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IDENTIFICATION OF POLYPHENOLS IN HOMOGENETIC AND HETEROGENETIC COMBINATION OF CHERRY GRAFTINGS

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Abstract

The aim of the study is to determine the role of phenolic compounds in different cherry grafting combinations. For this purpose, 5 graft combinations of the Regina cultivar (Regina/*Prunus avium*, Regina/*Prunus mahaleb*, Regina/Gisela 5, Regina/Tabel Edabriz and Regina/MaxMa 14) were used. The phenolic substances found in the bark samples collected above and below the graft union were investigated one year after the graft's application. The bark samples were extracted and then analyzed using high-performance liquid chromatography (HPLC). The presence of phenolic compounds, such as prunin, procyanidin B1 and chlorogenic acid, are thought to play an active role in the graft fusion process. The accumulation of phenolic substances in the homogenetic combination (Regina/*Prunus avium)* was found to be lower than that in the heterogenetic combinations (Regina/Gisela 5, Regina/Tabel Edabriz and Regina/MaxMa14). The Regina/Gisela 5 combination posed more risk in terms of graft healing, while the Regina/MaxMa 14 combination yields similar results to that of rootstocks of the same origin.

Key words: Grafting, Heterogenetic graft, Homogenetic graft, Phenolic compounds, *Prunus avium*, Rootstock.

Introduction

Modern sweet cherry production cannot be conceived without the use of rootstocks (Milinovic *et al*., 2016). Successful grafting is influenced by the plant genetics, growth characteristics and physiological and biochemical factors. Differentiation of the callus into vascular tissue (xylem and phloem vessels) is the result of a complex developmental process involving structural and physiological changes that aim to restore the transport system. During this process, lignin is synthesized in the cells to become part of the transport system (Bidabadi *et al*., 2017). Phenolic compounds are important because of their role in lignification, which occur during the graft fusion process, and their indirect effects on other plant growth regulators (Errea *et al*., 2001; Mng'omba *et al*., 2008; Ozdemir *et al.*, 2018). When plant tissue is damaged, it produces a chemical response involving either the oxidation of the inner phenolic compounds or the production of mono- or polyphenolic compounds. Healthy cells adjacent to the damaged cells start the repair process and become active in the accumulation of key enzymes, simple phenolic compounds (chlorogenic acid) and polymeric compounds (lignin) (Usanmaz *et al.*, 2018; Sarafi *et al.*, 2018). Phenolic compounds have recently gained importance in the detection of graft incompatibility because of their role in lignification and a variety of biochemical reactions (Prabpree *et al*., 2018; Okatan, 2018). Physiological studies have fallen short in explaining graft incompatibility and has led to a focus on anatomical studies, which are based on investigating the samples collected from rootstocks, scions and different graft regions over different periods of time. Although anatomical studies have maintained their importance, their lack of success in explaining graft incompatibility has also been discovered and this has led researchers to search for faster and more reliable methods to understand graft incompatibility. The current methods involve biochemical analyses, which were initially performed using lethal substances at the graft union sites. After revealing the importance of phenolic compounds in various physiological and biochemical events, the role of phenolic compounds in graft incompatibility was also investigated.

An early and accurate detection of graft incompatibility is of great importance because it can avoid the use of incompatible graft combinations and help toward the selection of compatible combinations (Gökbayrak *et al*., 2007; Cavusoglu, 2018). Stress situations can lead to the accumulation of phenolic compounds, which have been implicated in the different mechanisms regarding to scion-stock relationship (Usenik & Stampar, 2002). Previous studies have shown that the phenolic compounds found in the phloem tissues change seasonally with respect to the developmental stage of the tree (Schwalb & Feucht, 1999; Usenik & Stampar, 2002; Okatan & Çolak, 2019). Some previous studies have suggested that the grafting process is also comprised of biochemical alterations related to the synthesis of phenolic compounds. Indeed, the synthesis of phenolic compounds can be triggered by stress situations, such as wounding and infection as well as the planting mechanism (Canas *et al*., 2015).

Regina is an important sweet cherry variety grown worldwide. The planting area for the Regina variety has rapidly proliferated and its varieties are grown using different rootstocks. In the present study, the major phenolic compounds found in the phloem, which may cause graft incompatibility within the Regina variety grafted using different homogenetic and heterogenetic rootstocks were determined. In addition, a graft healing will be examined with details for this cherry combinations.

Materials and Methods

Plant materials: The study was carried out in 2017. Phenolic compounds found in 1-year old cultivars of Regina floem, which were grafted onto *Prunus avium*, *Prunus mahaleb*, Gisela 5 (*Prunus cerasus* × *Prunus canescens*), Tabel Edabriz (clone of *Prunus cerasus*) and MaxMa14 (Brokforest, *Prunus avium* × *Prunus mahaleb)*

were determined. The characteristics of the Regina variety are given below (Garcia-Quero *et al*., 2017).

Origin: Jork, Germany.
Parentage: 'Schneiders Spate Knorpel ×'Rube'Knorpel' × 'Rube'.
Tree growth: Pyramidal with spreading branches and vigorous.
S-alleles: *S1S3*.
Productivity: Very good.
Blooming time: Very late.
Ripening time: 28–35 d after 'Burlat'
Fruit characteristics: Flat-round to round, medium to large, very firm and dark red.
Resistance/specifics: Very tolerant to cracking and *Monilinia* blossom blight. Susceptible to bacterial canker in certain environments.

The Regina/*Prunus avium* combination is homogenic. The Regina/ *Prunus mahaleb*, Regina/ Gisela 5 and Regina/ Tabel Edabriz combinations are heterogenetic and the Regina/MaxMa14 combination is 50% homogenetic.

Sample preparation and extraction of the phenolic compounds: Nine trees were selected for each scion/stock combination. The samples (4 cm below and above the graft zone) were taken with the razor blade. And samples were frozen in liquid nitrogen.

The polyphenol analyses were performed according to the procedure of (Usenik *et al*., 2006). The samples were extracted using acetone-water (80:20, v/v) containing Triton X-100 (0.4%) for 10 d at 4°C according to the procedure of (Treutter & Feucht, 1988). Triton X-100 (TX100) is one of the most widely used nonionicnon-ionic surfactants used to lyse cells for the extraction of protein and other cellular organelles, and to permeabilize living cell membranes for transfection. In a mortar, 10 mg of dry plant material was homogenized with 2 mL of the extraction solution. After the extraction, the solvents were removed in vacuo at 40°C and the residue dissolved in 2 mL of methanol. The samples were clarified using centrifugation at 6000 × g for 15 min and then filtered through a 0.45 µm membrane filter.

Chemicals: The following standards were used to quantify the individual phenolic compounds: Catechin (98%), chlorogenic acid (95%), prunin (95%), *p*-coumaric acid (98%), quarcetin (98%) and prociyanidin B1 (90%). The standards were purchased from Sigma-Aldrich and were of HPLC grade.

High performance liquid chromatography (HPLC): The different phenolics compounds were seperated by Thermo Surveyor PDA HPLC. This system equipped with a diode array detector and Thermo Hypersil column (100 mm, 4.6 mm, 5 mm i.d.).

The HPLC analyses were performed using the procedure of Usenik *et al*., (2006) with some modification. The mobile phase comprised of CH_3COOH-H_2O (10%, v/v; solvent A) and CH_3COOH-MeOH (v/v; solvent B). The gradient duration was 90 min at a rate of 1 mL with the flow rate and injection volume were 1 ml/min and 20 µl, respectively and UV detection at 280 nm. A chromatogram of cherry tree tissue is presented in Fig. 1.

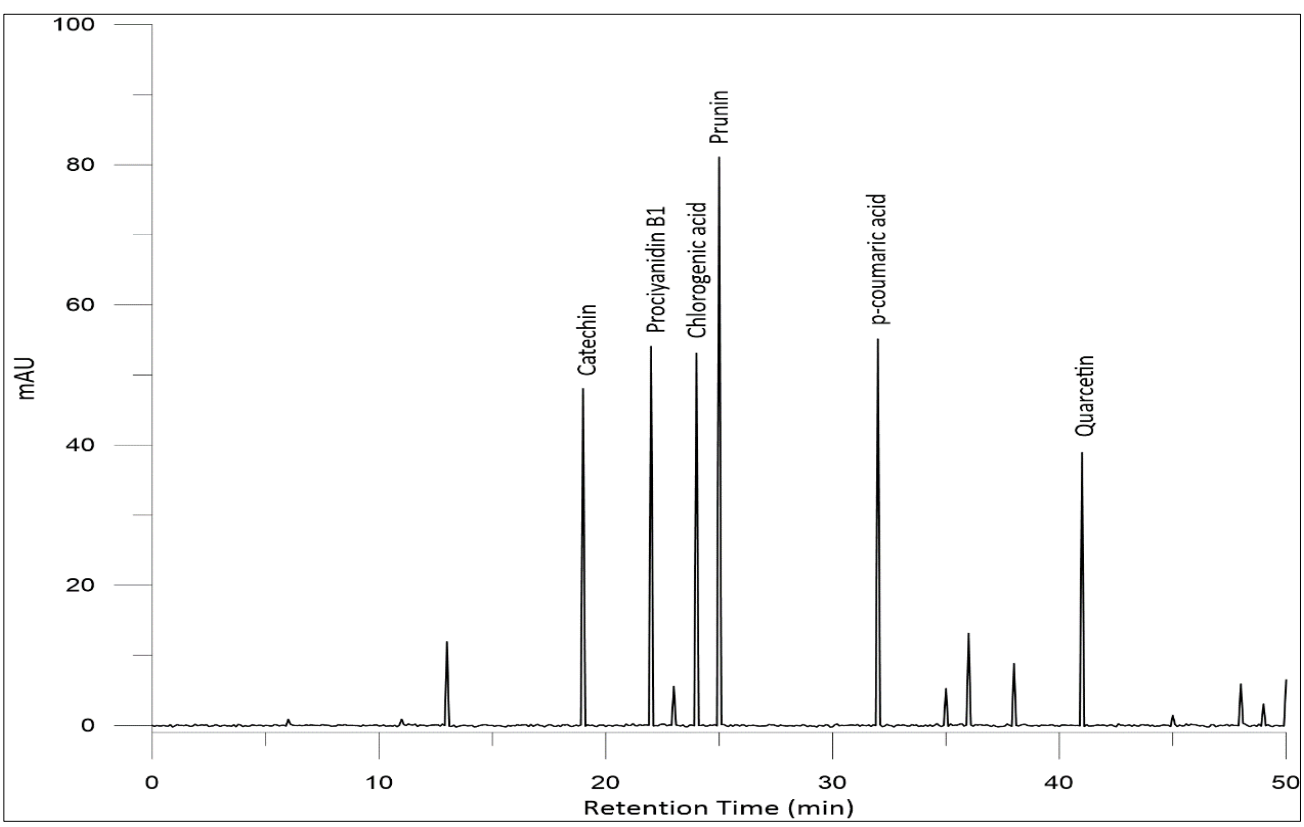
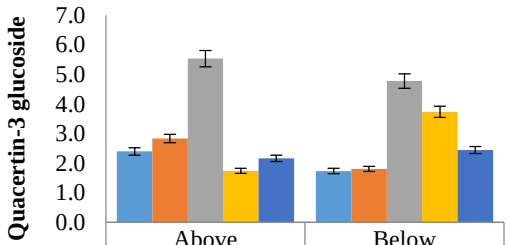
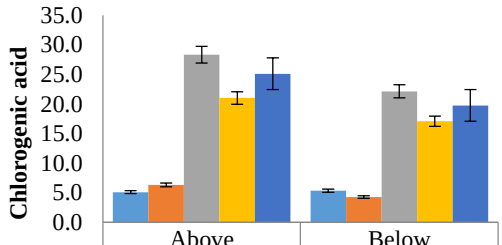


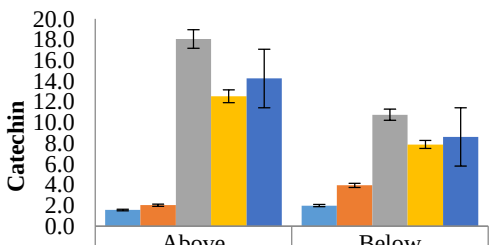
Fig. 1. A chromatogram of cherry grafting tissue. The mobile phase was comprised of CH_3COOH-H_2O (10%), %, v/v; solvent A) and CH_3COOH- MeOH. solvent B). The gradient duration was 90 min at a flow rate 1mlof 1 mL/min with UV detection at 280 nm.



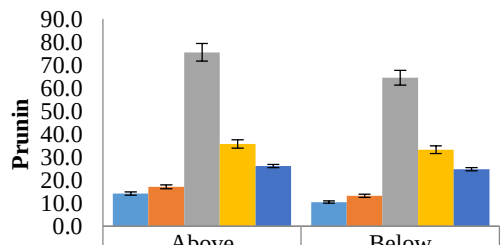
	Above	Below
Prunus avium	2.40 b*	1.74 d
Prunus mahaleb	2.84 b	1.81 d
Gisela 5	5.54 a	4.78 a
MaxMa14	1.75 d	3.74 b
Tabel Edabriz	2.17 c	2.45 c



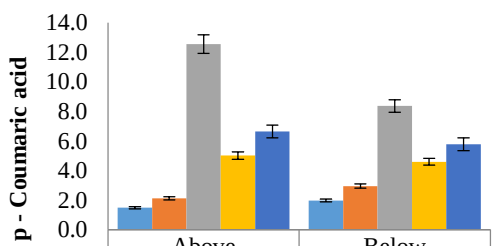
	Above	Below
Prunus avium	5.13 c	5.36 d
Prunus mahaleb	6.35 c	4.31 d
Gisela 5	28.36 a	22.15 a
MaxMa14	21.03 b	17.11 c
Tabel Edabriz	25.13 b	19.78 b



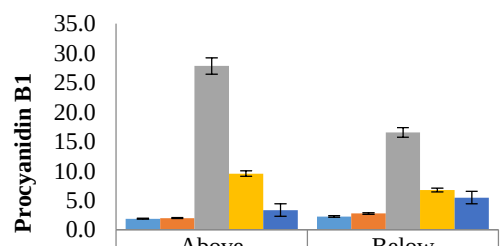
	Above	Below
Prunus avium*	1.56 d	1.98 d
Prunus mahaleb	2.02 c	3.94 d
Gisela 5	18.07 a	10.75 a
MaxMa14	12.54 b	7.88 c
Tabel Edabriz	14.25 b	8.62 b



	Above	Below
Prunus avium	14.11 e	10.45 d
Prunus mahaleb	17.01 d	13.16 d
Gisela 5	75.51 a	64.48 a
MaxMa14	35.71 b	33.22 b
Tabel Edabriz	26.13 c	24.75 c



	Above	Below
Prunus avium	1.48 d	1.97 d
Prunus mahaleb	2.12 d	2.95 d
Gisela 5	12.55 a	8.36 a
MaxMa14	5.01 c	4.59 c
Tabel Edabriz	6.64 b	5.78 b



	Above	Below
Prunus avium	1.87 c	2.25 d
Prunus mahaleb	1.98 c	2.76 d
Gisela 5	27.83 a	16.54 a
MaxMa14	9.54 b	6.75 b
Tabel Edabriz	3.34 c	5.46 c

Fig. 2. The content (mg/L) of quercetin, *p*-coumaric acid, prunin, catechin, chlorogenic acid and procyanidin B1 in the phloem above and below the graft union on the different rootstocks. The average values and standard error are presented. Sampling location: Above and below the graft union. * The values within a column followed by different letters are significantly different ($p<0.05$).

Statistics: The present study used a randomized block design (ANOVA) with three replicates; 3 saplings were used in each replicate. The statistical analyses were performed by SPSS software (V.18; Statistical software, SPPS. Inc., USA). Duncan's multiple range test was used to determine the significant differences between the mean values obtained for each group.

Results

Amount of phenolic contents are shown in Fig. 2. Quercetin-3 glucoside was present in significant amounts in all of the different combinations studied. The amount of *p*-coumaric acid observed in the homogenetic combination (Regina/*Prunus avium*) was quite low, while it was relatively high in the heterogenetic combinations studied.

A comparison between the different combinations of Regina cultivar with the different rootstocks studied showed that the highest values were obtained in the combination produced using the Gisela 5 rootstock. In particular, high amounts of certain phenolic substances such as prunin (75.51, 64.48 mg/L), chlorogenic acid (28.36, 22.15 mg/L) and catechin (18.07, 10.75 mg/L) were observed above and below the graft union, and are thought to play a key role in the graft incompatibility observed in the Regina/Gisela 5 combination.

The Regina/*Prunus avium* combination showed no difference in the phenolic compounds observed in the different bark samples. Among the phenolic compounds, prunin was the most common compound both below and above the graft union. Both procyanidin acid and chlorogenic acid have the second highest values in both graft partners. The total amount of phenolic compounds found in the Regina/Gisela 5 combination was high with prunin, procyanidin, chlorogenic and catechin found in high amounts (75.51, 28.36 and 27.83 mg/L, respectively), especially above the graft union.

Prunin and chlorogenic acid were determined to be the major phenolic compounds found in the Regina/Maxma 14 combination, both above and below the graft union. In the case of the homogenetic combinations, the amount of phenolic compounds deemed appropriate for use as markers in the MaxMa 14 rootstock was low. Unlike in the other combinations, catechin was the major phenolic compound in the Regina/Tabel Edabriz combination (26.13, 24.32 mg/L). Chlorogenic acid and prunin were found to be important for the detection of graft incompatibility. A general comparison of the graft combinations showed that the accumulation of phenolic compounds in the homogenetic combination was lower than that found in the heterogenetic combinations.

Discussion

The high tree mortality found in intensive orchard practices is directly related to lower yields and lower income. Several biochemical pathways near the graft union site, such as the metabolism of phenolic compounds, show strong effects (Feucht & Treutter 1991). *p*-Coumaric acid is an auxin-oxidase co-factor that plays an indirect role in graft healing. It is thought that, under stress conditions, prunin and genistein are synthesized from *p*-coumaric acid. Prunin and genistein accumulation interrupts ATP synthesis and thereby causes problems in indole-3-asetic acid (IAA) transport. In addition to inhibiting plant growth and development, phenolic acids inhibit IAA transport. The problems encountered in the differentiation of the sieve-tubes in incompatible combinations are linked with a decrease in the concentration of IAA. Externally, these metabolic events are reflected as symptoms of graft incompatibility and tree death (Usenik *et al*., 2006). Accordingly, the auxin-oxidase enzyme, a decomposer of IAA that has an important role in explaining the mechanism of dwarfing in plants, is encouraged by the increasing the amount of *p*-coumaric acid. Small quantities of decomposed auxin are carried to the graft elements, which results in even shorter trees.

In this study, the highest amount of *p*-coumaric acid was obtained in the dwarfing Regina/Gisela 5 combination. Among the investigated phenolic compounds, prunin was determined to be the most important phenolic compound and plays an important role in graft incompatibility, followed by chlorogenic acid and catechin, respectively (Figs. 1 and 2). In a previous study on grapes, the level of chlorogenic acid in the low-compatibility Sy383/ 110 R combination was low (Assuncao *et al.*, 2016) and this finding was also observed in this study. Phenolic compounds were deposited higher level at the above the graft zone. Güçlü & Koyuncu (2016), reported that in the combinations obtained upon grafting the '0900 Ziraat' cherry cultivar with different rootstocks, the amount of phenolic substances increased upon grafting and prunin could be used as a graft incompatibility indicator for cherry trees. In a previous study on the early detection of graft incompatibility in apricots, Luizet and Moniqui varieties used the scions of three apricot cultivars (Marianna 2624, Myrobalan 605 Ad and apricot rootstock A 843) as rootstocks and observed the amount of phenolic substances in the compatible combinations was very low, while it was quite high in the incompatible combinations (Errea *et al*., 2001). The high amount of *p*-coumaric acid in this combination caused dwarfing in the trees and difficulty in the differentiation of the sieve-tubes in the graft elements. The growth differences found between the graft elements and over-swelling at the graft union were attributed to the excess amount of *p*-coumaric acid in the Regina/Gisela combination. This difference between the graft elements occurs after grafting and disappears when the lignification process is completed. In other words, lower amounts of phenolic compounds are synthesized when the lignification process is completed, while higher amounts of phenolic compounds are synthesized during the lignification process because of the higher requirement for them. Thus, no problems hindering IAA transport are encountered and the transport process occurs normally. Graft combinations obtained by grafting rootstocks or scions of the same genetic origin are defined as homogenetic grafts. The combinations obtained upon grafting rootstocks and scions from different origins are defined as heterogenetic grafts; this approach has gained importance during the investigations on fruit tree graft incompatibility.The grafting partners often belong to the same species or genus, however, the use of genetically divergent genotypes is also common (Errea, 1998; Usenik *et al*., 2006). (Usenik & Stampar, 2001) have reported that the use of dwarfing rootstocks may be limited due to the incompatibility issues connected with grafting scions from different genetic origins. The concentrations of the polyphenol compounds found in heterogenetic *Prunus* grafts are different from those observed in their corresponding homogenetic grafts (Geibel & Feucht, 1982; Usenik & Stampar, 2000). In their study on pear trees, (Hudina *et al*., 2014a) reported that heterospecific grafts can be incompatible and the death of these seedlings may occur a few years after a successful grafting when using these combinations, which was in agreement with the results obtained in the present study. Another study on cherry trees reported that grafting the '0900 Ziraat' cherry cultivar with homospecific grafts was more successful (Güçlü, 2010). It has been reported that the amount of *p*-coumaric acid in the graft shoots of the homogenetic combinations obtained upon grafting the Lapins cultivar on F12/1 (*Prunus avium*) was lower than that found in the heterogenetic combinations (Usenik & Štampar, 2000). In addition, Mng'omba *et al*., (2008)

reported that the accumulation of *p*-coumaric acid found in the region above the graft union of heterogenetic combinations can cause incompatibility during their study on *Uapaca kirkiana*. In a previous study on *Prunus* phloem, the Royal Glory peach cultivar was grafted on rootstocks of various origins and it was reported that the flavanols were the most commonly phenolic substance found in the *Prunus persica* and *Prunus domestica* rootstocks. In the same study, it was found that, in terms of their graft incompatibility, the heterospecific combinations posed more risk than the homospecific combinations (Hudina *et al*., 2014b), which was in agreement with the results obtained in the present study.

In another study conducted on apricot trees by Usenik *et al*., (2006) an unknown phenolic compound was observed. The identity of this compound could be prunin, which has been shown to be an important phenolic compound in graft incompatibility during the present study. Graft incompatibility is a complex, anatomical, physiological and biochemical process that has been disputed for many years, however, its mechanism is not yet fully understood (Güçlü & Koyuncu, 2012). In the present study, prunin, chlorogenic acid and procyanidin B1, and subsequently catechin, were determined to be phenolic compounds suitable for determining the graft incompatibility of the combinations studied. Gainza *et al*., (2015) reported that the amount of flavonols, especially catechins and proanthocyanidins, increased shortly after grafting, which lead to the disruption of the growth of certain tissues (xylem and phloem). In addition, lower amounts of phenolic substances are accumulated in the homospecific combinations when compared to heterogenetic combinations. *Prunus avium* (mazzard) and *Prunus mahaleb* (mahaleb) are classical rootstocks. The accumulation of phenolic compounds in rootstock these rootstocks was lower when compared with the clonal rootstocks (Fig. 2). However, the phenolic content of *Prunus avium* and *Prunus mahaleb* were very similar with some phenolics of the phenolic compounds being found in lower amounts in *Prunus avium*. Graft incompatibility has been observed in *Prunus mahaleb* in the later growing periods, however, *Prunus avium* does not display any graft incompatibility. These differences may be attributed to the presence of an excessive amount of phenolic compounds. The study of the phenolic compounds in relation to clonal rootstock graft incompatibility has demonstrated the low accumulation of phenolic compounds in Maxma 14 and Tabel Edabriz (Fig. 2), whereas Gisela 5 showed increased levels of all the phenolic compounds studied.

The combination of the Regina cultivar with the Gisela 5 rootstock has been determined to pose a risk in terms of graft incompatibility. The results obtained from the bark samples collected above and below the graft union of the Regina/MaxMa 14 combination were close to the results obtained from the homogenetic combinations, which shows that this rootstock was compatible with the Regina cultivar. As mentioned above, since the response of each combination to grafting can differ, the present study should be continued under field conditions and further studies should be carried out using more cultivar/ rootstock combinations with further investigations carried out on the different phenolic compounds present.

Conclusions

Rootstocks for cherries are chosen from among taxa showing appropriate graft compatibility. Considering this criteria, *Prunus avium Prunus cerasus Prunus mahaleb* and *Prunus fruticosa* as well as their hybrids and some related taxa can be used as rootstocks (Hrotko & Rozpora, 2016). Graft incompatibility is a complicated anatomical and physiological process, and extensive studies to unravel its mechanism still continue (Gulen *et al*., 2005). Especially in *Prunus*, there is little knowledge and very few methods used to understand the metabolic events that occur during the development of the scion/rootstock union (Gainza *et al*., 2015). Methods developed for the early detection of possible incompatible combinations will prevent financial loss and delays. Moreover, a specified technique will allow early rootstock selection. In fruit breeding, these studies are focused on the combination of pear and quince. To the best of our knowledge, this study is the first for these cherry combinations and is expected to contribute significantly to science and fruit breeding in this area.

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