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Effects of Rootstock and Scion Interaction in Providing Nutrients and Phenyl Propanoid Pathway Products in Two Red Flesh Apple Genotypes and Red Delicious Cultivar

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ABSTRACT

Internal conditions and external display of grafted fruit trees are the results of rootstock effects on nutrient uptake, type and amount of rootstock inductive effects on the scion, and scion consumption. Effect of grafting combination on nutrient content, phenyl propanoid pathway enzymes and their effect on secondary metabolites production of apple leaves was investigated in two consecutive years. Two Iranian red flesh apple cultivars and 'Red delicious' cultivar grafted on M9, B9 and one red flesh apple colony rootstock of Bekran were tested in a factorial randomized complete block design. To make a noticeable difference in the amount of nutrients in samples, the treatments in the second year were fertilized. Nitrogen, phosphorus, potassium and calcium were measured in leaves for two years. Also PAL (Phenylalanine ammonia-lyase) and UFGT (UDP-glucose: flavonoid 3-O-glucosyltransferase) enzymes activity, phenolic compounds and flavonoids were determined by high-performance liquid chromatography (HPLC) in vegetative tissues. According to results of this study, among studied rootstocks, M9 was able to accumulate and maintain high concentration of minerals in leaves. Red flesh apples are capable of production of secondary metabolites if they grafted on rootstocks with higher absorption rate. In this way, they can meet the goal of secondary metabolites production. Also, our results showed that calcium was effective in production of all phenolic and flavonoid compounds and induction of UFGT enzyme activity.

Abbreviations: N, Nitrogen**; P,** Phosphorus; **K**, Potassium; **Ca,** Calcium; **Fe,** Iron; **Zn,** Zinc**; PAL**, Phenylalanine ammonia-lyase; **UFGT,** UDP-glucose: flavonoid 3-O-glucosyltransferase; **HPLC,** High-performance liquid chromatography; **BA**, Bastam; **BE**, Bekran

Introduction

Rootstocks regulate the uptake and transport of nutrient in plants but justifying that each element directly affects the activity of each enzymes and reactions or products requires precise investigations. Many reports showed that nutritional status of plants affect production of secondary metabolites (Awad et al. 2000, Keski-Saari and Julkunen-Tiitto 2003, Ruhmann and Treutter 2003). For instance, in several crops and model plants, N deficiency increases the concentration of phenolic compounds (Feyissa et

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al., 2009; Fritz et al., 2006; Hilbert et al., 2003; Lővdal et al., 2010). In tobacco, transcript levels of the phenyl propanoid pathway gene, PAL (phenylalanine ammonia-lyase), induced by N deficiency (Fritz et al., 2006). Fritz et al. (2006) identified that the nitrate tissue concentration is a signal triggering for phenolic accumulation in nitrogen-deficient tobacco. Phosphorus (P) deficiency, also increase anthocyanin content in plants and Potassium (K) plays as a cofactor for many enzymes and activates of several enzymes by changing their conformation through the stabilization of pH (Mengel, 2007; Siddique et al., 2012) and affects Root/Shoot ratio via phloem transport (Bate, 1994). Micronutrient as cofactors are involved in production of compounds such as various enzymes, plant hormones, proteins, and especially phenolic compounds (Sobhana et al., 2000; Satisha et al., 2005). Some researchers reported that calcium (Ca) plays regulatory role in plant cell metabolism, signal transduction and in nutrient absorption across the cell membranes (Talukdar, 2012; El-Beltagi and Mohamed, 2013).

According to the carbon/nitrogen balance hypothesis (Bryant et al., 1983), when nitrogen availability in soil is low, the low resource availability limits the growth of the plants more than the photosynthesis, and plant allocates the extra carbon that cannot be used for growth to the production of carbon based secondary metabolites. In the same way, dwarfing rootstocks with less absorption of nutrient can affect growth and the amount of leaf secondary metabolites. Influence of rootstock on phenol accumulation in lemons (Gil-Izquierdo et al. 2004), peaches and apricots (Scalzo et al. 2005), cherry (Usenik and Štampar 2000) and apples (Mainla et al. 2011) have been reported. Also many studies have emphasized that the rootstock has a significant effect on the amount of flavonoids in plants (Darjazi and Jaimand 2017, Mashayekhi et al. 2013, Ganji Moghadam et al. 2007, Treutter and Feucht 1987, Mainla et al. 2011, Foster et al. 2017, Kviklys et al. 2015). Although a negative correlation between tree vigor and the concentration of phenolic compounds has been reported by Mainla et al. (2011). The genotype of rootstock plays more important role in the accumulation of phenolic compounds than tree vigor (Kviklys et al., 2015). Tsao et al. (2003) reported that, typically, cultivar type is an important factor in determining the amount of phenolic compounds in apples. Red flesh apples have distinct traits that can be used as specific markers, with good amounts of secondary metabolites. In this study, the effect of rootstock, scion cultivar and their interaction on nutrient content, PAL (Phenylalanine ammonia-lyase) and UFGT (UDP-glucose: flavonoid 3-O-glucosyltransferase) enzymes and their effects on secondary metabolites production in apple leaves were investigated.

Materials and Methods

Plant materials

The present study was established at Horticulture Department of Ferdowsi University of Mashhad, Iran with geographical coordinates: 59° 31′ 46′′ E longitude and 36° 18′ 21′′ N latitude and 1038 m altitude. Three-year-old trees including grafting combination of three scion cultivars of two Iranian red flesh apple cultivars - 'Bekran' and 'Bastam' and 'Red delicious' as a control variety on three clonal rootstocks include B9 and M9 that are commonly used and Bekran as a new red flesh apple genotype were used for leaf sampling. Depending on seasonal conditions and temperature, the irrigation cycle was between 8 and 12 days. After rapid growth stage, mature leaves from the middle of branches were taken randomly in late August of 2016 and 2017. No fertilization was performed in the first year but in the second year of experiment, trees were fertilized with 100 g of a fertilizer containing NPK (20:20:20) for each trees and spraying with a micro fertilizer (2 g/1000 mL) containing a variety of micronutrient including Iron, Calcium and Zink. Leaves packed in polyethylene bags and stored at 4 ° C until sending to laboratory.

Extraction

About 5 g of leaves samples was homogenized in 40 mL of cold (4 °C) Hepes buffer (50 mM pH 7.5 + 2 mM dithioerithritol) with a mortar and pestle. The homogenate was centrifuged with (10 min at 19000 g at 4 °C) and the resulting supernatant was centrifuged again for 1 min at 19000 g. The final supernatant was used for assaying protein content and enzyme activity (Ju et al., 1995).

PAL activity

PAL activity was assayed in the partially purified enzyme extracts by an adaptation of the method of Zucker (1965) as reported by McCallum and Walker (1990). The assay mixture consisted of 0.06 M borate buffer (875 mL) and crude enzyme (250 mL). The reaction was initiated by the addition of L-phenylalanine (250 mL of 10 mg/mL, to give a final concentration of 11 mM). Tubes were incubated at 30 °C for 30 min (or one h when activity was low) and the reaction

stopped by addition of 35% w/v trifluoroacetic acid (125 mL). Tubes were then centrifuged for 5 min at 5000 ×g to pellet the denatured protein. PAL activity was determined from the yield of cinnamic acid, estimated by measuring A290 of the supernatant in 10 mm quartz cuvettes. PAL activity was defined as the amount of cinnamic acid that produced in 1 h under specific condition.

UFGT activity

The UFGT activity was assayed by the method of Gerats et al. (1984). Reaction mixture contained 40-130 µL of enzyme extract, 1 mM UDP Gal in 20 µL of a 330 PM solution of quercetin in methanol and additional extraction buffer to produce 200 µL total volumes. Samples were incubated for 15 min at 30 °C. Reaction was terminated by adding 800 µL of a 2: l mixture of chloroform: methanol (with 1% HCl) and mixing. The mixture was centrifuged at 15 000 g for 30 s, and in the biphasic partition the flavonoids were concentrated in the upper 400 µL phase. Reaction products were separated by high-performance liquid chromatography equipped with a C18 column in Unicam, crystal-200 HPLC system. Using an acetic acid: methanol: water mixture (10:25:65) as eluting solvent, quercetin and its galactoside, quercetin-3-galactoside, were detected at 254 nm. The flow rate was 3 mL min^{-1}, which resulted in retention times of 2.3 min for quercetin-3-galactoside and 4.3 min for the aglycone. The concentration of quercetin-3-galactoside was determined by comparing the peak area with that of the standard quercetin-3-galactoside solution. Enzyme protein was determined by the method of Bradford (1976) using bovine serum albumin, fraction V, as the standard.

Total flavonoids

A slightly modified version of the spectrophotometric method was used to determine the flavonoid content (Chang et al. 2002). Fresh leaves (1 g) were weighed and ground with 20 mL of 80% aqueous methanol in a mortar and pestle. The ground sample was filtered with Whatman filter paper No. 42 and a clear filtrate was obtained. Aliquot of this sample (0.5 Ml) was taken in a test tube and 3 mL of distilled water and 0.3 mL of 5% sodium nitrite were added. The solution was mixed well and allowed to stand at room temperature for 5 min. To this solution, 0.6 mL of 10% aluminum chloride was added. After 6 min, 2 mL of 1 M sodium hydroxide was added to the test tube. The solution was then diluted with distilled water to make the final volume up to 10 Ml. The absorbance was read at 510 nm. The amount of total flavonoids was calculated and expressed as equivalent to mg catechin per g of fresh weight.

Total phenolic content

The total phenolic content of leaves was determined by a modified Folin-Ciocalteu reagent method. About 2 g of fresh leaf tissue was macerated in liquid N_2 with mortar and pestle and mixed with 3 ml 80% (v/v) acetone. The sample was placed into a 1.5 mL tightly covered micro-tube and incubated in darkness at 4 °C overnight. Subsequently, the sample was centrifuged at 1000 rpm for 2 min and the supernatant was used as phenolic extract. A mixture of 135 µL distilled water, 750 µL 1/10 dilution Folin- Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) and 600 µL 7.5% (w/v) Na_2CO_3 was added to 50 µL of phenolic extract in a 1.5 mL micro-tube. After vortexing for 10 s, the mixture was incubated at 45 °C in a water bath for 15 min. Samples were allowed to cool at room temperature before measuring the absorbance at 765 nm by a spectrophotometer (U-1100, Hitachi Ltd. Japan). A blank was prepared using 50 µL 80% (v/v) acetone. A gallic acid standard curve was prepared from a freshly made stock solution of 1 mg/mL gallic acid (Acros Or-ganics, Belgium) in 80% (v/v) acetone.

Nutrient elements

To evaluate the amount of elements in the leaf samples, 0.3 g of plant sample was weighed and transfer to a 250 mL balloon. Then 2.3 mL of the acid mixture (18 mL of water was poured into a 250 mL Erlenmeyer flask and 100 mL of concentrated sulfuric acid was added to the water; then six g of salicylic acid was added to the solution) was added to the plant sample and then shaken carefully to mix thoroughly. The solution was kept in this condition for 24 hrs. The sample temperature was then increased to 180 °C and after cooling, five drops of H_2O_2 (30%) were added to it. Then the sample temperature was increased to 280 °C to evaporate and white steam was observed. After cooling again, five drops of H_2O_2 were added to the sample. The heating started again and this process continued until the samples became colorless. After the samples were cooled, 10 mL of water was added to them. The resulting sample was thoroughly mixed by shaking and finally brought to desired volume and smoothed. The amount of nitrogen elements was measured by Kjeldal method (Bremner, 1996), phosphorous and calcium by Spectrophotometer and potassium by flame photometry (Emami, 1996).

Statistical analysis

The statistical design was a randomized complete block design (RCBD) with three replications. Statistical analysis was performed with SPSS 16.0 software (SPSS, Inc., Chicago, Illinois). The three-way factorial analysis of variance (ANOVA) was applied to evaluate the significant difference in the parameters studied in different treatments. Data were reported as means ± standard error of the mean (SE) and differences between means were determined using Duncan's significant difference tests. Significant differences were accepted at $p < 0.05$ and represented by different letters. For better comparison of the results data were standardized with Z-score indicates that a given data how many standard deviations was far from the population average. The formula for calculating the Z-score is: $z = (x-\mu) / \sigma$. Where "x" is the raw score, "μ" is the mean of the population, and "σ" is the standard deviation of the population. If a Z-score is zero, it is in average. A positive Z-score indicates that the raw score is higher than average and a negative Z-score indicates that the raw score is below average. Also to determine the effect of nutrient on secondary metabolites a stepwise regression analysis was performed.

Results

Rootstock and scion effects on nutrient levels, enzyme activity and secondary metabolites

Although, both B9 and M9 are considered as dwarfing rootstocks, M9 was slightly more vigorous than B9 and the growth effect of Bekran genotype as rootstock was not significant. Among scions, Bastam genotype was vigorous than the others. In the present study, the trend of changes in nutritional status in each series of treatments instead of reviewing each treatment alone was monitored. Each series included one of scions on all three rootstocks. The difference in results was significant for all factors in two consecutive years.

In the first series by grafting three scion on the B9 rootstock, in the first year, the lowest amount of all nutrient was recorded in leaf samples of Bastam and Red on B9 (Table 1, Fig. 1). Also the activity of PAL and UFGT enzymes and total phenolic compounds were significantly lowest in these treatments while in second year, the Bekran on B9, showed the lowest values of elements and secondary metabolites (Table 2, Fig. 2).

In the second series, including grafting of three cultivars on M9, when Bastam scion grafted on M9 as efficient absorption rootstock, we recorded the lowest amount of N and K in the second year, that can be attributed to its higher consumption in the growth process of Bastam genotype and coordinates with the lowest activity of enzymes and measured secondary metabolites. Also, when Bekran scion grafted on M9 the balance between absorption and consumption of nutrient was in favor of more absorption by the rootstock, since the data of both years indicate more absorption by M9 and less consumption by Bekran. The third treatment of this series, Red Delicious on M9, showed a greater amount of element absorption and secondary metabolites in the first year but with continuing the growth and production of more shoots in the second year it seems that the balance had been towards more nutrient consumption in trees.

Table 1. Effect of Rootstock and Scion interaction on the amount of nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), Phenylalanine ammonia-lyase (PAL) and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT) enzymes activity and secondary metabolites in apple leaves (First year)

Scion/ rootstock	N (mg/kg)	P (mg/kg)	K (mg/kg)	CA (mg/kg)	PAL (µmol Cinamic acid/gr fw h)	UFGT (nmol/min/ mgr.protein)	Total Phenol (mgr Galic acid/ gr.dw)	Total flavonoid (mg catechin/100 gr fw)
BA/B9	1912.0±78.44f	832±32.42f	1869.7±62.44de	5.74±0.090c	1.07±0.074d	23.20±1.12c	98.43±5.27c	25.69±0.62bc
BE/B9	2554.5±131.5bc	1278.5±23.5ab	2208±124bc	5.77±0.065c	1.54±0.026c	22.23±1.08cd	119.70±4.40b	29.01±0.46a
RED/B9	2114.0±0.00ef	982±0.00e	1720±0.00e	4.25±0.00e	0.89±0.00d	8.14±0.00f	62.70±0.00e	18.44±0.00e
BA/M9	2286.0±61.24cde	1046.3±28.88de	2264±71.10bc	6.41±0.061b	2.07±0.088b	35.13±1.20b	137.33±6.38ab	24.98±1.07bc
BE/M9	2682.0±48.04ab	1326±21.17a	2833±60.29a	6.39±0.071b	1.69±0.051c	24.77±1.10c	126.33±4.57b	23.96±0.31c
RED/M9	2884.0±49.86a	1196.3±32.20bc	2231±36.96bc	5.52±0.031c	2.75±0.054a	18.81±0.79d	89.80±2.04c	21.16±0.60de
BA/BE	2520.0±77.78bcd	988±25.78de	2440±85.95.0b	7.11±0.050a	2.45±0.11a	39.80±0.97a	145.33±4.06a	26.94±0.64ab
BE/BE	2125.0±29.72ef	1082±20.13cde	2044±66.61cd	5.12±0.038d	1.19±0.053d	14.10±0.50e	87.50±3.37cd	23.12±0.17cd
RED/BE	2259.5±75.5de	1110±51cd	1875±59.5de	4.97±0.080d	1.55±0.069c	12.24±0.37ef	70.40±3.10de	19.83±0.06e

Values are means ± standard errors (n=3). The different letters indicate significant differences between the values (P

< 0.05). Bastam (BA), Bekran (BE) and Red delicious (RED), Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), PAL (Phenylalanine ammonia-lyase) and UFGT (UDP-glucose: flavonoid 3-O-glucosyltransferase).

Table 2. Effect of Rootstock and Scion interaction on the amount of nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), Phenylalanine ammonia-lyase (PAL) and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT) enzymes activity and secondary metabolites in apple leaves (Second year)

Scion/rootstock	N (mg/kg)	P (mg/kg)	K (mg/kg)	CA (mg/kg)	PAL (µmol Cinamic acid/gr fw h)	UFGT (nmol/min/mgr.protein)	Total Phenol (mgr Galic acid/ gr.dw)	Total flavonoid (mg catechin/100 gr fw)
BA/B9	2106.3±37.69d	709.7±36.08e	1936.7±90.78bcd	5.89±0.43ab	1.52±0.053abc	31.20±0.73a	98.6±1.12a	35.12±0.64a
BE/B9	2334±15.04bc	940±10.82ab	2014.7±11.85ab	4.92±0.059bcd	1.36±0.053cd	15.7±0.38de	78.5±0.99d	20.52±0.72cd
RED/B9	2317±0.57bc	869±0.00bc	1759±0.00de	4.33±0.00cd	1.12±0.060e	13.62±0.065ef	65.7±1.20f	21.13±0.28cd
BA/M9	2098±16.29d	734±9.71e	1920±16.65bcd	6.12±0.045ab	1.36±0.035cd	27.96±0.88b	92.8±0.90b	30.74±0.69ab
BE/M9	2524.5±12.5a	1004±8.00a	2219.5±14.5a	6.26±0.11a	1.67±0.015a	20.87±0.24c	86.9±0.45c	25.03±3.80bc
RED/M9	2240±15.72cd	835±11.79cd	1686±15.5e	4.03±0.064d	0.94±0.029f	10.21±0.60g	59.4±1.39g	17.31±0.80d
BA/BE	2136.3±66.87d	777.3±9.39de	2006±99.19abc	6.38±0.55a	1.56±0.040ab	30.29±0.40ab	97.3±1.00ab	34.50±0.74a
BE/BE	2408±13.10ab	975±7.51a	2082±13.89ab	5.40±0.043abc	1.50±0.051bc	17.42±0.47d	82.6±1.12cd	23.74±0.69bcd
RED/BE	2339.3±76.16bc	875.3±25.12bc	1780±34.36cde	4.53±0.42cd	1.20±0.070de	11.70±0.80fg	72.3±2.20e	23.74±1.11bcd

Values are means ± standard errors (n=3). The different letters indicate significant differences between the values (P < 0.05). Bastam (BA), Bekran (BE) and Red delicious (RED), Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), PAL (Phenylalanine ammonia-lyase) and UFGT (UDP-glucose: flavonoid 3-O-glucosyltransferase).

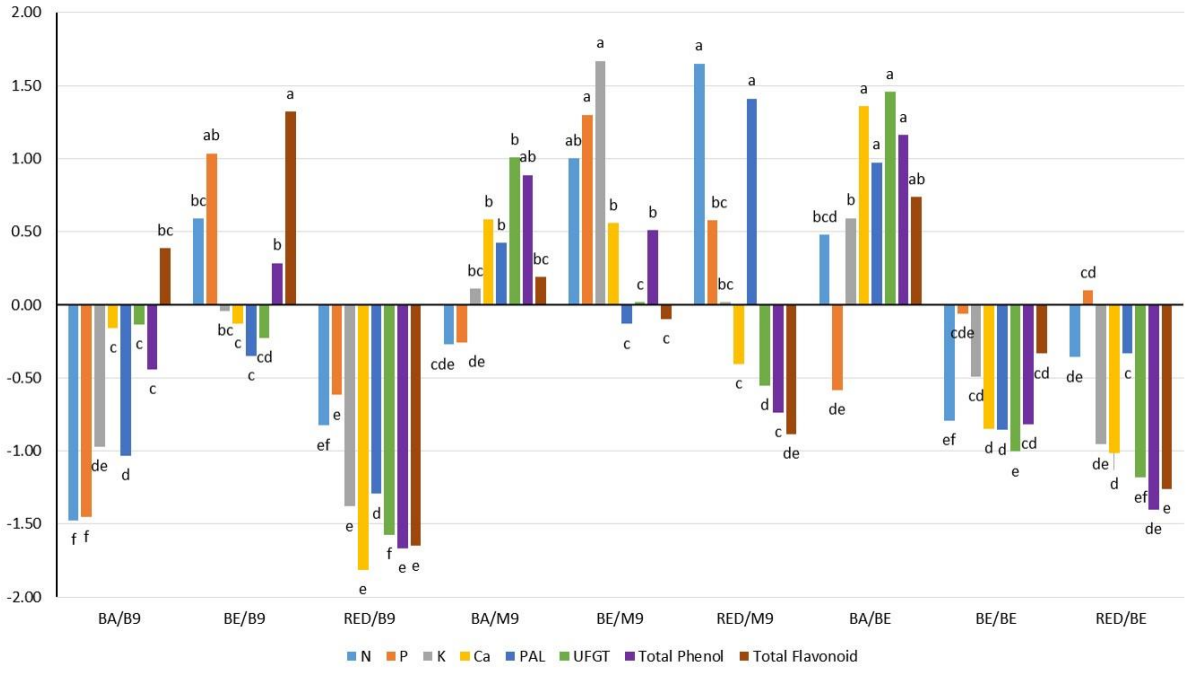

Fig. 1. Effect of Rootstock and Scion interaction on the amount of nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), Phenylalanine ammonia-lyase (PAL) and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT) enzymes activity and secondary metabolites in apple leaves (First year). Data were standardized with Z-score indicates that in a given data how many standard deviations was far from the population average. The different letters indicate significant differences between the values (P < 0.05). Bastam (BA), Bekran (BE) and Red delicious (RED)

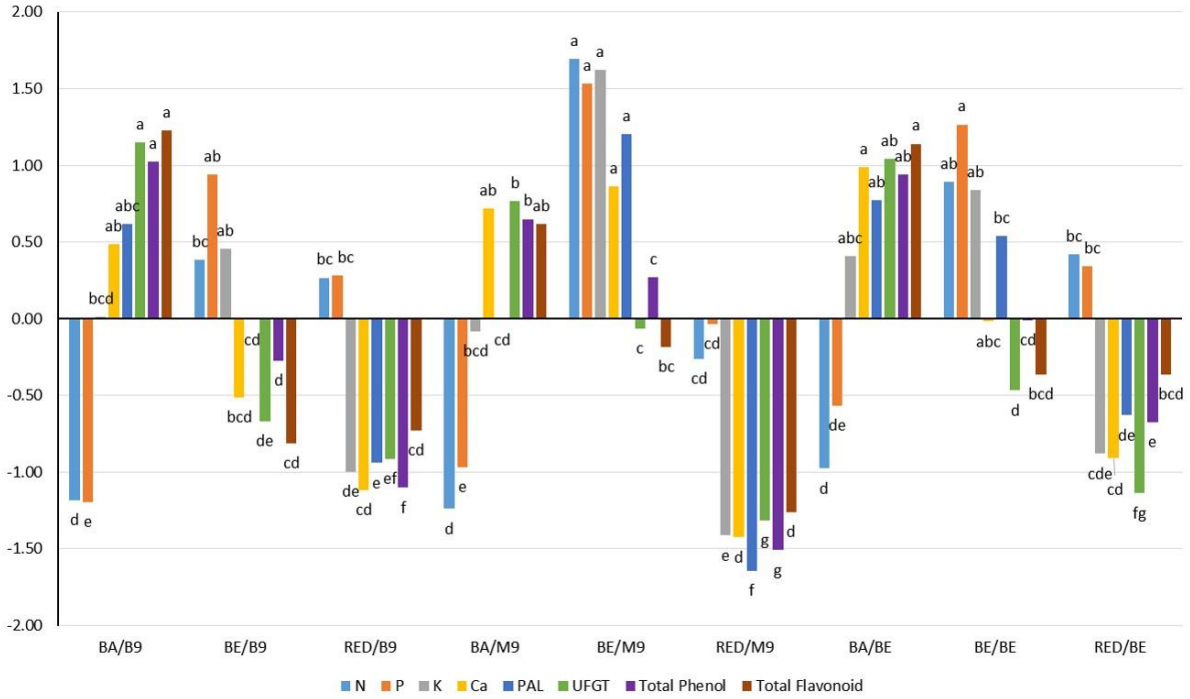

Fig. 2. Effect of Rootstock and Scion interaction on the amount of nitrogen (N), phosphorous (P), potassium (K), calcium (Ca), Phenylalanine ammonia-lyase (PAL) and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT) enzymes activity and secondary metabolites in apple leaves (Second year). Data were standardized with Z-score indicates that a given data how many standard deviations was far from the population average. The different letters indicate significant differences between the values ($P < 0.05$). Bastam (BA), Bekran (BE) and Red delicious (RED).

Although the behavior of Bekran's genotype as a rootstock is completely unknown, but the highest amounts of nitrogen, which is known as the most significant element contributing to growth, and on the other hand, recording the highest amount of secondary metabolites in this treatment shows that the interaction of Bekran's rootstock and Bastam's scion, that both of them are red flesh apple genotypes, led to production of more secondary metabolites. Also, our results showed that compared to grafting of Bekran scion on M9 and its own rootstock, if this genotype is grafted on a rootstock with higher absorption such as M9, it easily meet the goal of secondary metabolites production. The latest treatment was Red Delicious cultivar on Bekran rootstock, which has increased the amount of secondary metabolites in second year in this cultivar, and again emphasizes that the choice of rootstock according to the purpose of production.

Nutrient effect on PAL and UFGT enzymes and secondary metabolites

Stepwise regression analysis showed that in the first year of experiment 58% of PAL enzyme changes were dependent on nitrogen; while N and P together explained 72% changes of PAL activity (Table 3). Similarly, in the second year of our study N and K entered the model and potassium was more important while 54% of PAL activity depended on K and 63% of its effects depended on both K and N (Table 4).

UFGT enzyme activity in the first year depended on calcium and phosphorous. However, Ca alone justified approximately 88% of enzyme activity and Ca and P together justified 91% of UFGT activity (Table 3); whereas in the second year the determinants for UFGT activity were Ca and N. Ca alone 65% and Ca and N together affected 90% of UFGT enzyme activity. Interestingly, in both years, Ca played a key role in activity of the UFGT (Table 4).

However, our results showed that Ca was effective in all phenolic and flavonoid compounds and UFGT enzyme except PAL (Table 3 and 4). Calcium affected almost 83% on total leaf phenol production. In the second year, it had the greatest impact on total phenol production (70%). With addition of N, this effect was further increased by 10%, and with addition of K, the effect of all three elements increased by 86% on the total phenol content of apple leaves. Also in the second year, N and K without Ca were equally effective on production of phenolic compounds (Table 4).

Stepwise regression analysis showed that in the first year, only Ca had a significant role in production of flavonoids (38%) and in the second year Ca and P entered the model so that Ca 60% and Ca and P together accounted for 80% of total flavonoid content (Table 3).

Table 3. Variables that justify secondary metabolites changes in apple leaves (first year)

Dependent variable	Model	Predictors	Adjusted R Square	P-value	B	β	T
PAL (µmol Cinamic acid/gr fw h)	1	N	0.58	0.000	-1.73	0.775	5.612
	2	N	0.72	0.000	0.002	1.150	7.254
		P		0.003	-0.002	-0.535	-3.373
UFGT (nmol/min/mgr.protein)	1	Ca	0.88	0.000	11.94	0.939	12.51
	2	Ca	0.92	0.000	11.95	0.940	14.615
		P		0.008	-0.012	-0.189	-2.932
Total Phenol (mgr Galic acid/ gr.dw)	1	Ca	0.83	0.000	32.47	0.913	10.259
Total Flavonoid (mg catechin/100 gr fw)	1	Ca	0.38	0.001	2.498	0.641	3.832

Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), PAL (Phenylalanine ammonia-lyase) and UFGT (UDP-glucose: flavonoid 3-O-glucosyltransferase).

Table 4. Variables that justify secondary metabolites changes in apple leaves (second year)

Dependent variable	Model	Predictors	Adjusted R Square	P- value	B	β	T
PAL (µmol Cinamic acid/gr fw h)	1	K	0.54	0.00	0.001	0.750	5.197
	2	K	0.63	0.00	0.001	0.939	6.282
		N		0.02	0.001	-0.375	-2.505
UFGT (nmol/min/mgr.protein)	1	Ca	0.65	0.00	6.85	0.816	6.47
	2	Ca	0.90	0.00	6.55	0.78	11.55
		N		0.00	-0.27	-0.495	-7.33
Total Phenol (mgr Galic acid/ gr.dw)	1	Ca	0.70	0.00	11.89	0.85	7.25
	2	Ca	0.80	0.00	11.57	0.822	8.517
		N		0.004	-0.029	-0.317	-3.287
	3	Ca	0.86	0.32	2.92	0.208	1.014
		N		0.00	-0.086	-0.751	-4.844
		K		0.00	0.061	0.771	3.256
	4	N	0.86	0.00	-0.080	-0.878	-9.563
		K		0.00	0.078	0.992	10.806
Total Flavonoid (mg catechin/100 gr fw)	1	Ca	0.60	0.00	5.29	0.787	5.707
	2	Ca	0.81	0.00	4.66	0.694	7.121
		P		0.00	-2.027	-0.465	-4.766

Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), PAL (Phenylalanine ammonia-lyase) and UFGT (UDP-glucose: flavonoid 3-O-glucosyltransferase).

Discussion

Aguirre et al. (2001) suggested that one of the possible cause of mineral deficiencies is the low rate of mineral uptake by some dwarfing rootstocks. Given that among the rootstocks, B9 was a dwarfing rootstock our results are consistent with those of Jones (1976) that reported N content on dwarfing apple rootstocks were lower than the other rootstocks. Also Abdalla et al. (1982) reported that the K content in fruit of Delicious cultivar on dwarfing rootstocks was lower than that on vigorous rootstocks. Amiri and Fllahi (2009) reported that cultivars grafted on M9 had higher leaf content of N, Mg, Fe, and Mn concentrations than other rootstocks. Aguirre et al. (2001) reported that trees grafted on M9 rootstock were more efficient at N uptake, Dong et al. (1998) also observed high N uptake in M9 EMLA rootstocks. However Fallahi et al. (1984) suggest that the higher mineral concentration in leaf blades may

have resulted from greater uptake by roots and rapid transport from root system (xylem) to leaves. It can be inferred that M9 rootstock differs greatly in its mineral uptake and its ability to transport mineral nutrient.

Most of researchers have focused on the rootstock role without paying close attention to the role of scion, whereas according to our results and other reports the lower mineral consumption due to a lower rate of growth and yield suggest that M9 rootstock may be able to accumulate high concentration of minerals in leaves (Amiri and Fallahi, 2009; Parvaneh et al., 2019). Similarly Davis (2009) reported that increases in dry-matter accumulation, resulting from application of fertilizers under optimal environmental conditions often will be accompanied by decreases in plant mineral concentrations. This inverse relationship between growth and mineral concentration, termed dilution effect (Jarrell and Beverly, 1981) that occurs when dry-weight accumulation increases at a faster rate than mineral-nutrient accumulation. Conversely, increased mineral nutrient concentration will occurs when mineral-nutrient accumulation increases at a faster rate than dry weight accumulation. This relationship has been defined as a synergism effect (Jarrell and Beverly, 1981).

Several reports indicate that the nutritional status may affect secondary metabolism in plants (Awad et al., 2000; Keski-Saari and Julkunen-Tiitto, 2003; Ru¨hmann and Treutter 2003). Of course, many of these studies have been done in nutrition deficiency condition, while we studied the activity of enzymes, secondary metabolites production and their relationship in normal plant growth conditions. It seems that increasing the amount of nutrition as long as does not lead too much changes in growth, can increase the concentration of secondary metabolites. The results show that in genotypes that are inherently responsible for secondary metabolites production, larger amounts of nutrient have contributed to this goal. Specifically, N was more important factor in plant metabolism due to nitrogen role in growth and plants tendency in young trees is toward more growth. Some researcher suggested that activity of PAL is usually increased in nitrogen deficient plants (Kováčik and Bačkor, 2007). In contrast Ahenger et al. (2017) reported that an obvious increase in PAL activity was observed in Wheat (*Triticum aestivum* L) seedlings treated with K that exposed to water stress or controls.

Although Gomez et al. (2009) and Francisco et al. (2013) reported that Ca triggers a sharp inhibition in UFGT enzyme activity in grape cells, they suggested that Ca can block anthocyanin biosynthesis by inhibiting UFGT at transcriptional and enzyme activity levels.

Ahenger et al. (2017) reported that added K increase total phenols and tannins thereby strengthening the components of both the enzymatic as well as non-enzymatic antioxidant system. Tomar and Agarwal (2013) reported that K in verity amounts increase the synthesis of phenolics including tannins and phytic acid in *Jatropha curcas* L. Also significant increase in flavonoids content was observed in plants supplied with Ca and K individually as well as in combination (Bais et al., 2002; Li et al., 2005), which is in agreement with the results of this study. Increased production of total phenol and flavonoids with increased K levels may be due to an increase in non-structural carbohydrates because K is effective in stimulating photosynthetic activity and increasing the transfer of carbohydrates to different parts of the plant. Ibrahim et al., (2011) reported that increase in transmission indirectly increased the biosynthesis of total phenol and flavonoids in *Labisia pumila* Benth, which was treated with K fertilizer. Similarly Xu et al. (2014) suggested that Ca and K-mediated increase in the total phenols and flavonoids can be possible since Ca has a role in signaling and helps in up regulation of respective genes for polyphenols biosynthesis in strawberry. Similar to our results there are some reports that showed P limitation can affect some flavonoid compounds synthesis in *nla* mutant Arabidopsis that is unable to accumulate this flavonoids under N limitation (Chalker-Scott, 1999; Steyn et al., 2002).

Except for fluctuations related to momentary motivations, continuity of these variables including deficiency, presence of optimal amounts or excess of available nutrients affects the growth and physiology of plants. Nutrients directly affect plant growth and development but they are also important in secondary metabolite production and resistance process to adverse environmental conditions. However, because plants are collections of metabolic pathways and many variables are involved in controlling and advancing them, it is not possible to examine the effect of each element alone and only when that element is restrictive it can be examined with trigger situation. Studying the complexities and relationships between nutrition and secondary metabolites may help to determine the stress thresholds in each plant species, rootstock, and cultivar according to the tree age, growth capacity or specific goals for production of various materials. This study used red flesh apple genotypes that are efficient in secondary

metabolites production and by following the values of a set of nutrients in these plants for two consecutive years clarified some physiological aspects of these genotypes.

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Conflict of interest

The authors indicate no conflict of interest for this work.

References

Abdalla O.A, Khatamian H, Miles N.W. 1982. Effect of rootstocks and interstems on composition of 'Delicious' apple leaves. Journal of the American Society for Horticultural Science 107,730-733.

Aguirre P.B, Al-Hinai Y.K, Roper T. R, Krueger A. R. 2001. Apple tree rootstock and fertilizer application timing affect nitrogen uptake. HortScience 36, 1202-1205.

Ahenger M.A, Agarwal R.M. 2017. Potassium up-regulates antioxidant metabolism and alleviates growth inhibition under water and osmotic stress in wheat (*Triticum aestivum* L). Protoplasma 254, 1471-1486.

Amiri M.E, Fallahi E. 2009. Potential of Mineral Uptake Efficiency by Some Apple Rootstocks. UC Davis: Departtment of plant science.

Awad M.A, de Jager A, van Westing L.M. 2000. Flavonoid and chlorogenic acid levels in apple fruit: characterisation of variation. Scientia Horticulturae 83, 249-263.

Bais H.P, Walker T. S, Schweizer H.P, Vivanco, J.M. 2002. Root specific elicitation and antimicrobial activity of rosmarinic acid in hairy root cultures of *Ocimum basilicum.* Plant Physiology and Biochemistry 40, 983–995.

Bate N.J, Orr J, Ni W, Meromi A, Nadler-Hassar T, Doerner P.W, Dixon R.A, Lamb C.J, Elkind Y. 1994. Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. Proceedings of the National Academy of Sciences 91, 7608–7612.

Bryant, J.P, Chapin, F.S, Klein, D. R. 1983. Carbon nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40, 357–368.

Bradford, M. M, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72, 248–254.

Bremner J. M. 1996. Nitrogen-total. Methods of soil analysis: Part 3 Chemical methods 5, 1085-1121.

Chalker-Scott L. 1999. Environmental significance of anthocyanins in plant stress responses, Photochemistry and Photobiology 70, 1–9.

Chang C.C, Yang M. H, Wen H. M, Chern J.C. 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. Journal of food and drug analysis 10, 178-182.

Darjazi B.B, Jaimand K. 2017. The Effect of Rootstocks on the Peel Phenolic Compounds of Clementine Mandarin (*Citrus clementina*). Journal of Medicinal Plants and By-products 6, 173-180.

Davis D.R. 2009. Declining fruit and vegetable nutrient composition: What is the evidence?. HortScience 44, 15-19.

Dong S, Cheng L, Fuchigami L.H. 1998. Nutrient uptake by new roots of six clonal apple rootstocks. HortScience 34,492-493.

El-Beltagi H.S, Mohamed H. I. 2013. Alleviation of cadmium toxicity in *Pisum sativum* L. seedlings by calcium chloride. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 41, 157-168.

Emami A. 1996. Plant decomposition methods. Soil and Water Research Institute.

Fallahi E, Westwood M.N, Chaplin M.H, Richardson D. G. 1984. Influence of apple rootstocks and K and N fertilizers on leaf mineral composition and yield in a high density orchard. Journal of plant nutrition 7, 1161-1177.

Feyissa A.M, Chandran A, Stockmeier C.A, Karolewicz B. 2009. Reduced levels of NR2A and NR2B subunits of NMDA receptor and PSD-95 in the prefrontal cortex in major depression. Progress in Neuro-Psychopharmacology and Biological Psychiatry 33, 70-75.

Foster T.M, McAtee P.A, Waite C.N, Boldingh H.L, McGhie T.K. 2017. Apple dwarfing rootstocks exhibit an imbalance in carbohydrate allocation and reduced cell growth and metabolism. Horticulture research 4, 1-13.

Francisco R.M, Regalado A, Ageorges A, Burla B.J, Bassin B, Eisenach C, Zarrouk O, Vialet S, Marlin T, Chaves M.M, Martinoia E. 2013. ABCC1, an ATP binding cassette protein from grape berry, transports anthocyanidin 3-O-glucosides. The Plant Cell 25, 1840-1854.

Fritz C, Palacios-Rojas, N, Feil R, Stitt, M. 2006. Regulation of secondary metabolism by the carbon–nitrogen status in tobacco: nitrate inhibits large sectors of phenylpropanoid metabolism. The Plant Journal 46, 533–548.

Ganji Moghadam E, Talaie A, Mokhtarin A. 2007. Relationships between total phenol content, mineral nutrition and vigor of some selected dwarf Iranian mahaleb (*Prunus mahaleb* L.) genotypes. Journal of Plant Sciences 2, 82-88.

Gerats A.G.M, Bussard J, Coe E.H, Larson R. 1984. Influence of B and pl on UDPG: Flavonoid-3-O-glucosyltransferase in Zea mays L. Biochemical genetics 22, 161–1169.

Gil-Izquierdo A, Riquelme M. T, Porras I, Ferreres F. 2004. Effect of the rootstock and interstock grafted in lemon tree (*Citrus limon* L. Burm.) on the flavonoid content of lemon juice. Journal of agricultural and food chemistry 52, 324-331.

Gomez C, Terrier N, Torregrosa L, Vialet S, Fournier-Level A, Verries C, Souquet J.M, Mazauric J.P, Klein M, Cheynier V, Ageorges A. 2009. Grapevine MATE-type proteins act as vacuolar H+ - dependent acylated anthocyanin transporters. Plant Physiology 150, 402-415.

Hilbert G, Soyer J, Molot C, Giraudon J. 2003. Effects of nitrogen supply on must quality and anthocyanin accumulation in berries of cv. Merlot. Vitis 42, 69–76.

Ibrahim M.H, Jaafar H.Z, Rahmat A, Rahman Z.A. 2011. Effects of nitrogen fertilization on synthesis of primary and secondary metabolites in three varieties of kacip Fatimah (*Labisia pumila* Blume). International Journal of Molecular Sciences 12, 5238-5254.

Jarrell W.M, Beverly R.B. 1981. The dilution effect in plant nutrition studies. Advances in agronomy 34, 197-224.

Jones O.P. 1976. Effect of dwarfing interstocks on xylem sap composition in apple trees: effect on nitrogen, potassium, phosphorus, calcium and magnesium content. Annals of Botany 40, 1231-1235.

Ju Z, Liu C, Yuan Y. 1995. Activities of chalcone synthase and UDPGal: flavonoid-3-oglycosyltransferase in relation to anthocyanin synthesis in apple. Scientia Horticulturae 63, 175–185.

Keski-Saari S, Julkunen-Tiitto R. 2003. Resource allocation in different parts of juvenile mountain birch plants: effect of nitrogen supply on seedling phenolics and growth. Physiologia Plantarum 118, 114-126.

Kviklys D, Liaudanskas M, Janulis V, Viškelis P, Rubinskienė M, Lanauskas J, Uselis N. 2015. Rootstock genotype determines phenol content in apple fruits. Plant, Soil and Environment 60, 234-240.

Li W, Koike K, Asada Y, Yoshikawa T, Nikaido T. 2005. Rosmarinic acid production by Coleus forskohlii hairy root cultures. Plant cell, tissue and organ culture 80, 151–155.

Mainla L, Moor U, Karp K, Pussa T. 2011. The effect of genotype and rootstock on polyphenol composition of selected apple cultivars in Estonia. Agriculture 98, 63-70.

Mashayekhi K, Sadeghi H, Akbarpour V, Atashi S, Mousavizadeh S.J, Abshaei M, Nazari Z. 2013. Effect of some citrus rootstocks on the amount of biochemical composition of Parson Brown and Mars Oranges in Jiroft. Journal of Horticultral Science 27, 9-17.

Mccallum J.A, Walker J.R. 1990. Phenolic biosynthesis during grain development in wheat: changes in phenylalanine ammonia-lyase activity and soluble phenolic content. Journal of cereal science 11, 35–49.

Parvaneh T, Abedi B, Davarynejad G, Ganji Moghadam E. 2019. Correlation of Nutrient Content, Activity of Phenylpropanoid Pathway Enzymes and Secondary Metabolites in Two Iranian Red Flesh Apple Genotypes and Red delicious Cultivar on Different Rootstocks. Journal of Crops Improvement 22, 149-163.

Ruhmann S, Treutter D. 2003. Effect of N-nutrition in apple on the response of its secondary metabolism to prohexadione-Ca treatment. European Journal of Horticultural Science 68, 152-159.

Satisha J, Prakash G.S, Murti G.S.R, Upreti, K.K. 2005. Response of grape genotypes to water deficit: Root, shoot, growth and endogenous hormones. Indian journal of plant physiology 10, 225- 230.

Scalzo J, Politi A, Pellegrini N, Mezzetti B, Battino M. 2005. Plant genotype affects total antioxidant capacity and phenolic contents in fruit. Nutrition 21, 207-213.

Sobhana P, Thomsa M, Krishnakumar R, Jacob J. 2000. Can there be possible genetic conflict between genetically divergent rootstocks and scions in bud grafted plants. National Symposium on Recent Trends in Plant Science Research, University of Kerala, Trivandrum, India.

Steyn W.J, Wand S.J.E, Holcroft D.M, Jacobs G. 2002. Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytologist 155, 349-361.

Talukdar D. 2012. Exogenous calcium alleviates the impact of cadmium-induced oxidative stress in Lens culinaris Medic. Seedlings through modulation of antioxidant enzyme activities. Journal of Crop Science and Biotechnology 15, 325-334.

Tomar N.S, Agarwal R.M. 2013. Influence of treatment of *Jatropha curcas* L. leachates and potassium on growth and phytochemical constituents of wheat (*Triticum aestivum* L.). American Journal of Plant Sciences 4, 1134–1150.

Treutter D, eucht W. 1987. Accumulation of the flavonoid prunin in *Prunus avium/P. cerasus* grafts and its possible involvement in the process of incompatibility. nternational Symposium on Vegetative Propagation of Woody Species 227, 74-78.

Tsao R, Yang R, Young J.C, Zhu H. 2003. Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC). Journal of agricultural and food chemistry 51, 6347-6353.

Usenik V, Stampar F. 2000. Influence of various rootstocks for cherries on p-coumaric acid, genistein and prunin content and their involvement in the incompatibility process. Gartenbauwissenschaft 65, 245-250.

Xu W. Peng H, Yang T, Whitaker B, Huang L, Sun J, Chen P. 2014. Effect of calcium on strawberry fruit flavonoid pathway gene expression and anthocyanin accumulation. Plant physiology and biochemistry 82, 289-298.

Zucker M. 1965. Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. Plant Physiology 40, 779–784.