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Changes in Phytohormone and Antioxidant Enzyme Activities during the Healing Process of Different Crabapple Rootstock–Scion Combinations

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Abstract: By measuring the changes in phytohormones and related enzyme activities of *Malus* 'Huabiao' scions and different rootstocks during the healing process of grafting, this study aims to analyze the effects of different rootstocks on the physiological metabolism of the grafting healing site, thereby providing reference for the selection of *M.* 'Huabiao' grafting rootstocks. The live seedlings of 1-0 *M. hupehensis*, *M. robusta* and *M. baccata* were chosen as rootstocks, and *M.* 'Huabiao' scions were grafted with these seedlings in spring by pasting method. The dynamic changes in activities of indole acetic acid, zeatin riboside, methyl jasmonate content and peroxidase, polyphenol oxidase and L-phenylalanine ammonia-lyase at the healing site of grafting were measured, and the effects of different grafting combinations on the phytohormone and related enzyme activities at the grafted site were investigated. It was found that the *M.* 'Huabiao'/*M. hupehensis* and *M.* 'Huabiao'/*M. robusta* grafting combinations had significantly higher survival rate than *M.* 'Huabiao'/*M. baccata* which only had a success rate of 63%. At the beginning of graft healing, the contents of three phytohormones in healing sites of all the three grafting combinations exhibited a significant decrease. However, in the mid and late stages, great differences were shown in phytohormone content. During the grafting healing process, the changes in three enzyme activities in healing sites of the three grafting combinations showed some regularities. In conclusion, *M. hupehensis* and *M. robusta* have strong grafting compatibility with *M.* 'Huabiao'; thus, they are suitable to be selected as rootstocks for grafting and propagation of *M.* 'Huabiao'. In addition, the changes in phytohormone content and enzyme activity in different grafting combinations reflect the strong affinity relationship between rootstock and scion grafting.

Keywords: *Malus* 'Huabiao'; graft; healing; phytohormones; related enzyme activity

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1. Introduction

As an important asexual propagation technique, grafting has the advantages of maintaining the excellent genetic characteristics and high reproduction coefficient of the parent; therefore, it is widely used in the production of woody ornamental plant seedlings [1]. In the process of grafting propagation, it is of vital importance to select rootstocks and scions with high affinity. However, such affinity can be affected by various factors. Two of these are phytohormones and related enzymes, and their relationship with grafting affinity has been extensively reported [2–6]. Phytohormones are one of the important factors affecting the affinity of rootstock grafting [2], among which, indoleacetic acid IAA is the main hormone affecting graft healing [3], and other hormones such as gibberellin GA_3, abscisic acid ABA and zeatin riboside ZR have direct or indirect impacts on the graft healing process. Plant-related enzyme activities can reflect the strength of grafting affinity to some extent [4], among which, the activities of peroxidase POD, polyphenol oxidase

PPO and phenylalanine aminolytic enzyme PAL can be regarded as reference standards for judging rootstock and scion affinity at the early stage of grafting [5,6].

Malus 'Huabiao' is an excellent narrow-crowned ornamental crabapple variety independently selected and bred by Nanjing Forestry University. In December 2022, it was certified as a good forest tree species by the Forest Tree Judging Committee of Jiangsu Province (Su S-SV-MH-011-2022). With outstanding narrow-crown characteristics, it has an ornamental value of peculiar tree shape and enables co-appreciation of flowers and fruits. In addition, the angle between its branches and main branches is not more than 30°. Therefore, it can be cultivated as a flowering hedge and has great potential for popularization and application. Grafting can be adopted to promote the propagation of *Malus* 'Huabiao'; however, it also faces the problem of rootstock selection. At present, there have been some literature reports on the effects of different rootstock combinations and grafting methods on the survival rate of crabapples [7], but few were found on the interaction between plant hormones and related enzyme activities during graft healing of crabapples. In this study, seedlings of *M. hupehensis*, *M. robusta* and *M. baccata* were used as rootstocks for grafting *M.* 'Huabiao' scions, and the changes in phytohormone contents and related enzyme activities in the healing parts of different grafting combinations were compared to explore their relationship with the affinity of rootstock–scion grafting. It is hoped that this study can provide a scientific basis for the selection of grafting rootstocks for *M.* 'Huabiao'.

2. Materials and Methods

2.1. Test Site Overview

Crabapple grafting experiments were conducted at the teaching and research base of Nanjing Forestry University in Baima (Baima Town, Lishui District, Nanjing), which belongs to a transition zone from the northern subtropics to the central subtropics, with an average annual temperature of 15.4 °C, an annual frost-free period of 237 d, an average annual sunshine time of 2420 h and an average annual rainfall of 1087.4 mm.

2.2. Materials

The rootstocks used for grafting were seedlings of 1-0 *M. hupehensis*, *M. robusta* and *M. baccata*. The seedlings of *M. hupehensis* were from Linyi City, Shandong Province, with an average height of 90 cm and an average ground diameter of 0.8 cm. The seedlings of *M. robusta* originated from Shijiazhuang City, Hebei Province, with an average height of 60 cm and an average ground diameter of 1.0 cm, and those of *M. baccata* were from Yingkou City, Liaoning Province, with an average height of 60 cm and an average ground diameter of 0.6 cm. In late December 2021, these three kinds of grafted rootstock seedlings were planted in rows 10 cm apart and with 40 cm spacing between the rows in the experimental site. After planting, the seedlings were watered and were cut off to keep 50 cm in length. The grafting scions were selected from branches drawn from *M.* 'Huabiao' in the same year, with an average length of 60 cm and an average thickness of 0.5 cm. Collected in early January of 2022, they were wrapped with wet newspaper and clingfilm, and stored in a freezer at 3–5 °C for backup. On 7 March 2022, two skilled grafting workers were selected and the grafting was carried out by bud grafting method.

2.3. Experimental Design

The grafting experiments were conducted in a single-factor randomized complete block design, and three grafting combinations of *M.* 'Huabiao' /*M. hupehensis* (hereinafter referred to as 'HH'), *M.* 'Huabiao'/*M. robusta* (hereinafter referred to as 'HR') and *M.* 'Huabiao'/*M. baccata* (hereinafter referred to as 'HB') were arranged. In total, 600 plants were grafted in each combination, among which half were used for survival rate counting and half for physiological indexes measurement. The survival rate of the three combinations was counted on the 40th day after grafting, and 100 grafts were counted in each combination with three repetitions. The criteria for a successful grafting should be well-healed scions and full and swollen buds which started to sprout (Figure 1). At 9:00 a.m. on 1st, 4th, 7th, 10th, 13th, 16th, 19th, 22nd, 25th and 28th days after grafting, the grafted parts of each combination were cut and used as test samples. For each sampling, 30 grafted sections of each combination were cut and brought back by preservation in liquid nitrogen to the seed center laboratory of Nanjing Forestry University at 3:00 p.m. and stored in a refrigerator at −80 °C for storage materia.

(**a**) (**b**)

Figure 1. (**a**) Successful grafting. (**b**) Failed grafting.

2.4. Methods for the Determination of Physiological Indicators

In each grafting combination, 15 grafted segments from each sampling were randomly selected, and the grafting healing site was cut into small pieces with scissors and ground with liquid nitrogen as a mixed sample to test relevant phytohormone. IAA, methyl jasmonate MeJA and ZR contents were determined by enzyme-linked immunosorbent assay and conducted by relevant laboratories of China Agricultural University. The extraction, purification and determination of endogenous levels of IAA, MeJA and ZR by an indirect ELISA technique were performed as follows. The samples were homogenized in liquid nitrogen and extracted in cold 80% methanol with butylated hydroxytoluene (1 mmol·L^{-1}) overnight at 4 °C. The extracts were collected after centrifugation at 10,000× g (4 °C) for 20 min, passed through a C_{18} Sep-Pak cartridge and dried in N_2. The residues were dissolved in PBS (10 mmol·L^{-1}, pH 7.4) in order to determine the levels of IAA, MeJA and ZR. Microtitration plates were coated with synthetic IAA, MeJA and ZR conjugates in $NaHCO_3$ buffer (50 mmol·L^{-1}, pH 9.6), and left overnight at 37 °C. After incubation for 30 min at 37 °C, standard IAA, MeJA and ZR samples and antibodies were added and incubated for a further 45 min at 37 °C. Then, horseradish-peroxidase-labeled goat antirabbit immunoglobulin was added to each well and incubated for 1 h at 37 °C. Finally, the buffered enzyme substrate (orthophenylenediamino) was added and the enzyme reaction was carried out in the dark at 37 °C for 15 min, then terminated using 3 mol·L^{-1} H_2SO_4. The absorbance was recorded at 490 nm. The logit curve was used for ELISA result calculation. The abscissas of the curve represent the natural logarithm of each concentration (ng·mL^{-1}) of hormone standard sample and the ordinates represent the logit value of each concentration absorbance. The calculation method for logit value is as follows:

$$\text{Logit}\left(B\cdot B_0^{-1}\right) = \ln\frac{B\cdot B_0^{-1}}{(1-B)\cdot B_0^{-1}} = \ln\frac{B}{B_0 - B}$$

B_0 is the absorbance of the 0 ng·mL^{-1} well and B is the absorbance of other concentrations. The natural logarithm of the hormone concentration (ng·mL^{-1}) of the sample to be tested can be found from the graph according to the logit value of its absorbance and then the concentration (ng·mL^{-1}) of the hormone can be known through the inverse logarithm. Finally, the content (ng·g^{-1}) of the sample is calculated by the concentration.

In this study, the crossreactivity of IAA antibody with 1-naphthlcetic acid was 16.7% and the crossreactivity with methyl 2-(1H-indol-3-yl)acetate, indole-3-butyric acid, 1-naphthlcetic acid was less than 1.3%. The crossreactivity of ZR antibody with zeatin was 47% and the crossreactivity with DL-dihydrozeatin, 6-furfurylamino purine, 6-Benzylamino purine was less than 0.05%. The crossreactivity of MeJA antibody with jasmonic acid and 12-oxy-phytodienoic acid was 2.3% and 1.2%, respectively. The other analytes showed less than 0.5% crossreactivities. The percentage recovery of each hormone was calculated by adding known amounts of standard hormone to a split extract. The recoveries of IAA, ZR and MeJA were 95.1%, 86.1% and 92.2%, respectively.

In each grafting combination, 15 grafted segments from each sampling were randomly selected, and the peripheral shoot tissues, phloem and cambium of the grafting healing site were scraped with a scalpel blade as a mixed sample to test relevant enzyme activities. POD activity was determined by the method proposed by Wang Xuekui [8]. The sample weighing 500 mg was homogenized in ice-water bath with 6 mL phosphate buffer (pH 7.0, 4 °C, 100 mmol·L^{-1}), then centrifuged at 4 °C, 10,000× g for 10 min, the supernatant was collected and then the sample was quickly cooled in liquid nitrogen for analysis of POD enzyme activity. The POD activity was determined by measuring the increase in absorbance at 470 nm using guaiacol as a phenolic substrate with hydrogen peroxide. The reaction mixture contained 0.5 mL of 1.5% guaiacol, 0.2 mL of 1% H_2O_2, 2.1 mL of 100 mmol·L^{-1} phosphate buffer (pH 6.0) and 0.2 mL of the enzyme extract. The maximal initial reaction velocity was calculated over 20 to 320 s linear increase in absorbance. The amount of enzyme required for absorbance change of 0.01 per 1min was used as an active unit. PPO activity was carried out by Liu Wei [9]. The sample weighing 500 mg was homogenized in ice-water bath with 10 mL phosphate buffer (pH 6.8, 4 °C, 200 mmol·L^{-1}), then centrifuged at 4 °C, 10,000× g for 20 min and the supernatant was collected. The reaction mixture included 0.2 mL of 20 mmol·L^{-1} catechol and 2.6 mL of 100 mmol·L^{-1} phosphate buffer (pH 6.8), which were incubated at 30 °C. Then, 0.2 mL of enzyme solution was added to the reaction mixture to initiate the enzyme reaction. The mixed solution was allowed to stand for 5 min at 30 °C. The absorbance of the mixture within 300 s at 420 nm was monitored immediately. One unit of enzyme activity was defined as the amount of enzyme causing a change of 0.001 in absorbance at 420 nm per minute at fixed temperature. PAL activity was monitored by the method put forward by Qiu Feng [10]. A 100 mg of sample was ground in 3 mL potassium borate buffer (pH 7.8 4 °C, 200 mmol·L^{-1}). The homogenate was then centrifuged at 4 °C, 10,000× g for 10 min, and the supernatant constitutes the enzymatic extract. The reaction mixture consisted of 0.5 mL of enzymatic extract, 2 mL of 50 mmol·L^{-1} potassium borate buffer (pH 8.7) and 1 mL of 10 mmol·L^{-1} L-phenylalanine. After incubation at 37 °C for 30 min, absorbance was determined at 290 nm. The amount of enzyme required for absorbance change of 0.01 per 1 hour was used as an active unit. The above measurements were conducted for three times.

2.5. Data Processing and Analysis

Excel 2016 and SPSS 26 software were employed for statistics and analysis of data. Origin 2022 software was used for plotting.

3. Results

3.1. Variations in the Survival Rates of Seedlings from Different Rootstock–Scion Grafting Combinations

On one hand, the rootstock–scion affinity is a key factor in grafting success and, on the other, the survival rate is a key indicator for the determination of rootstock–scion grafting affinity. Therefore, the survival rate can be regarded as an indicator for the affinity of rootstock–scion grafting. There were significant differences ($P < 0.05$) in the survival rates of seedlings from different rootstock–scion grafting combinations and the survival rates followed the order of HR > HH > HB. HR has the highest survival rate of 83.33%, which was significantly higher than that of HB. The difference between HR and HH was not so significant as that between HH and HB (Figure 2). In short, different rootstocks show different affinities when grafted with *M.* 'Huabiao' scions and the survival rate tells that HR has the strongest grafting affinity, followed by HH and HB.

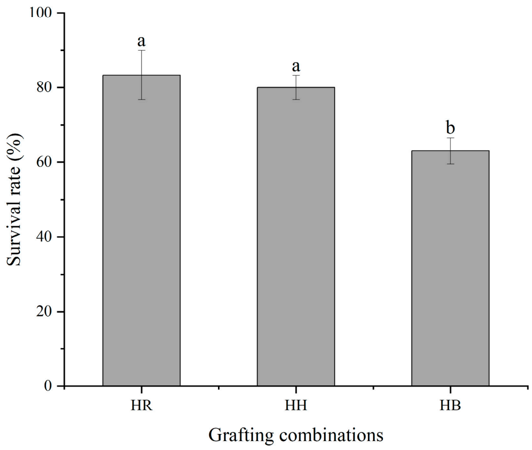

Figure 2. Statistical results for the survival rate of different grafting combinations. (Note: lower case letters indicate differences at the level of 0.05.)

3.2. Determination of Phytohormone Content in Healing Parts of Different Rootstock–Scion Combinations

To determine the IAA, MeJA and ZR contents in basts, the healing sites of different grafting combinations were sampled regularly and the hormone measurements for each grafting combination at different sampling times were analyzed by ANOVA (Table 1).

Table 1. ANOVA for phytohormone contents of healing sites in different grafting combinations.

Types of Hormones	Source of Variation	Degree of Freedom	Mean Square	Value of F	Value of *P*
IAA	Grafting combinations	2	10.140	42.830 **	0.000
	Sampling time	9	12.471	52.674 **	0.000
	Grafting combinations × Sampling time	18	11.973	50.572 **	0.000
MeJA	Grafting combinations	2	308.924	4385.058 **	0.000
	Sampling time	9	15.803	224.315 **	0.000
	Grafting combinations × Sampling time	18	18.319	260.035 **	0.000
ZR	Grafting combinations	2	0.135	9.248 **	0.000
	Sampling time	9	0.892	61.023 **	0.000
	Grafting combinations × Sampling time	18	0.560	38.295 **	0.000

Note: ** $P < 0.01$. Same as below.

3.2.1. Changes in IAA Content in Healing Parts of Different Rootstock–Scion Combinations

The effects of grafting combination, sampling time, and the interaction between grafting combination and sampling time on IAA contents of the healing parts of scions reached a highly significant level ($P < 0.01$), indicating that the differences in rootstock–scion combinations had a highly significant effect on IAA contents of the healing parts and there were large differences in the changes in IAA contents of the healing parts during the grafting healing process (Table 1).

The IAA contents of the healing parts of three grafting combinations showed an overall w-type trend of decreasing, increasing, decreasing and increasing (Figure 3). HR showed a decreasing trend from the first to the seventh day after grafting till the lowest value of 16.86 ng·g^{-1} was reached. From the 7th to the 16th day, it had a fluctuated increase, during which a sharp rise happened from the 13th to the 16th day till the highest value of 21.69 ng·g^{-1} was achieved. After the 16th day, the IAA content of the healing site of HR showed a decreasing and increasing trend again and reached a second peak on the 25th day and remained at a high level after a slight decrease on the 28th day. The IAA content in the healing parts of HH showed a constant decreasing trend after grafting until the lowest value of 15.46 ng·g^{-1} was reached on the 10th day, since when it went through a sudden and sharp increase. On the 13th day, it reached a peak and remained at a high level until a significant drop on the 22nd day ($P < 0.05$). After the 22th day, it started to rise sharply again till the highest value of 24.18 ng·g^{-1} on the 25th day. On the 28th day, similar to that of HR, the change in IAA content of HH witnessed a slight decrease but still remained at a high level. In conclusion, compared to those of the other two combinations, the IAA content of HB went through a milder change in the early healing period. However, a sudden and significant decrease started from the 19th day for HB ($P < 0.05$) and soon the lowest value (15.67 ng·g^{-1}) was reached on the 25th day, which was 84.4% of the value measured on the 1st day after grafting (Table 2). The above analysis shows that the IAA contents of the healing parts of all three grafting combinations decreased significantly ($P < 0.05$) in the early stage of grafting healing and remained relatively high in the mid stage. However, when it comes to the later stage, the IAA contents of different grafting combinations vary greatly. That of HB decreased to a relatively low level, while those of HR and HH remained high.

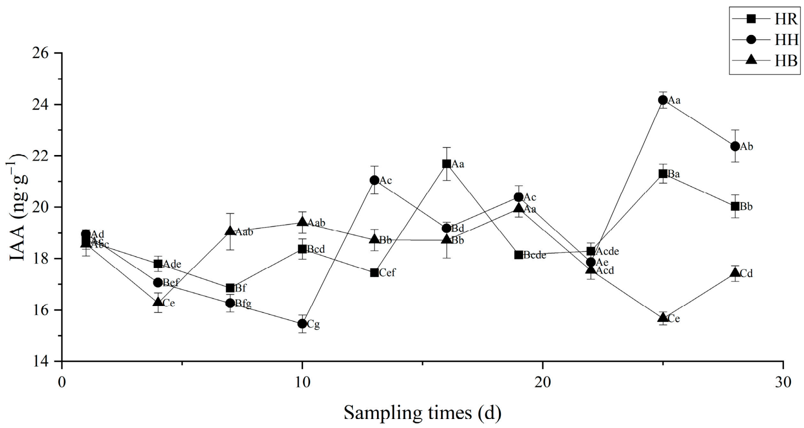

Figure 3. Results of IAA content in graft healing sites. (Note: capital letters indicate differences at the 0.05 level between physiological indicators of different grafting combinations for the same sampling time; lowercase letters indicate differences at the 0.05 level between physiological indicators of the same grafting combinations for different sampling times).

Table 2. Percentage changes in IAA content at different sampling times in relation to the first day.

Grafting Combinations	Percentage Changes in IAA Content at Different Sampling Times (%)								
	4 d	7 d	10 d	13 d	16 d	19 d	22 d	25 d	28 d
HR	95.1	90.1	98.2	93.3	116.0	97.0	97.8	113.9	107.1
HH	90.0	85.8	81.6	111.1	101.2	107.6	94.3	127.5	118.1
HB	87.7	102.6	104.6	100.9	100.9	107.4	94.5	84.4	93.8

3.2.2. Changes in MeJA Content in Healing Parts of Different Rootstock–Scion Combinations

The effects of grafting combination, sampling time, and the interaction between grafting combination and sampling time on the MeJA contents of the healing parts of scions reached a highly significant level ($P < 0.01$), indicating that the differences in rootstock–scion combinations had a highly significant effect on the MeJA contents of healing parts. During the process of grafting healing, the MeJA contents of the healing parts also varied greatly (Table 1).

The HR and HH combinations went through a significant decrease ($P < 0.05$) in the MeJA contents of the healing parts from the first to the seventh day and from the first to the fourth day after grafting. Afterwards, the MeJA contents of the healing sites of both grafting combinations started to increase till the 16th day of grafting, since when both continued to have a simultaneous decline and finally started to rise sharply on the 19th and 22nd day respectively till the 28th day (Figure 4). For HR and HH, the first peaks of MeJA content in the healing sites were achieved on the 16th day after grafting with the value of 12.56 ng·g^{-1} and 14.37 ng·g^{-1}, respectively. Their highest values occurred on the 28th day with 18.48 ng·g^{-1} and 15.14 ng·g^{-1}, respectively, which were 41.0% and 17.2% higher than those measured on the first day after grafting (Table 3). In terms of the HB combination, its MeJA content of the healing site exhibited a linear decrease from the first to the fourth day and a significant increase ($P < 0.05$) after the seventh day. After a continuous decline, it went through a significant rise and fall on the 16th and the 22nd day, respectively. From the 22nd day, there was a continuous decrease and it lasted till the 28th day when the lowest value of 3.86 ng·g^{-1} was reached, and it was only 32.6% of the value measured on the first day after grafting (Table 3). In conclusion, the MeJA contents of the healing parts of three grafting combinations went through a decrease in the early stage of grafting healing and a gradual increase in the mid stage. When it comes to the later stage, those of the HH and HR combinations showed a rapid increase whereas that of HB showed a gradual decreasing trend. At all sampling time points, those of HH and HR were significantly ($P < 0.05$) higher than that of HB.

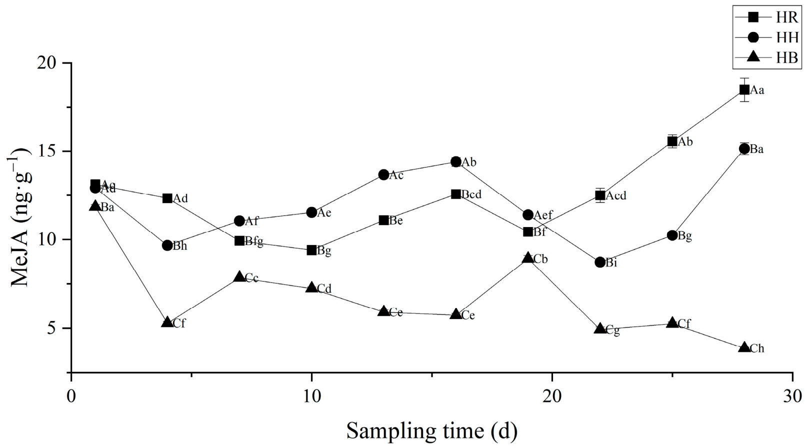

Figure 4. Results of MeJA content in graft healing sites. (Note: capital letters indicate differences at the 0.05 level between physiological indicators of different grafting combinations for the same sampling time; lowercase letters indicate differences at the 0.05 level between physiological indicators of the same grafting combinations for different sampling times).

Table 3. Percentage changes in MeJA content at different sampling times in relation to the first day.

Grafting Combinations	Percentage Changes in MeJA Content at Different Sampling Times (%)								
	4 d	7 d	10 d	13 d	16 d	19 d	22 d	25 d	28 d
HR	94.0	75.7	71.7	84.6	95.8	79.6	95.3	118.6	141.0
HH	74.8	85.6	89.3	105.8	111.3	88.3	67.6	79.3	117.2
HB	44.5	66.2	61.1	49.8	48.4	75.4	41.6	44.3	32.6

3.2.3. Changes in ZR Content in Healing Parts of Different Rootstock–Scion Combinations

The effects of grafting combination, sampling time, and the interaction between grafting combination and sampling time on the ZR contents of the healing parts of scions reached a highly significant level ($P < 0.01$), indicating that the differences in rootstock–scion combinations had a highly significant effect on the ZR contents of the healing parts.

In addition, there were large differences in the changes in ZR contents of the healing parts during the grafting healing process (Table 1).

For all three grafting combinations, the changes in ZR contents in the healing sites showed a fluctuating trend (Figure 5). From the first to the seventh day after grafting, all of the three grafting combinations exhibited a significant decrease ($P < 0.05$), during which both HR and HB reached the lowest values on the seventh day with 3.77 ng·g^{-1} and 3.55 ng·g^{-1}, respectively. From the 7th to the 22nd day after grafting, the ZR content of the healing site of HR showed an overall trend of increasing, decreasing and increasing (Table 4). The 22nd day witnessed the highest value of 5.45 ng·g^{-1}. The HH combination presented the same trend from the 7th to the 25th day after grafting and the 25th day marked the highest ZR value of 4.85 ng·g^{-1}. Likewise, HB went through a trend of increasing, decreasing and increasing from the 7th to the 19th day after grafting, and the highest ZR value (5.04 ng·g^{-1}) occurred on the 19th day. The 28th day witnessed a striking decrease for HH and HR ($P < 0.05$) and a remarkable increase for HB ($P < 0.05$). In short, the ZR contents of the healing parts of all three grafting combinations showed a gradual decrease in the early stage of graft healing, and a general trend of increasing, decreasing and increasing in the mid phase. At the late stage, the contents of HR and HH decreased significantly ($P < 0.05$), while that of HB maintained high.

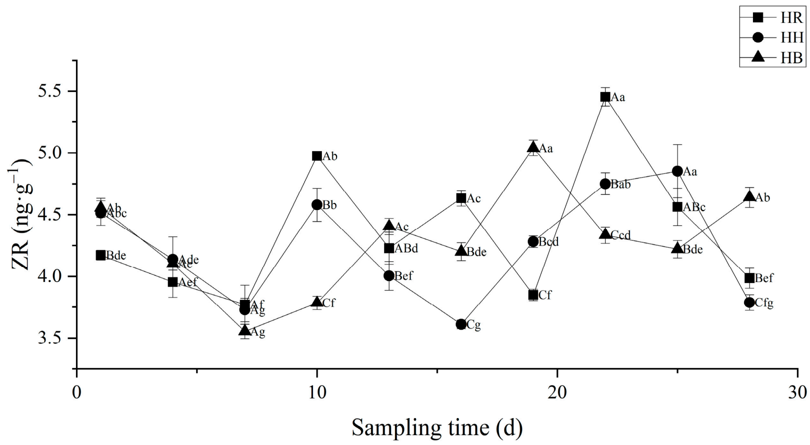

Figure 5. Results of ZR content in graft healing sites. (Note: capital letters indicate differences at the 0.05 level between physiological indicators of different grafting combinations for the same sampling time; lowercase letters indicate differences at the 0.05 level between physiological indicators of the same grafting combinations for different sampling times).

Table 4. Percentage changes in ZR content at different sampling times in relation to the first day.

Grafting Combinations	Percentage Changes in ZR Content at Different Sampling Times (%)								
	4 d	**7 d**	**10 d**	**13 d**	**16 d**	**19 d**	**22 d**	**25 d**	**28 d**
HR	94.8	90.3	119.3	101.3	111.6	92.2	130.7	109.4	95.5
HH	91.7	82.6	101.5	88.7	80.0	94.8	105.3	107.5	83.9
HB	90.0	78.0	83.0	96.7	92.2	110.6	95.1	92.6	101.9

3.3. Determination of Related Enzyme Activity in the Healing Parts of Different Rootstock–Scion Combinations

The healing sites of different grafting combinations were sampled regularly for determination of POD, PPO and PAL activity in the bast. In addition, the related enzyme activity measurements for each grafting combination at different sampling times were analyzed by ANOVA (Table 5).

Table 5. ANOVA for enzyme activities of healing sites in different grafting combinations.

Types of Enzyme	Source of Variation	Degree of Freedom	Mean Square	Value of F	Value of P
POD	Grafting combinations	2	35,051,076.01	572.535 **	0.000
	Sampling time	9	966,883.422	15.793 **	0.000
	Grafting combinations × Sampling time	18	348,802.409	5.697 **	0.000
PPO	Grafting combinations	2	1824.490	121.748 **	0.000
	Sampling time	9	282.366	18.842 **	0.000
	Grafting combinations × Sampling time	18	51.580	3.442 **	0.000
PAL	Grafting combinations	2	876.481	174.566 **	0.000
	Sampling time	9	90.902	18.105 **	0.000
	Grafting combinations × Sampling time	18	84.235	16.777 **	0.000

Note: ** $P < 0.01$.

3.3.1. Changes in POD Activity in Healing Parts of Different Rootstock–Scion Combinations

The effect of grafting combination, sampling time and the interaction between grafting combination and sampling time on the POD activity of the healing parts of scions reached a highly significant level ($P < 0.01$), indicating that the differences in rootstock combinations have a highly significant effect on the POD activity of the healing parts. In addition, during the process of grafting healing, the POD activity of the healing parts at different sampling times also varied greatly (Table 5).

The POD activity of the healing parts of HB combination showed an increasing trend from the 1st to the 10th day after grafting and the first peak (3642.80 $U \cdot g^{-1} \cdot min^{-1}$) occurred on the 10th day, which was 21.6% higher than the value measured on the 1st day (Table 6). From the 10th day after grafting, it began to decrease till the lowest value (3036.13 $U \cdot g^{-1} \cdot min^{-1}$) was reached on the 16th day and it was not significantly different from the value measured on the 1st day after grafting. Since then, the POD activity of the healing site began to increase slowly. On the 28th day, it reached the highest value which was slightly higher than the peak on the 10th day after grafting (Figure 6). The HR combination exhibited a significant increase from the 1st to the 10th day after grafting ($P < 0.05$). Since a slight decrease on the 13th day, it had begun to rise gently until the highest value (6417.87 $U \cdot g^{-1} \cdot min^{-1}$) on the 22nd day, which was 54.0% higher than that measured on the 1st day after grafting (Table 6). From the 22nd day, the POD activity of the healing site of HR started to decrease and remained stable till the end of sampling (Figure 6). From the first to the seventh day after grafting, the POD activity of the healing part of HH presented a decreasing trend. From the 7th to the 13th day, there was an increasing and decreasing process. From the 13th to the 25th day, it went through a steady increase till the highest was achieved (5145.33 $U \cdot g^{-1} \cdot min^{-1}$), which was 18.6% higher than that measured on the 1st day of grafting. From the 25th day to the end of sampling, there was a trend of significant decrease (Figure 6). The above analysis indicates that, at both early and late stages of grafting healing, the POD activity of the healing parts of all three grafting combinations would reach one peak each stage and the change was relatively flat in the mid stage. Those of HR and HH at all sampling time points were significantly ($P < 0.05$) higher than that of HB. HR has the highest mean value of POD activity, followed by HH and HB.

Table 6. Percentage changes in POD activity at different sampling times in relation to the first day.

Grafting Combinations	Percentage Changes in POD Activity at Different Sampling Times (%)								
	4 d	7 d	10 d	13 d	16 d	19 d	22 d	25 d	28 d
HR	118.2	138.7	128.2	131.0	136.4	144.6	154.0	140.2	141.6
HH	102.3	96.9	112.7	100.0	104.1	105.2	110.0	118.6	101.3
HB	105.7	112.6	121.6	115.3	101.3	111.3	121.5	121.9	122.6

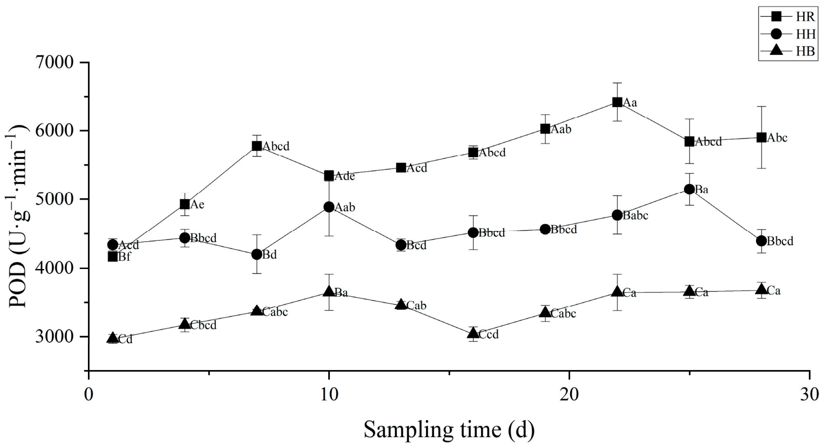

Figure 6. Results of POD activity in graft healing sites. (Note: capital letters indicate differences at the 0.05 level between physiological indicators of different grafting combinations for the same sampling time; lowercase letters indicate differences at the 0.05 level between physiological indicators of the same grafting combinations for different sampling times).

3.3.2. Changes in PPO Activity in Healing Parts of Different Rootstock–Scion Combinations

The effect of grafting combination, sampling time, and the interaction between grafting combination and sampling time on the PPO activity of the healing parts of scions reached a highly significant level ($P < 0.01$), indicating that the differences in rootstock combinations have a highly significant effect on the PPO activity of the healing parts. In addition, during the process of grafting healing, the PPO activity of the healing parts at different sampling times also varied greatly (Table 5).

The PPO activity of the healing parts of the HR and HH combinations showed a trend of rising, falling, rising and falling (Figure 7). The first peaks for both occurred on the seventh day after grafting with the value of 154.44 U·g^{-1}·min^{-1} and 145.83 U·g^{-1}·min^{-1}, respectively. Compared with that measured on the first day after grafting, the former was 8.3% higher, while the latter had no significant difference ($P > 0.05$) (Table 7). From the 7th day, the PPO activities for both exhibited a decreasing trend and a second increase happened on the 10th and the 16th day for HR and HH respectively. The highest values for both combinations occurred on the 22nd day after grafting, which were 16.8% and 10.3% higher than that measured on the 1st day, respectively (Table 7). From the 22nd day to the end of sampling, the PPO activity in the healing site of both combinations showed a trend of decreasing and then increasing. The HB combination showed gradual increases in the PPO activity in healing sites, with a significant ($P < 0.05$) one from the first to the seventh day and a relatively slow one after the seventh day. The 25th day marked a maximum value of 147.83 U·g^{-1}·min^{-1}, which was 17.0% higher than that measured on the first day after grafting (Table 7). In conclusion, HR and HH combinations went through a similar trend in the PPO activity of the healing parts. In the mid stage of grafting healing, both dropped to a low level which had no significant ($P > 0.05$) difference with that measured on the first day after grafting. In addition, the HB combination showed a steadily increasing trend throughout the graft healing process.

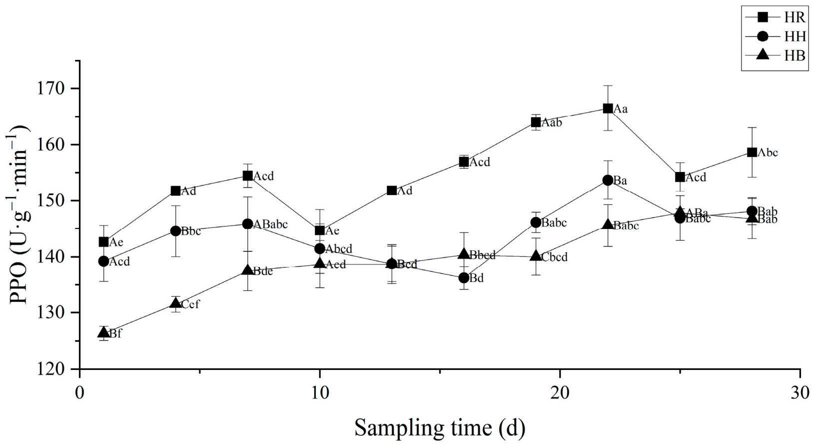

Figure 7. Results of PPO activity in graft healing sites. (Note: capital letters indicate differences at the 0.05 level between physiological indicators of different grafting combinations for the same sampling time; lowercase letters indicate differences at the 0.05 level between physiological indicators of the same grafting combinations for different sampling times).

Table 7. Percentage changes in PPO activity at different sampling times in relation to the first day.

Grafting Combinations	Percentage Changes in PPO Activity at Different Sampling Times (%)								
	4 d	7 d	10 d	13 d	16 d	19 d	22 d	25 d	28 d
HR	106.4	108.3	101.4	106.5	110.0	115.0	116.8	108.1	111.2
HH	103.8	104.7	101.6	99.6	97.8	104.9	110.3	105.5	106.4
HB	104.1	108.8	109.8	109.8	111.1	110.8	115.2	117.0	116.2

3.3.3. Changes in PAL Activity in Healing Parts of Different Rootstock–Scion Combinations

The effect of grafting combination, sampling time, and the interaction between grafting combination and sampling time on the PAL activities of the healing parts of scions reached a highly significant level ($P < 0.01$), indicating that the differences in the rootstock combinations have a highly significant effect on the PAL activity of the healing part. During the grafting healing process, there are also large differences in the changes in the PAL activity of the healing part of the graft at different sampling times (Table 5).

Compared with the activities of the former two enzymes, those of PAL went through a more dramatic change at the graft healing sites, with the values of most time points significantly different from those of the adjacent ones ($P < 0.05$) (Figure 8). From the beginning to the 16th day after grafting, the HR combination went through an up-and-down trend in the PAL activity of the healing site, during which the 13th day witnessed the lowest value of 49.67 U·g^{-1}·min^{-1}, which was only 85.2% of that measured on the first day after grafting (Table 8). From the 16th to the 19th day, an increase continued till a high value of 68.16 U·g^{-1}·min^{-1} which was 16.9% higher than that measured on the first day (Table 8). Since then, it started to decrease till the end of the sampling. The HH combination showed an up-and-down trend in PAL activities of the healing parts before the 10th day, since when it started to decrease significantly ($P < 0.05$) till the lowest value of 54.74 U·g^{-1}·min^{-1} was reached on the 16th day. Compared with that measured on the first day after grafting, there was no significant difference. From the 16th to the 22nd day, the PAL activity tended to increase rapidly till a maximum value of 68.16 U·g^{-1}·min^{-1} which was 21.2% higher than that measured on the first day (Table 8). Since then, it started to decrease till the end of the sampling. The HB combination presented a significant ($P < 0.05$) increasing trend in the PAL activity of the healing sites from the beginning to the fourth day after grafting. It then went through a sharp decline till a minimum value of 40.78 U·g^{-1}·min^{-1} was reached. After a fluctuated rise and fall, it remained at a high level from the 22nd to the 28th day, during which the 25th day marked the highest value of 57.89 U·g^{-1}·min^{-1}. In short, during the graft healing process, the HR and HH combinations had similar trend in the change in PAL activity in the healing parts, while the HB combination exhibited a smaller fluctuation. The highest values of PAL activity at the healing site of all three grafting combinations occurred at the late stage of grafting healing, with those of the HR and HH combinations being significantly higher than that of HB.

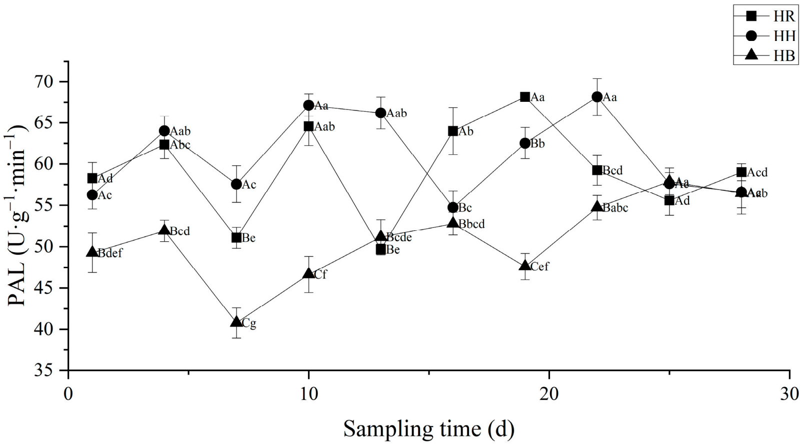

Figure 8. Results of PAL activity in graft healing sites. (Note: capital letters indicate differences at the 0.05 level between physiological indicators of different grafting combinations for the same sampling time; lowercase letters indicate differences at the 0.05 level between physiological indicators of the same grafting combinations for different sampling times).

Table 8. Percentage changes in PAL activity at different sampling times in relation to the first day.

Grafting Combinations	Percentage Changes in PAL Activity at Different Sampling Times (%)								
	4 d	7 d	10 d	13 d	16 d	19 d	22 d	25 d	28 d
HR	107.0	87.6	110.8	85.2	109.8	116.9	101.7	95.4	101.3
HH	113.7	102.3	119.3	117.6	97.3	111.1	121.2	102.4	100.6
HB	105.4	82.8	94.7	103.8	107.2	96.6	111.1	117.5	114.7

3.4. Correlation Analysis of the Survival Rate of Seedling Grafting, Enzyme Activity and Hormone Content

In order to comprehensively analyze the relationship of the phytohormone content or related enzyme activity of the graft with the survival rate of grafting, a correlation analysis was carried out (Table 9).

Table 9. Results of correlation analysis of the survival rate of seedling grafting, enzyme activity and hormone content.

Indicators	Survival Rate	POD	PPO	PAL	IAA	ZR	MeJA
Survival rate	1						
POD	0.856 **	1					
PPO	0.592 **	0.835 **	1				
PAL	0.623 **	0.512 **	0.454 *	1			
IAA	0.211	0.188	0.078	−0.022	1		
ZR	0.025	0.188	0.189	0.222	0.121	1	
MeJA	0.752 **	0.595 **	0.297	0.291	0.446 *	−0.087	1

Note: * Correlation at 0.05 level. ** Correlation at 0.01 level.

There was a highly significant positive correlation between survival rate of grafting, and POD, PPO and PAL activities in the grafting healing site ($P < 0.01$); the relationships were also significant between survival rate of grafting and MeJA content in the graft healing site ($P < 0.01$), between POD activity and PPO or PAL activities or MeJA content ($P < 0.01$), and between PPO activity and PAL activity in the graft healing site ($P < 0.01$). Moreover, there was a significant positive correlation between PPO activity and PAL activity in the grafted healing site ($0.01 < P < 0.05$); the relationship was also significant between IAA and MeJA content in the graft healing site ($0.01 < P < 0.05$) (Table 9). The above analysis shows that there is a strong relationship between the enzyme activity related to the grafting healing site and the grafting survival rate or grafting affinity, the correlation for which is a highly significant positive one. During graft healing, there was a significant positive correlation between the three enzymes, which indicated that these enzymes were closely related and played an interactive role in the graft healing process. Among the plant hormones, MeJA was strongly associated with graft survival and graft affinity, and it had a strong interaction with POD activity; therefore, the correlation for the above three reached a highly significant positive level. The plant hormones MeJA and IAA were also closely interlinked in the graft healing process.

4. Discussion

1. Effect of different rootstock–scion combinations on the survival rate of grafting;

High grafting affinity means that the rootstock and scion can be fully healed into one and will grow for a long time. Those who have close correlation between relatives generally have high grafting affinities; therefore, their grafting success is accordingly high [11]. In this study, on the 40th day after grafting, the survival rates of HR and HH reached 83.3% and 80.0% respectively, while that of HB was only 63.0% which was significantly lower than that of HH and HR ($P < 0.05$). As there have been no conclusive studies on the affinities between *M.* 'Huabiao', and *M. robusta*, *M. hupehensis* and *M. baccata*, whether the differences in grafting affinities among the three combinations are related to genetic relationship remains to be studied in depth. According to the statistical results of grafting survival rate, compared with *M. baccata*, the other two are more suitable to be selected as grafting rootstocks for *M.* 'Huabiao'.

2. Effect of different rootstock–scion combinations on phytohormone content;

IAA, which plays a role in regulating vascular tissue development and promoting cell division and elongation, can regulate the differentiation of three elements, i.e., guaiac tissue, formation layer and vascular tissue during graft healing, and it is an endogenous hormone that exerted the greatest influence on graft healing [3]. In this study, the IAA contents in the healing sites of all three grafting combinations maintained at a high level from the 10th to the 22nd day after grafting, from which it can be concluded that this stage could be a period of differentiation and formation of healing tissues, and connection of forming layers in the grafting healing process. However, after the 22nd day of grafting, the IAA contents of the healing parts of the HR and HH combinations kept increasing steadily, while that of HB exhibited a decreasing trend. Based on the statistical results of the survival rate of three grafting combinations, it is inferred that the reason for the above phenomenon could be that, when the vascular bundles are differentiated and connected within a combination with high grafting affinity, the IAA content of the healing site gradually increases. On the contrary, when the same happened within a grafting combination with poor affinity, the IAA content of the healing site will go through a gradual decrease. Such a conclusion is similar to the results obtained in Lu Shanfa et al.'s studies [2].

As a plant hormone and signaling molecule associated with damage, MeJA serves as a hormone which can increase the activity of enzymes such as POD, PAL and PPO [12]. However, there are few studies on the role of MeJA in the healing process of plant grafting. In this study, the healing site of the HR combination has the highest mean value of MeJA, followed by HH and HB, the order for which was the same as that of the mean activities of three enzymes in the three grafting combinations. However, at the late stage of graft healing, the MeJA content of the healing sites of the HR and HH combinations increased sharply, but that of HB showed signs of decreasing, which was the opposite of the change in the relevant active enzymes of the three grafting combinations at the late stage of graft healing. Combined with the results of the correlation analysis in (Section 3.4), this enzyme had a highly significant positive correlation with graft survival and POD enzyme activity, and a positive correlation with IAA content, based on which it can be inferred that MeJA might be involved in the regulation of related enzyme activities during the graft healing process, thereby affecting the process to a certain extent, but such a conclusion remains to be further studied.

ZR, which is widely found in the parts of ongoing cell division in plants, can promote cell division and enlargement, facilitate the formation and differentiation of healing tissue during graft healing, and exert a positive effect on healing success [13]. Cytokinins enhance the sensitivity of plants to growth hormone and the appropriate ratio of cytokinin to growth hormone can regulate the differentiation of healing tissue [14]. In this study, the changes in ZR content and IAA content in the healing sites of HR were basically the same. Especially in the late stage of graft healing, ZR and IAA synergistically induced the differentiation and connection of vascular bundles in the healing site. However, for the HH and HB combinations, there was no obvious regularity in the variation of IAA and ZR contents in the healing sites. Since the HR combination had better synergy between ZR and IAA in the healing sites, its survival rate was accordingly higher. This finding was similar to that of Su WenChuan [15].

3. Effect of different rootstock–scion combinations on the activity of related enzymes.

POD plays an important role in the grafting healing process. At the early stage of grafting, the graft often increases its POD activity to eliminate the H_2O_2 produced by mechanical damage during the process [16], which contributes to the healing of wounds [17]. On the other hand, the increased POD activity also helps to connect the healing tissue at the early stage of grafting [18]. At the later stage of grafting healing, POD participates in lignin synthesis, and catalyzes the dehydrogenation and polymerization of lignin monomers in the cells of the graft, which promotes the connection of vascular tissues in the graft [19]. In this study, all the three grafting combinations exhibited an increasing–decreasing–increasing trend of POD activity at the healing sites, which is similar to the results obtained by Huang Hang [20]. The first increase in enzyme activity was caused by scavenging free radicals generated at the grafting wound which accelerated wound healing and maintained hormone homeostasis. The second was generated out of the promotion of the connection of vascular tissue at the healing site [21]. The three grafting combinations showed similar trends in POD activity at the healing sites but went through two peaks at different times. To be specific, the HR combination reached its two peaks earlier than the other two. This is, precisely speaking, the speed of graft healing reflected in the changes in POD activity. During the graft healing process, the POD activities of different combinations were ranked in the following order, HR > HH > HB, which is the same as the ranking of grafting survival rate and consistent with the findings of Wang Ji Wei et al. [22], i.e., the higher the POD activity of the healing sites during the grafting healing process, the higher the survival rate.

PPO is a protective enzyme in plants that interacts with polyphenolic compounds to produce black and brown substances when the plant body is injured. It forms a barrier that protects the plant from bacteria and can inhibit graft healing [23]. Therefore, the higher the PPO activity at the grafting site, the lower the survival rate [24,25]. In this study, the HR combination had the highest PPO activity at the healing site compared with the other two but its survival rate remained the highest, which contradicts the findings of previous studies [24,25]. Through analysis, the reason could be that a higher PPO activity facilitates the synthesis of lignin, which promotes the differentiation and formation of ducts. Such an inference is consistent with the findings of Qu Yunfeng [26]. In the early stage of grafting, due to the role of PPO in promoting the formation of the isolation layer, the PPO activity of the healing parts of the three grafting combinations increased [23]. In the middle stage of grafting, out of the disappearance of the isolation layer, the PPO activity of the healing parts of the HR and HH combinations gradually decreased to their initial level. In the late stage of grafting, that of the HR and HH combinations increased again to a higher level, which was related to the involvement of PPO in regulating lignin synthesis. By contrast, that of

HB maintained a rising trend, the reason for which, through analysis, could be that the prolonged presence of an isolation layer in a graft with poor affinity facilitated the durable working of PPO. This conclusion is similar to the findings of ShuQiu Zhang et al. [27].

PAL is an important enzyme that regulates the metabolic pathway of secondary phenylpropanoid metabolism in plants. It is mainly distributed in vascular tissues and epidermal cells [28] and is involved in the lignification process together with PPO and POD. PAL catalyzes the removal of the amino group on L-phenylalanine to form cinnamic acid, and further forms structural units of lignin such as pineol, mustard alcohol and coumarin. These units are converted by POD into free radicals and spontaneously polymerized into lignin afterwards [29]. In the present study, the mean values of PAL activity in the healing parts of HR and HH were significantly higher than that of the HB combination. High PAL activity contributes to graft healing and thus improves the survival rate of grafting, which is in accordance with the findings of Feng Jinling et al. [29]. From the changes in POD, PPO and PAL activities during the grafting healing process, it can be seen that the highest values of these three enzymes occur after the 20th day of grafting, which should be the period of vascular tissue connection at the grafting healing site. Combined with the results of correlation analysis (Section 3.4), it is concluded that all three enzymes can promote vascular tissue connection during graft healing and that their effects overlap with each other [14].

5. Conclusions

In this study, different rootstocks were selected for grafting with the scions of *M.* 'Huabiao'. It was found that different combinations varied in their grafting survival rates and this was also reflected in the different grafting affinities of different combinations. Compared with *M. baccata*, *M. robusta* and *M. hupehensis* are more suitable to be used as grafting rootstocks for *M.* 'Huabiao' varieties, and the reasons for the poorer grafting affinity of *M. baccata* remain to be further studied. Moreover, since POD, PPO and PAL enzyme activities, and MeJA hormones are closely related to the grafting affinity of crabapples, the increase in these enzyme activities and MeJA content, which is beneficial to improve the survival rate of grafting, can be used as a characteristic indicator to determine the grafting affinity of different crabapple rootstocks. Phytohormones and related enzymes, which work on different mechanisms, often interact with each other to promote grafting healing on rootstocks by participating in or regulating the formation of healing tissue, cambium differentiation and vascular tissue connection at the healing site in direct or indirect ways, and jointly accelerating the healing process.

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