ lmol m}^{-2} \text{ s}^{-1}$] and 8 h dark photoperiod in a growth chamber (23–25_C) for 3 weeks. Every 4–5 weeks, microbial contamination-free explants were subcultured on fresh medium in magenta containers. In the end of fourth subculture, the explants are cultured on NRM (Nas and Read, 2004) environment that gelled with $5.5 \text{ g}\cdot\text{L}^{-1}$ agar and contains 4 different salt concentrations (0, 50, 100, 150 mM NaCl) and $1.0 \text{ mg}\cdot\text{L}^{-1}$ BA + $0.01 \text{ mg}\cdot\text{L}^{-1}$ IBA + $30 \text{ g}\cdot\text{L}^{-1}$ sucrose on the purpose of being subjected to different salt concentrations in tissue culture environment. Four explants are out in culture plate (Magenta plate) are left for growing under 16/8 light/dark photoperiod and cold fluorescence ($80 \text{ }\mu\text{molm}^{-2}\text{s}^{-1}$) in $23 \pm 2 \text{ }^\circ\text{C}$ heated climate chamber. NaCl test is applied twice. The experiments were set up as completely randomized designs. Four Replications (magenta containers each containing 4 axillary buds) are used for each treatment and whole test is repeated twice.

2.2. Comparison of Growth and Development in In Vitro

The explants that belong to genotypes which are exposed to salt stress are evaluated by using key criterions like multiplication factor in *in vitro* and quality of microshoots (length, thickness, color, callus status, necrosis, end drying and explant aliveness).

2.3. Determination of Chlorophyll Content

Chlorophyll content was determined by taking fresh leaf samples (0.5 g) from randomly selected three plants per each replicate. The samples were homogenized with 5 mL of acetone (80%, v/v) using pestle and mortar and filtered through a filter paper (Whatman, No. 2). The absorbance was measured with UV/visible spectrophotometer (Optima SP3000-Plus, Tokyo, Japan) at 663 and 645 nm and chlorophyll contents were calculated using the equations proposed by Güneş et al., (2007) given below. Total chlorophyll content was expressed as Chl a + Chl b

$$\text{Chlorophyll a (mg/g)} = 11.75 \times A_{663} - 2.35 \times A_{645}$$

$$\text{Chlorophyll b (mg/g)} = 18.61 \times A_{645} - 3.96 \times A_{663}$$

2.4. Determination of Proline Contents

Proline content was determined according to the method described by Bates et al. (1973), 0.25 gr fresh leaf sample is extracted with 5 ml 3% (w/v) 5 sulfa-salicylic acid in porcelain mortar which is taken from alive plants that are accidentally chosen. The extracts are filtered by Whatman No:2 filter paper. 1ml is taken from filtered extract and mixed in test tube with 1 ml acid ninhydrin + 1 ml glacial acidic acid. The test tubes are cooled in ice for 1 minute after being waited in $100 \text{ }^\circ\text{C}$ water-bath for 1 hour. The reaction mixture was extracted with 4 mL toluene and the chromophore containing toluene was aspirated, cooled to room temperature, and the absorbance was measured at 520 nm with UV/visible. The standard curve is generated by the help of solutions contain Proline in variable density and the appropriate proline standards were included for the calculation of proline in the sample

2.5. Determination of Malondialdehyd (MDA) Contents

The last product of Lipid Peroxidation is Malondialdehyde (MDA) is calculated to determine the degree of Lipid Peroxidation that resultant on leafs in the end of saltness application. 0.5 gr powder leaf sample is taken and homogenized by adding 6 ml 10% trichloroacetic acid (TCA) and centrifuged at $10,000 \times \text{g}$ for 15 min, 2 ml supernatant is taken from up part of homogenate and added 2 ml from 20% TCS included 0.6% thiobarbituric acid (TBA). Than the mixture was placed in a water bath set at $100 \text{ }^\circ\text{C}$ for 20 min, cooled quickly, and centrifuged at $10,000 \times \text{g}$ for 10 min. Than the absorbance was determined by spectrophotometer at 450, 532 and 600 nm and the content of MDA concentration was determined according to the method of Zhang et al. (2005)

$$\text{MDA} = 6.45 \times (A_{532} - A_{600})$$

2.6. Determination of Hydrogen Peroxide (H₂O₂) Activity

The activity of hydrogen peroxide was determined according to the method described by Özden et al., (2009). 0.5 gr fresh leaf samples are centrifuged at 10,000 × g at 4 °C for 10 minutes after homogenized by pestling on ice in 3 ml 1% TCA that are taken from each application. Then, 0.75 10mM potassium phosphate buffer solution (pH: 7,0) and 1,5 ml 1 M KI are mixed with 0.75 ml supernatant. After being read the absorbance of mixture in spectrophotometer at 390nm. Appropriate H₂O₂ standards were included for the calculation of H₂O₂ in the samples

3. Statistical Analysis

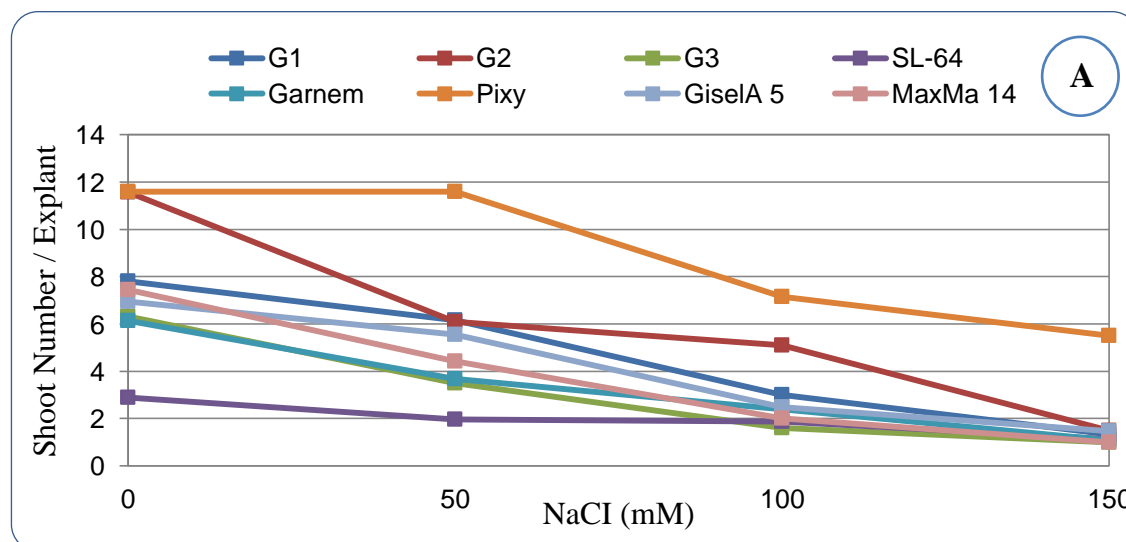
The research is settled down as being five each recidivisms in *ex vitro*, four each in *in vitro* based on randomized blocks (*in vitro*) and coincidence parcels (*ex vitro*). In the end of the test, obtained results are subjected to variance analysis by using SPSS statistical analysis packaged software(Standard version 16.0). Duncan’s multiple range test is made for reveal the importance degree of the differences between applications statistically. The statistical importance of the effects of factors on researched parameters mentioned on charts as followed: ÖD.: p<0.05 level unimportant, **: p<0.05 level important and ***: p<0.005 level important.

4. Results

4.1. Comparison of Growth and Development in In Vitro

There is observed a decrease in numbers of shoots with salt concentration in general of the genotypes of *Prunus microcarpa* and some Stone fruit rootstocks which are exposed to different salt concentrations *In vitro* circumstances (Figure. 4. 1. a). The highest decrease was seen in G2 genotype with 87,8% in shoot number for per explant in proportion to control group in 150 mM NaCl concentration as well as the increased salt concentration. The fewest decrease was seen on Pixy rootstock with 52,5%. Observed that the shoot number is not high in control group also when looking at SL-64 genotype. Likewise, there are recorded significant decreases for shoot numbers in 50, 100 and 150 mM concentrations in Colt cherry tree rootstock at *In vitro* conditions (Aricı and Eraslan, 2012). Significant decreases are reported for shoot numbers with salt stress in pear rootstock (Sotiropoulos et al., 2006), CAB-6P cherry rootstock (Chatzissavvidis et al., 2008), MM 106 apple rootstock (Bahmani et al., 2012).

The average shoot length seriously remained low *in vitro* depends on increased salt concentrations (figure 4.1.b). It is thought that this negative effect on shoot length resulted from extreme osmotic pressure of environment (Aricı and Eraslan, 2012). The highest decrease was realized in SL-64 genotype with in 150 mM NaCl when compare the control group in terms of shoot length. MaxMa14 rootstock with 66,6% followed this. Gisela 5 became the fewest decrease genotype by 26,6%. Chlorosis status in plants was clearly seen in parallel with the increased salt concentration. In particular, NaCl concentrations higher than 50 mM were found to cause severe chlorosis and damage . The end of the study, all the genotypes were browned in 100 and 150 mM NaCl concentrations and severe necrosis was observed at the leaf tips (figure 4.3). Developing in shoots on the medium containing one hundred and fifty (150) mM NaCl, was almost stopped, in fact many soots died.



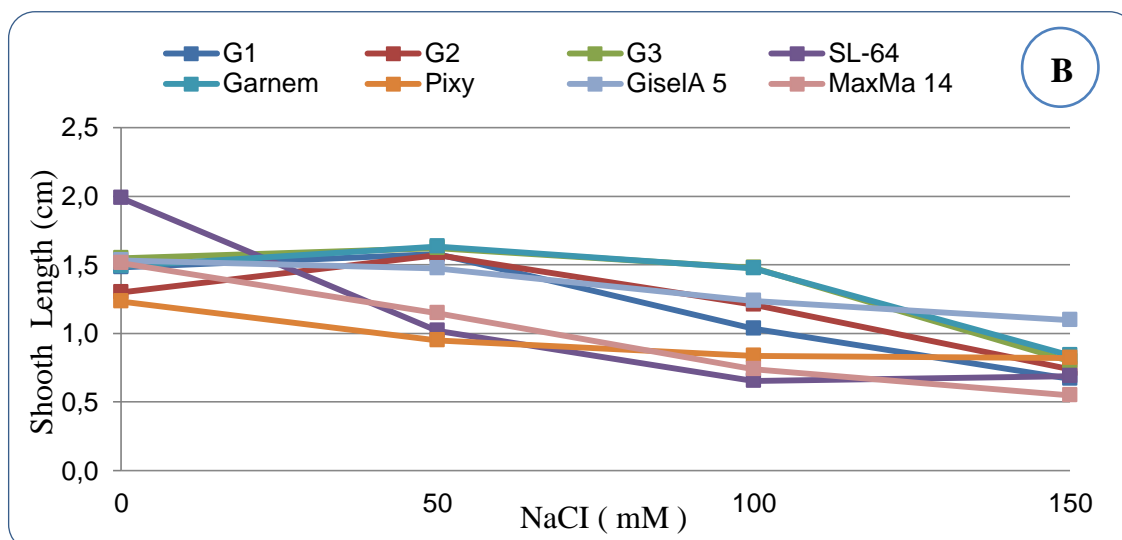


Figure 4.1. Mean numbers of shoots obtained from per cultured explant, (A) mean shoot lengths of rootstock (B) in salinity stress in in vitro

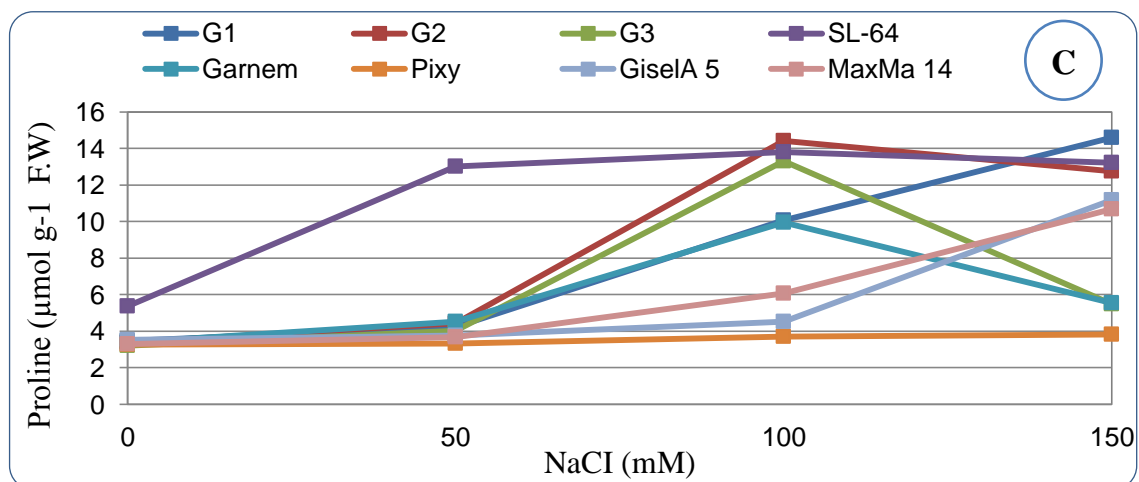
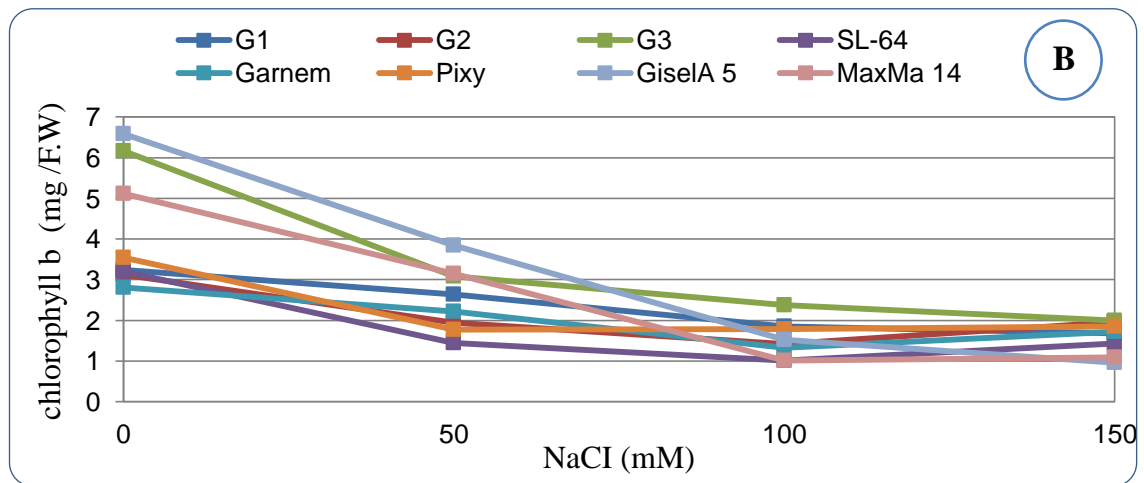
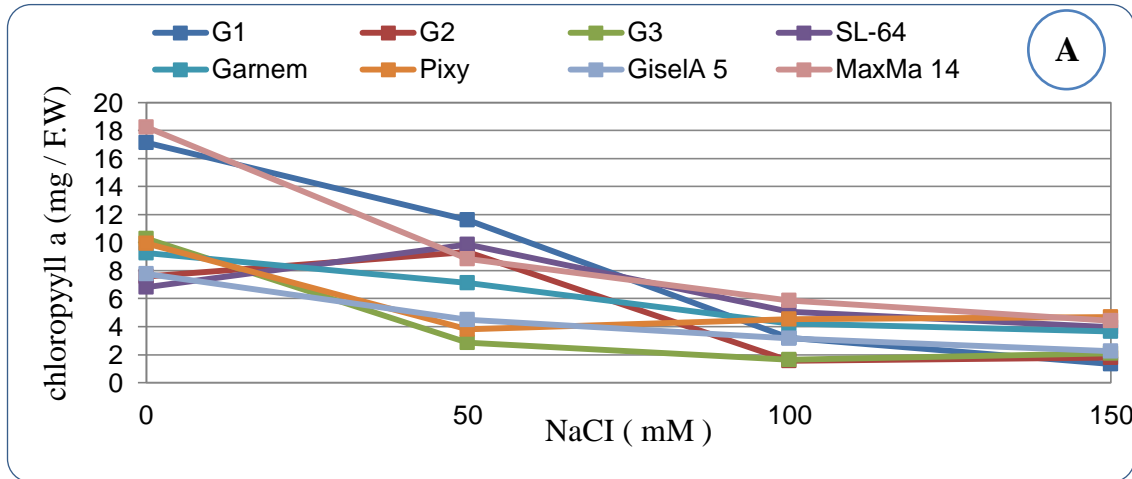
4.2. Determination of Chlorophyll a and b, Proline and Lipid Peroxidation (MDA) Content

There were seen significant decreases in general of genotypes of *Prunus microcarpa* and some stonefruit rootstocks that are exposed to different salt concentrations with salt concentrations in amount of chlorophyll a and b. The lowest chlorophyll a amount was seen in G1 genotype with 1,3 (mg/g) by 150 mM NaCl application. The highest chlorophyll a amount was seen in MaxMa 14 and G1 control groups respectively with 18.23 and 17.14 (mg/g). The highest decrease in chlorophyll a amount is seen respectively on genotypes of G1, G3 and G2 with 92.1%, 79.7%, 76.3% (Figure 4.2.a). This decline in chlorophyll a amount may result from genotypes of *Prunus microcarpa*'s trying to alive by dropping its leaves in stress conditions. Because, *Prunus microcarpa* generally drops its leaves in early periods of summer in natural spreading areas. Chlorophyll b amount is decreased as with being in chlorophyll a. The lowest chlorophyll b amount is observed in 150 mM NaCl concentration in genotypes of Gisela 5 with 0.96 mg/g fresh weight. The highest chlorophyll b amount is respectively observed in (6.58, 6.15, 5.11 mg/g) genotypes of Gisela 5, G3 and MaxMa 14 (Figure 4. 2.b).

In this study, proline amount increased by the increasing salt concentration. The highest proline amount (14,6 $\mu\text{mol g}^{-1}\text{F.W}$) was measured in genotype of G1 in 150mM NaCl application depends on the increasing salt concentration. There were seen regular increment in amount of proline in genotypes of Gisela 5 and MaxMa 14 with the increase in salt concentration besides G1 genotype. Observed declines in Proline amount of some genotypes with increasing salt concentration based on genotypes after a certain level. There was happened a linear increment in Proline amount in genotypes of G2, G3 and Garnem until 100mM NaCl application, but seen a decline in Proline amount after 100mM. The Proline amount in genotypes of G2, G3 and Garnem in 100mM NaCl concentration respectively became 14.41, 13.31 and 9.93 ($\mu\text{mol g}^{-1}\text{F.W}$). 5.35 ($\mu\text{mol g}^{-1}\text{F.W}$) Proline amount in control group of SL-64 genotype is measured as 13.02 ($\mu\text{mol g}^{-1}\text{F.W}$) at 50mM (Figure 4.2.c). The increment in amount of proline inhibits of being damaged of plant by salt by increasing the osmotic pressure in cell (Khalvati, 2001). The proline that commonly found in plants accumulate especially more than other amino acids and arranges the useable nitrogen amount (Abraham et al., 2003). Moreover, the Proline reduces the negative effect of NaCl on cell membrane by providing the membrane stability (Mansour, 1998; Yildiz et al., 2010).

It was observed that the salt stress is effective on MDA contents of genotypes which are exposed to salt stress. Firstly the increment was determined in generally MDA amount with the increasing stress. In some varieties, especially after application of 100mM NaCl, there was a decrease in the amount of MDA due to excessive damage to the plant. The highest MDA amount (2.44 $\mu\text{mol/g F.W}$) was seen in genotype of G2 in 100mM NaCl concentration. Especially in some genotypes (G2, Gisela5, Pixy, MaxMa 14), there were started decreases in some MDA amounts after 100mM NaCl. In

G1 genotype, the MDA amounts showed a constant decline by increasing salt stress when considering all genotypes in study, the lowest MDA amounts were observed in control group, the highest MDA amount was



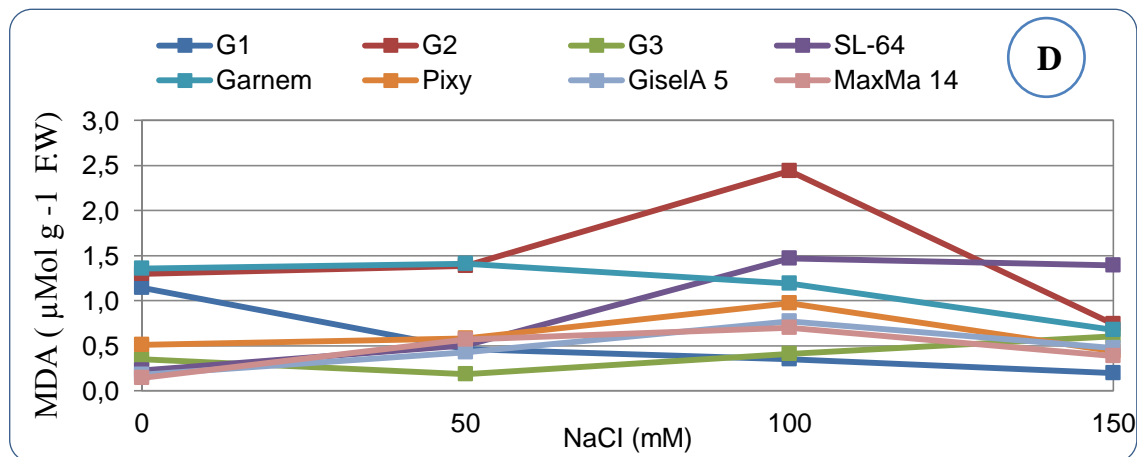


Figure 4.2. The effects of NaCl treatments on chlorophyll a (A), chlorophyll b (B), Prolin (C) MDA (D) contents in *in vitro* observed in plants on 100 mM NaCl concentration (figure 4.2.d). Negative effect of salt stress on MDA was observed in a research by Eraslan et al., (2009) where they applied salt on Gisela 5, MaxMa 14 and Colt cherry rootstocks and stated that 50 mM salt application enhances the MDA amount.

4.3. Determination of Hydrogen Peroxide (H₂O₂) Contents

There was happened an increment for H₂O₂ amount in all plants by increasing NaCl concentration when looking at the effect of NaCl concentration on Hydrogen Peroxide amount in explant leaves. Highest H₂O₂ amount is seen on G3 genotype in 150 mM NaCl concentration with 187.33 µMol g⁻¹ fresh weight. H₂O₂ amount shows increment in type of Pixy till 100mM NaCl concentration (59,8µMol g⁻¹) while shows a decrease in 150 mM NaCl concentration(45,6 µMol g⁻¹) in contrast with 100 mM NaCl concentration (figure 4.8). Observed an increment in H₂O₂ amount by increasing the salt concentration in general, but the increment in H₂O₂ amount remained low in genotypes of G2, MaxMa 14 and Pixy (figure 4.3) When evaluating the results collectively, the salinity stress is applied in *in vitro* conditions brought serious damages in much-used some drupe rootstocks and *P. microcarpa* genotypes. But the development differences between genotypes effected the getting harmed ratios by salt. The development of *Prunus microcarpa* genotypes (G1, G2, G3) which are in glasswort form are lower than other standard rootstocks. Thats why, discussed that getting visual harmed in green parts are stronger because of much salt amount per unit tissue area in these genotypes. Another reason could be the genotypes of *Prunus microcarpa* appeal to escape mechanism from stress by dropping their leafs in stress conditions. There are seen that respectively *P. microcarpa*, Pixy, MaxMa 14, Garnem genotypes become prominent in terms of tolerance for saltness stress. The result is concluded that the genotypes of *P. microcarpa* in this research are more tolerant than standard rootstocks or tolerant as much as these rootstocks for saltness stress. Especially it is possible to say the G2 genotype is more tolerant than other genotypes for salt stress.

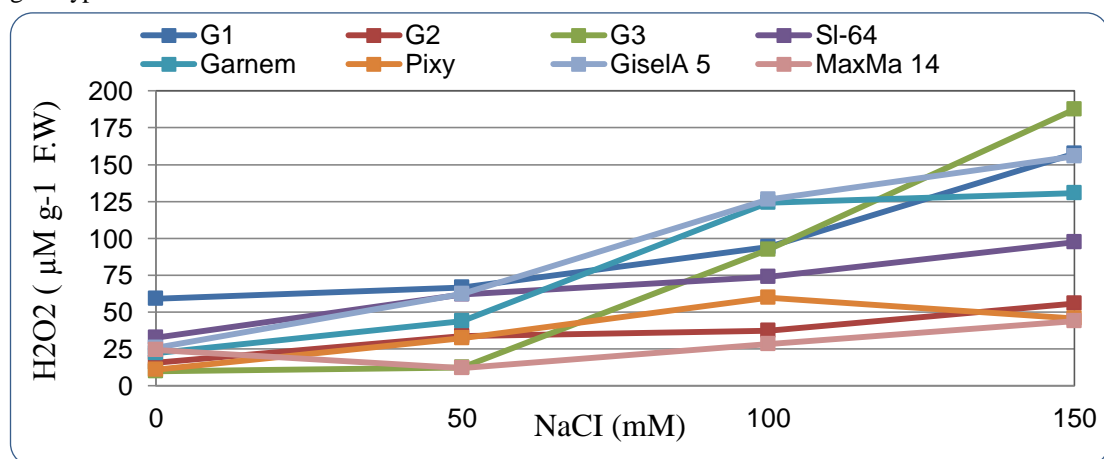


Figure 4. 3.The effects of NaCl treatments on H₂O₂ amount in *in vitro*

5.CONCLUSION

All rootstocks were affected by salinity stress. *P. microcarpa* in this research are more tolerant than standard rootstocks or tolerant as much as these rootstocks for salt stress. Especially it is possible to say the G2 genotype is more tolerant than other genotypes for salt stress. Consequently, the genotypes of *P. microcarpa* could be easily used as at least other standard rootstocks in partly saline soil or places where the irrigation water is partly saline.

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