MOLECULAR ANALYSIS OF PEROXIDASE GENES IN CHERRY ROOT STOCKS DEVELOPING *IN VITRO* STRESS CONDITIONS

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ABSTRACT

In this study, differences in peroxidase (POD) genes of cherry root stocks resistant to NaCl (0, 50, 100 and 150 mM), and boron (0, 1.5 and 3 mM) toxicity under in vitro conditions were investigated for 3 different cherry rootstocks [Cold (*Prunus avium* x P*runus pseudocerasus*), Gisela 5 (*Prunus cerasus* x *Prunus avium*) and MaxMa (*Prunus mahaleb* x P*runus avium*)]. The results showed that increasing concentrations of NaCl and boron reduced shoots growth and length. PCR analysis of cherry root stocks demonstrated bands of all primers and band sizes to be 80-1000 bp. Polimorphism was observed in base sequence of genes that play a role in peroxidase synthesis of cherry rootstocks cultivated in different salt concentrations, and it came up with a rate of similarity in the 0.88-0.97 range. Also, peroxidase enzyme synthesis showed differences in plants cultured in the MS medium with high concentrations of NaCl, and no groups could be determined among varieties.

KEYWORDS:
rootstock, peroxidase, salinity, *in vitro*, NaCl, boron

1 INTRODUCTION

Plants undergo stress conditions through their life. Development, metabolism and productivity of plants are severely affected by stress conditions. Major abiotic stresses are drought, undernourishment, over-nutrition, salinity, extremes in temperature, terrestrial and atmospheric pollution, and radiation which are all factors to limit yields of crop production [1]. Percentages of salt in irrigation water tend to increase due to supplies of salt and boron across the world with the result that dramatic losses of develop ment and productivity of crops appear. Almost all plants are exposed to oxidative damages under the above-mentioned stress circumstances. Mechanisms to avoid, eliminate or withstand stress conditions are of course varying, depending on their natural and environmental characteristics. Therefore, some plants are affected more severely by stress conditions whereas others manage to develop resistance to them. A series of variations have been observed in biochemical mechanisms of plants, resistant to stress, one of which is peroxidase enzyme which is intracellularly located, controlled by various genes and playing vital parts in such processes as relative interactions, pathogenic infections, insect tolerance, salt tolerance, aoxin deterioration, lignification in cellular walls, tissue suberisation and vegetative aging [2-7]. 25 multiple genes including peroxidase gene were found in herbaceous plants, and peroxidase alignments were discovered to be in the range of 53.1-90.9 [8]. Some studies reported that cherry rootstocks showed variations in levels of peroxidase enzymes, especially in stress factors [9-11]. In addition, molecular studies on peroxidase genes have hardly been encountered.

The present study was carried out to investigate differences in (POD) genes in cherry rootstocks resistant to salinity (NaCl) and boron (B) toxicity under *in vitro* conditions for three different cherry rootstocks.

2 MATERIALS AND METHODS

2.1 Plant samples

In this study, three different cherry rootstocks, namely Colt (*Prunus avium* x *Prunus pseudocerasus*), Gisela 5 (*Prunus cerasus* x *Prunus avium*), and MaxMa (*Prunus mahaleb* x *Prunus avium*) were used as plant material.

2.2 Disinfection of explants

In order to create surface disinfection, shoot tips were made by keeping them in 70% ethanol for 2 min, and later in a 5% NaCl solution containing 1-2 drops of Tween 20 for 10 min, followed by rinsing 3 times with sterile distilled water, for 5 min each.

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2.3 Nutrient media

Through every step of tissue culturing, Murashige and Skoog`s (MS) + 1 mg/L 6-benzyl aminopurine (BAP) + 0.02 mg/L α-naphthaleneacetic acid (NAA) hormones + 3% (w/v) sucrose with was used as nutrient medium [12]. Its pH was adjusted to 5.7.

2.4 Application of NaCl and boron to cherry stocks *in vitro*

Shoot tips (20 mm) of 3 different cherry rootstocks were used in tissue culture studies. NaCl (25 mM, 50 mM) and B (1.5 mM, 3 mM) were separately added in the MS medium as a stress factor. These salts were not added in the MS used for control in which plant material was placed. The experiments were established under coincidence plots design with 4 repetitions, each of which contained 15 explants.

2.5 DNA isolation

MaxMa, Gisela and Colt explants of 3 different cherry rootstocks cultured on MS media containing different boron and NaCl amounts were used for extraction and purification of genomic DNA according to [13], with some modifications. In brief, 100 mg tissue pieces were taken from plant leaves and placed in 1.5-ml Eppendorf tubes on which liquid nitrogen was added to pulverize them using plastic sticks. The prepared tissue suspension was incubated at 65 °C for 15-20 min, and an extraction buffer was added into the pulverized plant material. Then, phenol:chlorophorm:isoamyl alcohol in the ratio of 25:24:1, respectively, was added to the mixture with the plant suspension being gently mixed and centrifuged at 14,000 rpm for 10 min. The liquid part on top was transferred into another Eppendorf tube, and blended with RNase and proteinase K to incubate it in the incubator at 30 °C for 30 min. In order to precipitate the obtained DNA in a pellet form, 0.6 ml cold isopropanol and 0.3 ml sodium acetate solution were added to the solution. The solution was centrifuged with the DNA being accumulated in a pellet form. The DNA samples were stored in cold lockers at -20 °C until analysis.

2.6 PCR analyses

In this study 11 peroxidase primer pairs (POGP1, POGP2, POGP3, POGP6, POGP8, POGP9, POGP10, POGP11, POGP12, POGP13, POGP14) were used for PCR reaction [14]. A total volume of 15 µl (1.33 µM primer pair, 200 µM d NTPs, 1.5 µl 10 x PCR buffer, 2.5 mM $MgCL_2$, 1 U of Taq polymerase, 2.5 ng DNA) was run with one cycle for 3 min at 94 °C, in 34 cycles, 1 min at 94 °C, 1 min at 48-57 °C, 1 min at 72 °C, and, finally, 5 min at 72 °C. PCR product was visualized by staining with ethidium bromide in 2.5% agarose gel /1xTAE buffer).

2.7 Data analysis

Data were scored as 1 for presence, and 0 for absence of a DNA band, for each sample. The data matrix was entered by NTSYSpc Version 2.11f.

3 RESULTS

3.1 Application of NaCl and boron to cherry root stocks under *in vitro* conditions

In this study, development of cherry rootstocks in MS medium including different salt concentrations was observed. Developmental retardations, yellowing, browning and calli were found on cherry stocks. Over callus formation was seen in cherry rootstocks developed in the MS supplemented with 3 mM boron, with significant developmental retardation and dwarfism in them. (Figs. 1a, b).

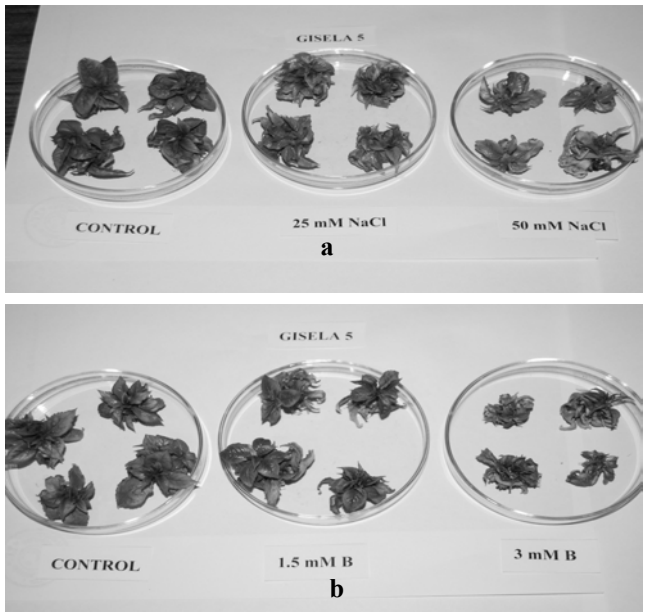

FIGURE 1 - Cherry root stocks applied with different concentrations of NaCl (a) and boron (b).

The higher percentage of NaCl and boron salts in the MS medium, the lesser lengths and numbers of shoots were observed (data not shown). Both salt and boron toxicity trials found the highest reduction in shoot length and number of MaxMa, followed by Colt and Gisela 5. As a result, the most resistant cherry rootstock to NaCl and boron toxicity was Gisela 5 while the most sensitive was MaxMa. Considering, salt concentration, decrease and yellowing in number of shoots, increase in callus formation is believed to have been due to toxicity of high salt concentration.

3.2 PCR analysis

PCR analysis using 11 peroxidase primer pairs was performed on all samples of the 3 different cherry rootstocks cultured in MS medium containing different NaCl and boron levels. All primers showed bands whose size ranged from 80 to 1000 bp (Fig. 2).

Results of PCR analysis indicated that cherry rootstocks cultured in different salt concentrations had a range of 0.88-0.97 in base alignment of the genes which played a role in peroxidase synthesis to determine polymorphism in the end. In spite of the fact that plants developed in

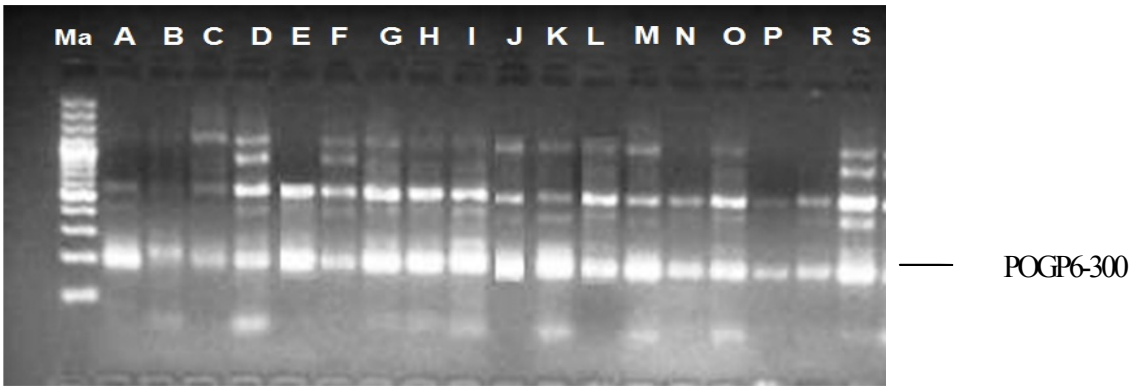

FIGURE 2 - POGP profiles amplified from DNA of cherry rootstock using primer POGP6; Ma: Marker; A: Control Gisela 5; B: 3 mM Boron Gisela 5; C: 1.5 mM Boron Gisela 5; D: Control MaxMa; E: 1.5 mM Boron MaxMa; F: 3 mM Boron MaxMa; G: Control Colt, H: 1.5 mM Boron Colt; I: 3 mM Boron Colt; J: Control Gisela 5; K: 25 mM NaCl Gisela 5; L: 50 mM NaCl Gisela 5; M: Control MaxMa; N: 25 mM NaCl MaxMa; O:50 mM NaCl MaxM; P: Control Colt; R: 25 mM NaCl Colt; S:50 mM NaCl Colt.

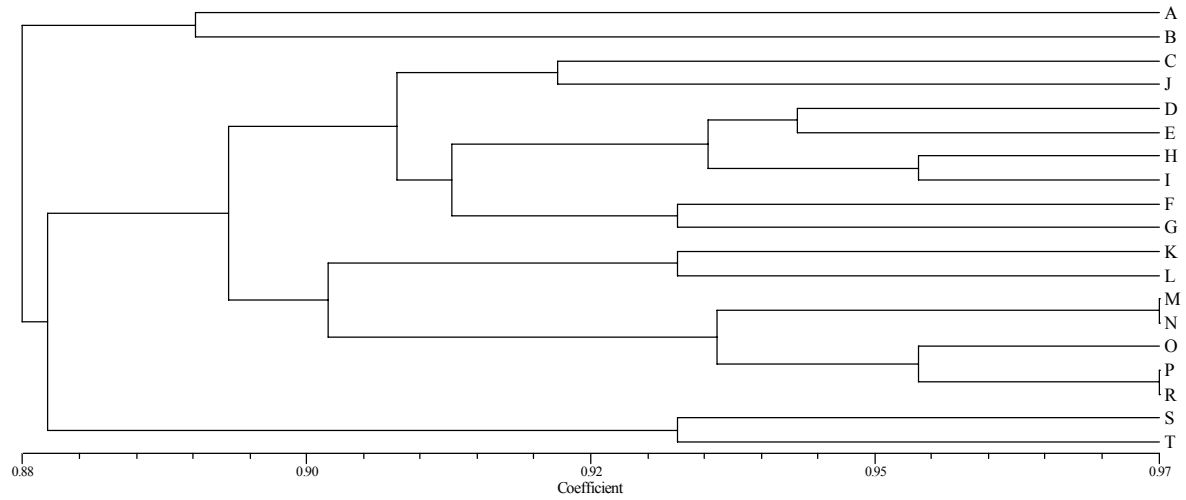

FIGURE 3 - Diversitiy of peroxidase genes by molecular analysis using POGP markers: The data matrix was entered by NTSYSpc Version 2.11f.Ma: Marker; A: Control Gisela 5; B: 3 mM Boron Gisela 5; C: 1.5 mM Boron Gisela 5; D: Control MaxMa; E:1.5 mM B Boron MaxMa; F: 3 mM Boron MaxMa; G:Control Colt, H:1.5 mM Boron Colt; I: 3 mM Boron Colt; J: Control Gisela 5; K: 25 mM NaCl Gisela 5; L:50 mM NaCl Gisela 5; M: Control MaxMa; N: 25 mM NaCl MaxMa; O:50 mM NaCl MaxM; P: Control Colt; R: 25 mM NaCl Colt; S:50 mM NaCl Colt

NaCl and boron-applied nutrient media, they showed differences in peroxidase enzyme synthesis, and specific groups were not established among varieties (Fig. 3).

4 DISCUSSION

The increasing NaCl and B concentrations decreased shoot length and shoot growth of cherry rootstocks in this study. Erturk et al. [11] cultured shoot tips of Gisela 5 cherry rootstock in MS medium supplemented with doses of 0, 50, 100 and 150 mM NaCl, and found that salt application decreased growth of shoot and chlorophyll content but did not affect water content. Molassiotis et al. [15] applied EM9 (*Mallus domestica* Borkh) in gradually increasing B doses of 0.1, 0.5, 1.0, 3.0 and 6.0 mM to an apple rootstock under *in vitro* conditions. The consequence of the trial pointed out that doses of 6 mM B caused oxidative strains, reducing dry weight of plants.

The result of PCR analysis, with NaCl application in cherry stocks cultivated in different salt concentrations, showed a similarity interval of 0.88-0.97 to determine polymorphism. Although polymorphism and variations were observed in peroxidase enzymes of cherry stocks developed at high salt concentrations, no different groups were found. It was clear from PCR analysis that bands were seen in all primers with band sizes of 80-1000 bp. Duroux and Welinder [10] examined peroxidase genes in 73 different plants, and finally reported that they had specific

and individual peroxidase genes. Turkish apple germ plasm was analyzed using 14 peroxidase specific primers [14].

These results demonstrate that primers targeting the peroxidase gene family can be used to study genotypic diversity and evolutionary relationships on an intra-and inter-specific basis. Peroxidase genes of monocotyledons and dicotyledons had different responses to biotic and abiotic factors, and 4 peroxidase genes existing in a range of 53.1-90.9 bp in the plants were reported by [10]. From the studies conducted in Iran [16], Syria [17], Nigeria [18], Spain [19] and Italy [20], it may be concluded that diversity relations of *Malus* spp. with peroxidase genes showed common DNA signs, alignment repetitions and random polymorphism. Molassiotis et al. [21] cultivated 106 mM of apple stocks in MS medium including mannitol, sorbitol, NaCl and KaCl, and observed increases in activities of non-enzymatic antioxidants, super oxidase dismutase (SOD) and peroxidase (POD) in leaves and sprouts of the plants. Antioxidant interactions caused by boron toxicity in two barley types were examined [22], Anadolu resistant and Hamidiye sensitive to boron, to determine plant dry weight, protein proline, MDA, hydrogen peroxide, membrane damage, super oxidase dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and gulutathion reductase (GR) enzyme activities in an experiment in which they applied 5 and 10 mM boric acid. In addition, [23] cultivated wheat plants in MS medium containing different NaCl concentrations. It was reported that an increase terminated SOD, CAT, and POD enzyme activities, as a salt stress response.

Considering the consequences of the study, we found that shoot tip developments of all rootstocks were negatively affected by salt stress and boron toxicity, with stress conditions creating significant variations involving physiological and biochemical structures of the rootstocks. Although there were differences and polymorphism of cherry rootstock peroxidases developed at high salt concentrations, no different groups were found. In determining tolerance to salinity and boron toxicity, development of shoot tips and POD enzyme activity were found to be suitable criteria of choice in mechanism of resistance to stress in primers.

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