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Comprehensive evaluation of tolerance to alkali stress by 17 genotypes of apple rootstocks

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Abstract

Alkaline soils have a great influence on apple production in Northern China. Therefore, comprehensive evaluations of tolerance to such stress are important when selecting the most suitable apple rootstocks. We used hydroponics culturing to test 17 genotypes of apple rootstocks after treatment with 1:1 Na_2CO_3 and $NaHCO_3$. When compared with the normally grown controls, stressed plants produced fewer new leaves, and had shorter roots and shoots and lower fresh and dry weights after 15 d of exposure to alkaline conditions. Their root/shoot ratios were also reduced, indicating that the roots had been severely damaged. For all stressed rootstocks, electrolyte leakage (EL) and the concentration of malondialdehyde (MDA) increased while levels of chlorophyll decreased. Changes in root activity (up or down), as well as the activities of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) were rootstock-dependent, possibly reflecting their differences in alkali tolerance. Using alkali injury index (AI), adversity resistance coefficients (ARC), cluster analysis, and evaluation of their physiological responses, we classified these 17 genotypes into three groups: (1) high tolerance: Hubeihaitang, Wushanbianyehaitang, Laoshanhaitang Ls2, Xiaojinbianyehaitang, and Fupingqiuzi; (2) moderate tolerance: Pingyitiancha, Laoshanhaitang Ls3, Hubeihaitang A1, Deqinhaitang, Balenghaitang, Maoshandingzi, Shandingzi, and Xinjiangyepingguo; or (3) low tolerance: Pingdinghaitang, Hongsanyehaitang, Xiaojinhaitang, and Sanyehaitang. These results will significantly contribute to the selection of the most suitable materials for rootstocks with desired levels of tolerance to alkali stress.

Keywords: alkali stress, apple rootstock, alkali tolerance

1. Introduction

Soil salinization is a widespread environmental problem. Among all of the areas cultivated around the world, approximately 0.34×10^9 ha (23%) are saline and 0.56×10^9 ha (37%) are sodic (Tanji 1990). In northwestern China, reduced rainfall combined with greater soil evaporation led to soil alkalization. This important agricultural contaminant has complex impacts on plant growth, metabolism, and economic yields. Whereas salt stress refers to the challenges

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associated with neutral salts, the term alkali stress applies to scenarios involving alkaline salts (Shi and Sheng 2005; Shi and Wang 2005; Yang *et al.* 2008a). The latter has more severe effects on plant development (Shi and Yin 1993; Yang *et al.* 2008a, b). In addition to osmotic stress and ion-induced injuries (Munns 2002), plants under alkali stress must cope with high pH levels (Keutgen and Pawelzik 2009) that not only affect normal root functioning in the rhizosphere and destroy cell structure, but also reduce their absorption capacity due to diminished root respiration (Yang *et al.* 2008a, b). These factors lead to reduced root growth (Bingham and Stevenson 1993; Alhendawi *et al.* 1997) and the precipitation of other mineral ions, which decreases the availability of essential nutrients (Shi and Zhao 1997; Li *et al.* 2010; Gong *et al.* 2014). Shoot development is also indirectly but significantly inhibited because stressed plants produce smaller leaves (Pearce *et al.* 1999). Consequently, growth and photosynthesis are negatively affected by alkaline conditions (Yang *et al.* 2009).

The effects of HCO_3^- have been investigated with several commercial crops, including bean (Valdez-Aguilar and Reed, 2008), cucumber (Rouphael *et al.* 2010), wheat (Yang *et al.* 2008b), maize, barley (Alhendawi *et al.* 1997), sunflower (Shi and Sheng 2005), tomato (Navarro *et al.* 2000; Bialczyk *et al.* 2004), pea (Zribi and Gharsalli 2002), and rice (Hajiboland *et al.* 2005). Decreased Na^+ exclusion and ion imbalances associated with alkali stress, along with an elevated pH, lead to toxic accumulations of Na^+, which induces osmotic stress (Yang *et al.* 2007). When the pH of a saline growth medium is increased, cell membranes are more severely damaged. However, research with roots from alkali-stressed tomato has shown that the activities of superoxide dismutase (SOD) and catalase (CAT), combined with the ascorbate-glutathione cycle, play important roles in alleviating oxidative stress (Gong *et al.* 2014). Because the physiological responses to these combined stresses are regulated by different pathways in various species, it is important that investigations should focus on how plants can adapt to high alkali stress over an entire life cycle.

Apple (*Malus*) is one of the most important temperate fruits, but its productivity is adversely affected by many environment factors. The arid and semi-arid regions in China are the optimal ecological zones for this crop because environmental conditions such as wide temperature fluctuations between day and night, deep soils, and adequate light support cultivation. However, increased levels of pH in the soil (i.e., alkali stress) influence fruit yield and quality, particularly in conjunction with drought and salt stresses in those regions, which further seriously affects the apple fruit industry. The susceptibility to damage from alkali stress is determined by the degree of tolerance by an apple rootstocks. Although dwarfing cultivation methods are becoming more popular, their need for certain environmental conditions and orchard management techniques mean that such practices are not necessarily suitable for arid and semi-arid apple production areas in other parts of China. Because dwarfed interstocks are still utilized there on a large scale, breeders require more comprehensive information about the characteristics of tolerant rootstocks. Abundant germplasm resources of apple rootstocks with strong tolerance to various environmental challenges are already available in China, but the alkali tolerance of some apple rootstocks has not yet been fully evaluated. Therefore, it is critical that researchers screen for that desirable characteristic in order to improve regional recommendations for appropriate rootstocks. Here, we examined the seedlings of 17 genotypes of apple rootstocks (Table 1) to determine their relative alkali tolerance based on growth parameters and morphological indexes.

Table 1 Apple rootstocks evaluated for alkali tolerance

Code	Genotype	Species	Apomictic	Origin in China
1	Pingyitiancha	*Malus hupehensis* Rehd.	Yes	Pingyi, Shandong
2	Laoshanhaitang Ls3	*M. hupehensis* Rehd.	Yes	Qingdao, Shandong
3	Wushanbianyehaitang	*M. toringoides* Rehd. Hughes	Yes	Xingcheng, Liaoning
4	Laoshanhaitang Ls2	*M. hupehensis* Rehd.	Yes	Qingdao, Shandong
5	Hubeihaitang A1	*M. hupehensis* Rehd.	Yes	Qingdao, Shandong
6	Hubeihaitang	*M. hupehensis* Rehd.	Yes	Xingcheng, Liaoning
7	Deqinhaitang	*M. sikkimensis* Koehne.	Yes	Xingcheng, Liaoning
8	Xiaojinbianyehaitang	*M. toringoides* Hughes.	Yes	Xingcheng, Liaoning
9	Pingdinghaitang	*M. micromalus* Makino.	No	Huailai, Hebei
10	Hongsanyehaitang	*M. sieboldii* Rehd.	Yes	Xingcheng, Liaoning
11	Balenghaitang	*M. robusta* Rehd.	No	Huailai, Hebei
12	Xiaojinhaitang	*M. tiaojinensis* Cheng et Jiang.	Yes	Xingcheng, Liaoning
13	Maoshandingzi	*M. mandshurica* Komarov.	No	Xingcheng, Liaoning
14	Sanyehaitang	*M. sieboldii* Rehd.	No	Qingdao, Shandong
15	Shandingzi	*M. baccata* Borkh.	No	Qingyang, Gansu
16	Fupingqiuzi	*M. prunifolia* Borkh.	No	Fuping, Shaanxi
17	Xinjiangyepingguo	*M. sieversii* Roem.	No	Yili, Xinjiang

2. Materials and methods

2.1. Plant materials and experimental design

The 17 genotypes of apple rootstocks evaluated here originated from different climate regions within China (Table 1). 4 materials, codes 2, 4, 5, 14, were provided by Sha Guangli (Qingdao Academy of Agricultural Science, Qingdao, China) and 7 materials, codes 3, 6, 7, 8, 10, 12, and 13, were provided by Wang Kun (Chinese Academy of Agricultural Sciences, Xingcheng, China). For the hydroponics experiments, all seeds were stratified in sand at 0 to 4ºC for 60 d. Afterward, seeds of uniform size and stage of germination were placed in plastic pots (9 cm×9 cm×10 cm; 3 seeds each) that contained sand. To ensure that the seedling responses were consistent when exposed to alkali treatment, we planted three times as many seeds as were needed for these trials. The pots were then moved outdoors for 60 d under natural lighting and temperature conditions in an experimental field. Beginning at the second true-leaf stage, the seedlings were irrigated every 4 d with a 1/2-strength Hoagland nutrient solution (Hoagland and Arnon 1950). Hydroponics culturing techniques were applied as described by Bai *et al.* (2008). Seedlings of similar size (with 6–8 leaves) were selected after 60 d of growth in the outdoor pots and transferred to plastic basins (52 cm×37 cm×15 cm), each containing 13 L of a 1/2-strength Hoagland nutrient solution. The basins were placed in a greenhouse under conditions of natural light and temperatures of 23 to 25°C/15 to 18°C (day/night). The nutrient solution was aerated each hour with an air pump and the dissolved oxygen concentration was maintained at 8.0 to 8.5 mg L^{-1}. The pH of the nutrient solution was adjusted to 6.0±0.1 by adding diluted H_2SO_4 and the solution was refreshed every 4 d.

The stress treatments were initiated after 10-d pre-cultivation (described above). Seedlings of each genotype were randomly assigned to two groups (n=54 plants per treatment): (1) control, standard 1/2-strength Hoagland nutrient solution+H_2SO_4 to adjust pH; and (2) alkali treatment, 1/2-strength Hoagland nutrient solution+Na_2CO_3 and $NaHCO_3$ (1:1 molar ratio). The pH of the control and treatment solutions was measured with a digital pH meter and then adjusted each day to 6.0±0.1 and 8.5±0.1, respectively.

2.2. Assay of alkali injury

On days 5, 10, and 15 of the experiment, 54 seedlings were randomly selected per treatment to investigate the extent of injury related to alkali stress. The presence of leaf necrosis was rated along a scale from 0 to 4. 0, no symptoms or lesions; 1, a few young leaves were yellow, but the yellow area was smaller than the green area; 2, a few young leaves were yellow, and the yellow area was larger than the green area and the leaf tips or edges were red; 3, most leaves were yellow, but the red area was larger than the yellow area; and 4, most older leaves were yellow while the new leaves were red.

The alkali injury index (AI) was calculated as AI=(0×S0+1×S1+2×S2+3×S3+4×S4)/54, where S0, S1, S2, S3, and S4 represented the numbers of plants receiving scores of 0, 1, 2, 3, and 4, respectively; and 54 was the total number of plants investigated per genotype per sampling date.

2.3. Assays of plant growth and adversity resistance coefficients (ARCs)

On d 15 of the experiment, 15 seedlings per treatment were harvested and washed with water before shoot and root lengths were measured and the number of new leaves was manually counted. After fresh weights (FWs) were determined individually for the root and shoot portions, their dry weights (DWs) were obtained by oven-drying the samples at 105°C for 15 min and then at 70°C for 24 h.

Adversity resistance coefficients were computed based on seven growth parameters: number of new leaves, root length, shoot length, root FW, shoot FW, root DW, and shoot DW. Corresponding values were used in these calculations, with ARC=Treatment value/Control value for each factor.

2.4. Assays of chlorophyll concentrations, electrolyte leakage, root activity, and levels of malondialdehyde

The leaves and roots were collected from 10 plants per treatment to analyze various physiological indexes. Briefly, 80% acetone was used for chlorophyll extractions and the concentrations were determined spectrophotometrically as described by Arnon (1949).

Electrolyte leakage (EL) in the leaves was measured according to the methods of Dionisio-Sese and Tobita (1998) by placing 10 uniformly sized leaf pieces (1 cm×1 cm) in a test tube containing 10 mL of distilled water. The initial electrical conductivity (EC0) was determined by using another test tube that contained 10 mL of distilled water but no leaf tissue. All EL measurements were made with an electrical conductivity analyzer (DDS-307; Shanghai Precision Scientific Instrument Co., Ltd., China). After 3.5 h of incubation in a water bath at room temperature (RT), the second round of electrical conductivity (EC1) of the medium was measured. Samples were then autoclaved at 100°C for 20 min to release all electrolytes and then cooled to RT before measuring the final electrical conductivity (EC2). Afterward, the percentage of electrolyte leakage was calculated as EL= (EC1–EC0)/(EC2–EC0)×100%.

Root activity was determined by the triphenyltetrazolium chloride (TTC) reduction method, as described by Comas

et al. (2000). Briefly, 0.5 g of root tissue was added to 10 mL of 0.1 mmol L^{-1} sodium phosphate buffer (pH 7.5) containing 1% (w/v) TTC. The reaction began in a water bath (37°C) under darkness for 2.5 h and was then stopped by the addition of 2 mL of 1 mol L^{-1} H_2SO_4. The red product of the reaction (triphenyl formazan) was extracted using acetic ether and root activity was determined as the reduction of TTC at 485 nm.

Lipid peroxidation was evaluated in terms of the total content of thiobarbituric acid-reactive substances, and was indirectly expressed as the malondialdehyde (MDA) concentration, as defined by Paradiso *et al.* (2008).

2.5. Assays of enzyme activity

Three essential enzymes — peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) — were extracted according to the methods of Li *et al.* (2008). Briefly, 0.5 g of frozen leaf tissue was ground in a chilled mortar with 5% (w/v) polyvinylpyrrolidone and homogenized with 1.2 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 7.0) containing 1 mmol L^{-1} EDTA-Na_2 and 0.3% Triton X-100. The homogenate was centrifuged at 13 000×g for 20 min at 4°C and the supernatant was used for the following assays.

Activity of SOD was determined as described by Li *et al.* (2008), with one unit defined as the amount of enzyme required to cause 50% inhibition of the reduction of nitrotetrazolium blue chloride (NBT) as monitored at 560 nm. POD activity was assayed at 470 nm using the reaction substrates of hydrogen peroxide and guaiacol (Gao 2006), while CAT activity was determined by monitoring the decrease in absorbance at 240 nm (Li *et al.* 2015).

2.6. Statistical analysis

All data were statistically analyzed with Excel and SPSS 16.0 software. Values were considered significantly different at $P<0.05$.

3. Results

3.1. Alkali Index and stress tolerance

After 5 d of alkali treatment (1/2-strength Hoagland nutrient solution+1:1 mixture of Na_2CO_3 and $NaHCO_3$; pH 8.5), the leaves of stressed seedlings from most rootstocks showed damage, with the exception of Laoshanhaitang Ls2, Hubeihaitang, and Deqinhaitang. By day 10, plants exhibited severe symptoms, with an average value of 2.39 calculated for the injury index, or AI (Table 2). After 15 d, the level of damage varied among genotypes, with AI values ranging from 0.30 to 2.96, which reflected different degrees of tolerance to alkali stress. The least damaged rootstocks were Hubeihaitang and Wushanbianyehaitang, indicating that they were the most tolerant. With AI values >2, the rootstocks with the lowest tolerance were Shandingzi (2.02), Sanyehaitang (2.14), Pingdinghaitang (2.44), Hongsanyehaitang (2.67), and Xiaojinhaitang (2.96).

Table 2 Alkali injury index (AI) and tolerance to alkali stress by 17 genotypes of apple rootstocks

Code	AI[1)]			Tolerance[2)]
	5 d	10 d	15 d	
6	0.00	0.20	0.30	H
3	0.04	0.27	0.43	H
4	0.00	0.30	0.72	H
16	0.04	0.31	0.83	H
8	0.19	0.63	0.93	H
17	0.50	0.74	1.26	M
5	0.08	1.00	1.35	M
7	0.00	0.33	1.43	M
13	0.16	1.22	1.56	M
2	0.19	1.02	1.64	M
11	0.36	0.71	1.65	M
1	0.71	1.68	1.84	M
15	0.22	1.87	2.02	L
14	0.72	0.90	2.14	L
9	0.49	1.98	2.44	L
10	0.70	2.50	2.67	L
12	0.89	2.39	2.96	L

[1)] AI=(0×S0+1×S1+2×S2+3×S3+4×S4)/54. S0, S1, S2, S3, and S4, degree of damage, ranked on scale from 0 to 4, respectively; 54, total number of investigated plants.
[2)] H, high tolerance; M, moderate tolerance; and L, low tolerance. The same as in Table 3.

3.2. Growth parameters

The values measured for all growth indicators were decreased to some extent for stressed seedlings (Fig. 1-A–E). After 15 d of treatment, the number of new leaves, root and shoot lengths, and total DW accumulations were reduced, and the reduction varied among rootstocks. This reflected differences in the capacity of each genotype to tolerate such challenges. For example, alkali treatment had little impact on leaf numbers or shoot and root lengths in samples from Laoshanhaitang Ls2 and Hubeihaitang (Fig. 1-A–C). However, Pingdinghaitang and Sanyehaitang were significantly affected, and plants had 65 and 55% fewer leaves, 72 and 67% shorter roots, and 55 and 72% shorter shoots, respectively, when compared with the normally grown control plants (i.e., 1/2-strength Hoagland nutrient solution and a pH of 6.0, as adjusted with H_2SO_4). All rootstocks except Hubeihaitang and Fupingqiuzi showed small increments in total FW but only slight changes in total DW when compared

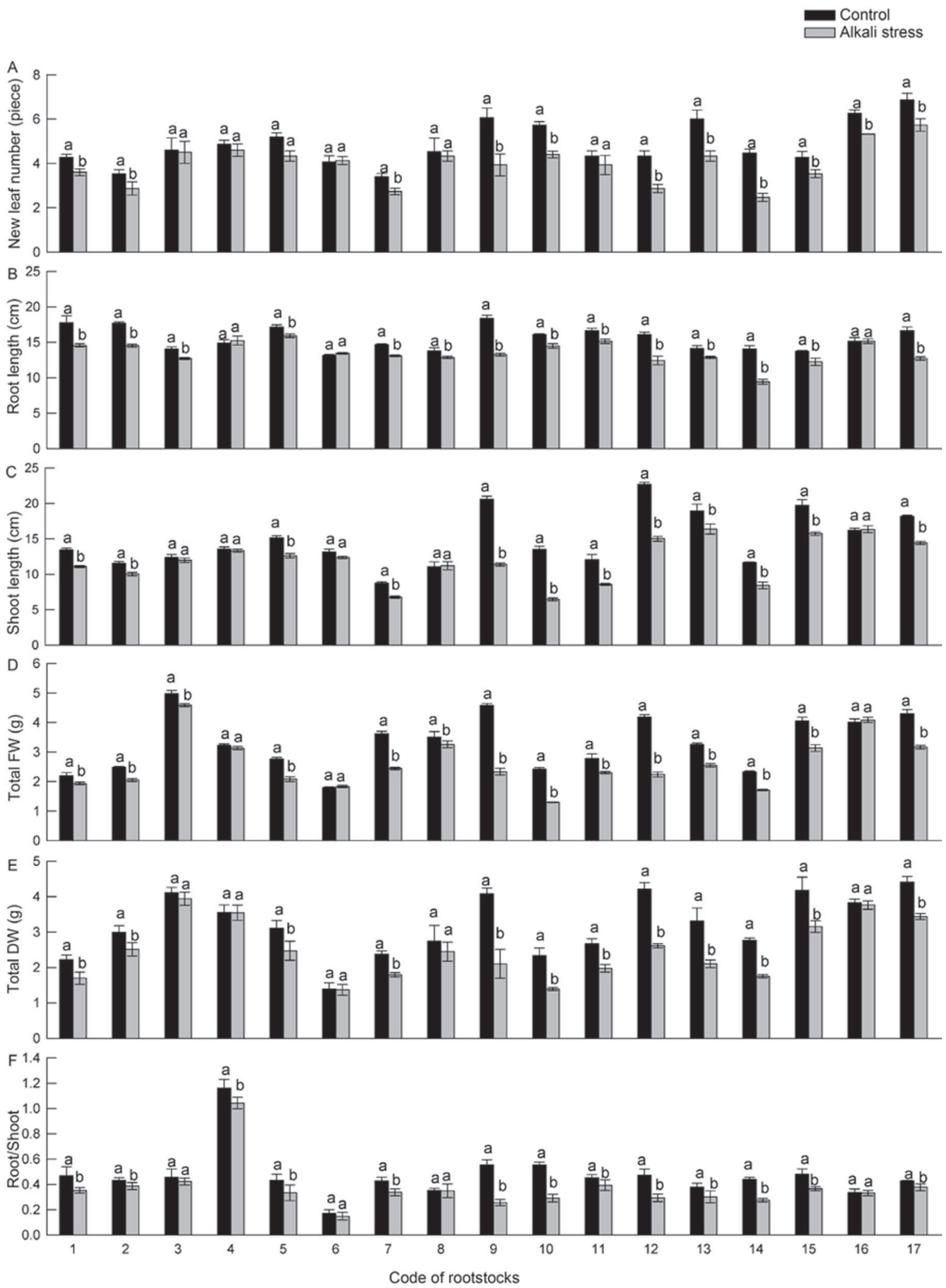

Fig. 1 Comparisons of growth parameters among 17 genotypes of apple rootstocks during 15 d of alkali stress. A, number of new leaves. B, root length. C, shoot length. D, total FW. E, total DW. F, root/shoot ratio. Data are means±standard errors (n=15). For each genotype, values not followed by same letters indicate significant differences between control and alkaline treatments at P<0.05, based on LSD tests. Rootstock codes are explained in Table 1. The same as in Fig. 3.

with the control. The remaining rootstocks responded to varying degrees, i.e., reductions in total FW ranged from 2.9 to 49.1% and total DW declined by 0.3 to 48.9% relative to the control (Fig. 1-D, E). For all other growth parameters, the roots appeared to be more sensitive than the shoots to alkali stress (Fig. 1-F).

3.3. ARCs and cluster analysis of alkali tolerance

ARCs varied among rootstocks (Table 3). Higher values indicated greater tolerance. For examples, the average ARC did not differ significantly for the highly tolerant Laoshanhaitang Ls2 (0.99), Hubeihaitang (0.99), Fupingqiuzi (0.98), Wushanbianyehaitang (0.95), and Xiaojinbianyehaitang (0.93). For more vulnerable rootstocks, including Pingdinghaitang, Hongsanyehaitang, Xiaojinhaitang, and Sanyehaitang, the average ARCs were less than 0.78. Compared with the very tolerant rootstocks with shoot length ARCs≥0.94 and shoot DW ARCs≥0.95, the rootstocks with low tolerance had root length ARCs≤0.72 and shoot DW ARCs≤0.66. The remaining rootstocks were classified as moderately tolerant to alkali stress, and their ARC values did not differ significantly for each parameter. Therefore, based on cluster analysis of new leaf numbers, root and shoot lengths, FWs, and DWs, we categorized these rootstocks into three groups: (1) high tolerance: Hubeihaitang, Wushanbianyehaitang, Laoshanhaitang Ls2, Xiaojinbianyehaitang, and Fupingqiuzi; (2) moderate tolerance: Pingyitiancha, Laoshanhaitang Ls3, Hubeihaitang A1, Deqinhaitang, Balenghaitang, Maoshandingzi, Shandingzi, Xinjiangyepingguo, and Sanyehaitang; and (3) low tolerance: Pingdinghaitang, Hongsanyehaitang, and Xiaojinhaitang (Fig. 2).

3.4. Physiological responses to alkali stress

Alkali treatment decreased the concentrations of total chlorophyll (Chl *t*) in Pingdinghaitang, Hongsanyehaitang, Xiaojinhaitang and Sanyehaitang to 91.0, 75.0, 78.4, and 74.4%, respectively, of the level measured in the untreated control. However, levels of Chl *t* in the highly tolerant rootstocks, especially Hubeihaitang, Xiaojinbianyehaitang, and Fupingqiuzi, were only slightly affected (Fig. 3-A). Similar trends were noted for chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) (Fig. 3-B, C).

Plant roots are sensitive to stress signals and changes in those systems directly determine overall growth. Here, root activity was significantly increased (17.3–92.62%) in the more tolerant rootstocks but was significantly reduced (29.46–47.46%) in rootstocks with lower tolerance. As important indicators of stress-related damage in leaf membranes, EL and concentrations of MDA were decreased after 15 d of alkali treatment when compared with the control. Enzyme activities differed significantly among genotypes. Levels of POD, SOD, and CAT were markedly increased in most rootstocks, especially for the more tolerant Laoshanhaitang Ls2, Fupingqiuzi, Wushanbianyehaitang, and Xiaojinbianyehaitang, but were only slightly elevated or even reduced in the less tolerant Pingdinghaitang, Hongsanyehaitang, Xiaojinhaitang, and Sanyehaitang (Table 4).

Table 3 Adversity resistance coefficients (ARCs) and alkali tolerance by 17 genotypes of apple rootstocks under 15 d of alkaline treatment

Code	New leaf number	Root length	Shoot length	Root DW	Shoot DW	Root FW	Shoot FW	Average ARC[1)]	Tolerance
4	0.95±0.07	1.02±0.01	0.98±0.01	1.00±0.08	1.00±0.05	0.98±0.06	0.97±0.02	0.99±0.02 a	H
6	0.97±0.04	1.02±0.01	0.94±0.03	0.98±0.29	1.02±0.08	1.00±0.13	1.03±0.01	0.99±0.03 a	H
16	0.85±0.02	1.00±0.03	1.01±0.04	0.99±0.05	0.97±0.04	0.98±0.04	1.04±0.09	0.98±0.06 a	H
3	0.99±0.12	0.91±0.02	0.97±0.04	0.95±0.03	0.97±0.06	0.87±0.05	0.97±0.03	0.95±0.04 a	H
8	0.97±0.09	0.93±0.04	1.02±0.11	0.99±0.07	0.75±0.24	0.88±0.06	0.98±0.10	0.93±0.09 a	H
1	0.84±0.01	0.82±0.05	0.82±0.02	0.76±0.10	0.79±0.12	0.85±0.09	0.90±0.03	0.83±0.04 b	M
2	0.81±0.09	0.82±0.01	0.87±0.04	0.87±0.18	0.77±0.09	0.89±0.05	0.80±0.03	0.83±0.04 b	M
5	0.83±0.06	0.93±0.03	0.83±0.01	0.82±0.30	0.77±0.13	0.81±0.06	0.73±0.04	0.82±0.06 b	M
11	0.91±0.14	0.91±0.03	0.71±0.04	0.71±0.08	0.82±0.08	0.89±0.07	0.80±0.05	0.82±0.09 b	M
13	0.72±0.03	0.91±0.02	0.86±0.03	0.60±0.02	0.79±0.11	0.87±0.03	0.75±0.02	0.79±0.11 b	M
15	0.83±0.02	0.89±0.03	0.80±0.03	0.79±0.12	0.70±0.07	0.74±0.02	0.79±0.02	0.79±0.06 b	M
17	0.84±0.07	0.76±0.03	0.79±0.01	0.75±0.09	0.86±0.04	0.70±0.06	0.75±0.02	0.78±0.06 b	M
7	0.81±0.06	0.89±0.01	0.78±0.03	0.76±0.02	0.73±0.05	0.77±0.04	0.58±0.01	0.76±0.09 b	M
14	0.55±0.04	0.67±0.04	0.72±0.04	0.66±0.05	0.58±0.02	0.87±0.02	0.66±0.02	0.67±0.10 c	L
12	0.66±0.05	0.77±0.04	0.66±0.01	0.58±0.07	0.75±0.04	0.61±0.01	0.51±0.02	0.65±0.09 cd	L
10	0.77±0.04	0.90±0.02	0.48±0.03	0.59±0.13	0.62±0.07	0.48±0.04	0.57±0.04	0.63±0.15 cd	L
9	0.65±0.08	0.72±0.02	0.55±0.01	0.50±0.04	0.58±0.11	0.48±0.02	0.53±0.04	0.57±0.09 d	L

[1)] ARC=Treatment value/Control value. Data are means±standard errors (n=15). Values in ARC column not followed by the same letters indicate significant differences among genotypes at P<0.05, based on LSD tests.

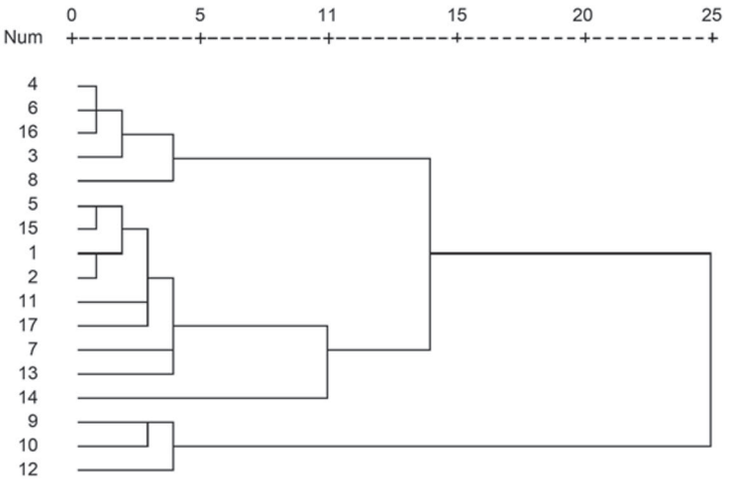

Fig. 2 Cluster analysis of alkali tolerance for 17 genotypes of apple rootstocks. Seven adversity resistance coefficients (new leaf number, root length, shoot length, root DW, shoot DW, root FW, and shoot FW) were used to determine average linkage clustering by Euclidean's distance tests. Numbers along left-hand side match rootstock codes explained in Table 1.

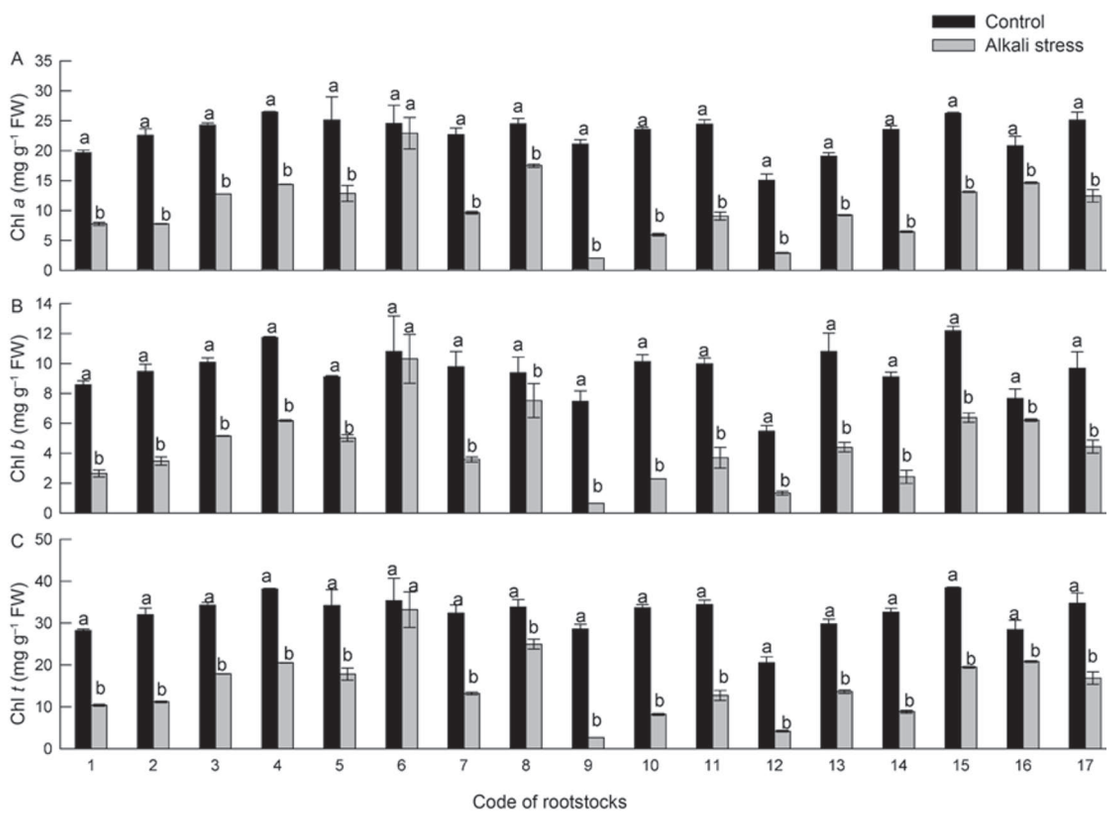

Fig. 3 Chlorophyll status (mg g^{-1} FW) for 17 genotypes of apple rootstocks during 15 d of alkali stress. A, Chl *a*. B, Chl *b*. C, Chl *t*. Data are means of 3 replicates±standard errors (n=3).

4. Discussion

4.1. Cell damage and alkali tolerance

Under abiotic stress, leaves display various symptoms of damage, such as leaf-tip necrosis due to excess NaCl (Wahome *et al.* 2001) or decreases in leaf area in response to salinity or alkalinity (Shi and Sheng 2005). The extent of such damage can be used to illustrate the level of resistance or tolerance by a plant. For example, the salt injury index

Table 4 Comparisons of physiological indicators (% variation) for leaves from 17 genotypes of apple rootstocks under 15 d of alkaline treatment

Code	Root activity	MDA	EL	POD	SOD	CAT
1	11.74±2.34 bcd	57.15±12.56 bcde	12.70±2.10 ab	38.70±7.74 abcd	7.29±2.15 bcd	41.36±8.27 ab
2	46.35±9.27 b	70.27±14.05 de	60.93±5.45 bc	31.28±5.44 abcd	17.59±2.13 abc	24.71±3.51 ab
3	29.32±4.99 bc	3.02±0.53 a	0.06±0.01 a	78.20±2.93 ab	41.00±8.20 a	74.08±14.82 a
4	92.62±18.52 a	22.06±4.41 abc	2.22±0.47 a	84.49±14.38 ab	31.38±6.28 ab	84.88±7.50 a
5	–17.84±3.79 cde	12.47±2.49 ab	11.12±1.11 ab	35.75±7.15 abcd	20.72±5.99 abc	17.83±3.57 ab
6	18.03±4.63 bcd	18.55±2.78 abc	15.53±2.21 ab	84.39±8.59 ab	42.86±7.54 a	24.81±4.96 ab
7	–29.46±5.89 de	55.81±19.45 bcde	24.56±4.91 ab	63.73±12.75 abc	25.38±4.76 ab	22.95±4.59 ab
8	28.26±5.65 bc	6.06±1.36 a	22.64±4.53 ab	89.69±17.94 a	46.49±6.86 a	44.36±9.41 ab
9	–29.86±7.04 de	85.51±12.97 de	74.67±11.66 c	–8.14±3.90 d	–26.87±5.37 e	–31.84±6.83 b
10	–29.72±6.67 de	83.45±10.38 de	80.77±0.89 c	–77.71±4.64 e	5.66±1.13 bcd	–43.96±10.83 b
11	–10.00±2.00 cde	77.36±15.52 de	23.12±5.39 ab	36.86±7.37 abcd	23.26±4.65 ab	16.00±3.20 ab
12	–33.19±6.64 de	103.14±42.81 f	73.16±23.79 c	6.67±1.33 cd	–8.53±2.90 cde	–46.22±12.43 b
13	–25.79±5.67 de	64.96±12.99 cde	25.41±5.08 ab	60.68±12.14 abc	25.99±5.20 ab	41.26±0.83 ab
14	–47.46±5.45 e	83.35±9.38 de	57.49±10.68 bc	–66.51±10.01 e	–15.87±2.57 de	–28.79±0.56 b
15	–29.70±5.62 de	39.60±9.95 abcd	42.59±12.79 abc	65.02±13.00 abc	9.26±0.63 bcd	30.18±6.28 ab
16	17.31±4.16 bcd	20.88±4.18 abc	18.67±3.73 ab	75.03±5.61 abc	9.25±1.85 bcd	83.19±16.64 a
17	–3.79±0.78 bcde	37.17±1.94 abcd	27.19±4.08 ab	13.33±12.49 bcd	19.63±2.48 abc	33.80±6.76 ab

Variation percentage=(Tr−CK)/CK×100, where, Tr is physiological indicator of tested apple rootstocks under 15 of alkali stress (pH 8.5±0.1); CK is physiological indicator of tested genotypes under 15 d of control treatment (pH 6.0±0.1). –, decrease in value over time. MDA, malondialdehyde; EL, electrolyte leakage; POD, peroxidase; SOD, superoxide dismutase; CAT, catalase. Within each column, values not followed by same letters indicate significant differences among genotypes at $P<0.05$, based on LSD tests ($n=3$).

(SI) serves as an important marker of relative resistance to the effects of salinity (Yin *et al.* 2010; Li *et al.* 2015). We calculated values for an AI to represent how tolerant our 17 apple rootstocks are to alkali stress. The lowest values were obtained for the most tolerant genotypes, i.e., Hubeihaitang, Wushanbianyehaitang, Laoshanhaitang Ls2, Fupingqiuzi, and Xiaojinbianyehaitang. In contrast, the highest AI values were calculated for the least tolerant Shandingzi, Sanyehaitang, Pingdinghaitang, Hongsanyehaitang, and Xiaojinhaitang rootstocks.

4.2. The relationship between plant growth and alkali tolerance

Plant growth can be seriously inhibited under alkaline conditions (Navarro *et al.* 2000; Hajiboland *et al.* 2005; Shi and Sheng 2005; Yang *et al.* 2008b). This was also true for the apple rootstocks tested here, as evidenced by lower values for new leaf numbers, root and shoot lengths, and DW accumulations. However, similar to the trends reported previously for SI, those responses differed among our rootstocks, depending upon their relative tolerance to alkali stress. For example, in the highly tolerant Hubeihaitang, plant growth was only slightly inhibited while performance was severely suppressed in the low-tolerance Xiaojinhaitang. This has also been shown with salt-tolerant species, where plant growth is only slightly or moderately inhibited, or even stimulated by salinity (Cramer *et al.* 1986; Marcum 1999). In addition, the decrease in root to shoot ratios found here suggested that the growth of *Malus* roots was inhibited to a greater extent than the shoots under alkali stress, similar to what has been described from the study with tomato (Wang *et al.* 2011).

4.3. ARCs and cluster analysis of alkali tolerance

Under abiotic stress, the physiological and biochemical processes in the cells must adapt to altered environmental conditions to ensure plant survival and development. Although these physiological and biochemical indicators can be used to demonstrate genetic differences, it is not easy to determine which indicators are most accurate and convenient for expressing how tolerant a species or genotype is (Du *et al.* 2002). However, observing a series of physiological and biochemical changes as well as any adjustments to morphological characteristics provides insight into the stress response. Therefore, those methods remain the most commonly used (Bai *et al.* 2008).

By conducting a comprehensive analysis of several growth and morphology indicators under alkali stress, we were able to classify our 17 apple rootstocks into three groups of tolerance, based on ARCs and AI values. However, we did find some irregularities in the results. For example, Shandingzi was considered moderately tolerant *via* ARCs but had only low tolerance when AI was calculated. To obtain more consistent results, we combined the ARCs approach with cluster analysis. Except for Sanyehaitang and Shandingzi, which were classified as moderately tolerant, the groupings of the other rootstocks followed those based on ARCs and AI values.

4.4. The relationship between physiological responses and alkali tolerance

Alkali stress reduced the concentrations of Chl *t*, Chl *a*, and Chl *b* in almost all apple rootstocks (Fig. 3). This was especially true for Pingdinghaitang, Hongsanyehaitang, Xiaojinhaitang, and Sanyehaitang, whereas those reductions were small in Hubeihaitang, Xiaojinbianyehaitang, and Fupingqiuzi. These findings demonstrated that, although alkaline conditions can affect chloroplast structure and the photosynthetic process, the extent of the damage differs among genotypes and influences their level of stress tolerance. Similar effects of alkalinity on chlorophyll have been reported with *Medicago sativa* (Li *et al.* 2010). A combination of alkali stress and high pH can cause Mg^{2+} to precipitate and inhibit chlorophyll synthesis (Shi and Zhao 1997) or can possibly stimulate the activity of a Chl-degrading enzyme, chlorophyllase (Reddy and Vora 1986). Those responses modulate electron transport in the leaves and restrict photosynthesis, leading to a decrease in biomass production (Li *et al.* 2010; Wu *et al.* 2014).

Previous studies have shown that alkali stress is associated with direct structural damage that is induced by elevated pH, which then results in greater lipid peroxidation, less root activity, and an ion imbalance (Gong *et al.* 2014). The reduction in root activity is due to weakened capacity for metabolism, which has a direct impact on stress tolerance (Yang *et al.* 2002). We found that root activity was either increased or decreased after 15 d of treatment, depending upon rootstock (Table 4). For the highly tolerant Hubeihaitang, Wushanbianyehaitang, Laoshanhaitang Ls2, Xiaojinbianyehaitang, and Fupingqiuzi, this activity was not obviously affected but was slightly increased. However, root activities of alkali-sensitive Xiaojinhaitang and Sanyehaitang declined relative to the untreated control. This implied that damage associated with long-term alkalinity, in combination with an elevated pH, makes plants less tolerant of such stress because it is harmful to the root cell structure, causes an internal and external pH imbalance, and irreversibly damages the roots of plants from those genotypes.

When the cell membrane system and its structure are severely interrupted by alkali stress, membrane permeability is increased and $O_2^{\cdot-}$ and H_2O_2 begin to accumulate. This leads to the accumulation of MDA and an increase in EL. Enzymatic antioxidants such as POD, SOD, and CAT are synthesized in plants to reduce the accumulation of reactive oxygen species. EL is a good indicator of the loss of membrane integrity under stress conditions while measurements of MDA levels are an effective means for assessing membrane damage (Mandhania *et al.* 2006; Ahmad *et al.* 2012a, b). Using both parameters, we found that MDA concentrations and EL values were relatively increased over control amounts, indicating that all rootstocks were directly or indirectly damaged by alkali stress. However, those increments were relatively smaller in the highly tolerant rootstocks. Activities of POD, SOD, and CAT were stimulated by stress for almost all rootstocks but were reduced for Pingdinghaitang and Sanyehaitang. The same pattern has been reported by Sun and Hong (2011) with the halophyte *Leymus chinensis*. However, we observed that enzyme activities were negatively correlated with MDA levels and EL, clearly demonstrating that the more alkali-tolerant rootstocks (Laoshanhaitang Ls2, Hubeihaitang, and Xiaojinbianyehaitang) were able to synthesize large amounts of enzymatic antioxidants to counteract the variety of oxidants generated by alkali stress, thereby maintaining the stability of cell membranes. The opposite phenomenon was found with the stress-sensitive Pingdinghaitang and Sanyehaitang.

5. Conclusion

The integrated application of three classification methods — AI, average ARC, and cluster analysis — together with our comparisons of growth and morphology indicators and physiological responses to alkali stress made our final classifications more reliable. Those groupings included (1) high tolerance: Hubeihaitang, Wushanbianyehaitang, Laoshanhaitang Ls2, Xiaojinbianyehaitang, and Fupingqiuzi; (2) moderate tolerance: Pingyitiancha, Laoshanhaitang Ls3, Hubeihaitang A1, Deqinhaitang, Balenghaitang, Maoshandingzi, Shandingzi, and Xinjiangyepingguo; and (3) low tolerance: Pingdinghaitang, Hongsanyehaitang, Xiaojinhaitang, Sanyehaitang. These results will significantly contribute to the selection of apple rootstocks that will have the most appropriate level of tolerance to alkali stress when grown in specific regions.

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