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# Physiological and biochemical changes during graft union formation in *Carya illinoinensis*

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## Abstract

To reveal the physiological and biochemical mechanism underlying graft union formation in pecan (*Carya illinoinensis*), dynamic changes in content of nutrients, tannin, and phytohormones together with key enzyme activities were investigated 0, 3, 5, 7, 10, 14, 18, 22, 31, and 40 d after grafting (DAG), in homograft unions. During graft union formation, peroxidase (POD) activity increased at 7 - 10 DAG compared with that at 0 DAG. Polyphenol oxidase (PPO) activity was higher in grafted than ungrafted pecan seedlings (control) at 22 DAG, which was similar to POD activity. The tannin content exhibited a decreasing trend with grafting relative to the control. Indole-3-acetic acid (IAA) and zeatin riboside (ZR) content increased from 7 - 10 DAG, with higher average content than in the control at 14 - 31 DAG. Abscisic acid (ABA), soluble sugar, starch, and soluble protein content was generally lower in grafted plants than in the control. Combined with our previous anatomical observations, these results suggested that, during graft development, some enzymes and growth promoting hormones might be required for callus proliferation at early stage and for vascular reconnection at the later stage. Nutrients provided energy for the whole graft development process. In contrast, some polyphenols and growth inhibiting hormones seemed to have negative effects on this process.

*Keywords*: ABA, grafting, IAA, nutrients, pecan, peroxidase, polyphenol oxidase, tannin, zeatin riboside.

## Introduction

The use of grafting has expanded greatly for the production of woody and herbaceous plants. During grafting, a specific organ of one plant (scion) is adhered to another rooted plant (rootstock), and then a series of biological changes take place in the graft union so that a complete plant is formed. This clonal propagation technique allows people not only to reduce the damage caused by stress, but also to influence metabolism and development, such as shortening the long juvenile phase of some woody plants (Zhang *et al*. 2013, Duan *et al*. 2017, Tsobeng *et al*. 2017). In general, graft union formation can be divided into five stages: initial adhesion of the grafted partners; wound response of the graft surface; callus formation and proliferation; new vascular cambium establishment; and xylem and phloem reconnection of the rootstock and scion derived from the new vascular cambium (Aloni *et al*. 2010, Baron *et al*. 2019).

Graft union formation is a highly complex process that involves various physiological and biochemical changes. Grafting, as a wounding stress, triggers a high accumulation of reactive oxygen species (ROS) at the early stage of development, which is associated with a less efficient detoxification system on the graft interface (Irisarri *et al*. 2015). To maintain ROS at a moderate level, plants usually initiate an enzyme-dependent defence system, in which peroxidase (POD) plays an important role. Previous

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*Abbreviations*: ABA - abscisic acid; DAG - days after grafting; ELISA - enzyme-linked immunosorbent assay; IAA - indole-3-acetic acid; POD - peroxidase; PBS - phosphate buffered saline; PPO - polyphenol oxidase; ROS - reactive oxygen species; ZR - zeatin riboside.

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studies have shown that POD activity was significantly increased 1 d after grafting (DAG) in grafted cucumber because of the wounding response (Miao *et al*. 2019). On the graft interface, phenolic compounds are released from injured vacuoles into the cytoplasmic matrix, and then oxidized by polyphenol oxidase (PPO) into highly toxic quinine, which defends against invading pathogens in early stages of graft union development (Hartmann *et al*. 2011, Mohammadi *et al*. 2002). On the other hand, quinines actively react with polymerized proteins and other cellular components, then produce dark-colored pigments (He *et al.* 2009). These pigment compounds form a necrotic layer, which limits the formation of tubes and calli, and results in a low grafting compatibility (Xu *et al*. 2015). The regeneration of the vascular bundle can re-establish the continuity of the transport system, thus, it plays a critical role in graft healing. As one of the main signalling molecules, indole-3-acetic acid (IAA) accumulation directly activates vascular differentiation and reconnection (Yin *et al*. 2012, Melnyk *et al*. 2018). Vessels and sieve tubes are normally differentiated from the vascular cambium, and cambial activity is largely regulated by cytokinins (Nieminen *et al*. 2008, Aloni *et al*. 2010). Studies on grafted apple have shown that abscisic acid (ABA) can effectively alter xylem development and reduce hydraulic conductance, which induces dwarfing (Tworkoski and Fazio 2011, 2015). Aside from endogenous hormones, POD is also involved in differentiating the new vasculature. The synthesis of lignin is mainly located in plant cells for the transport system. The last step in lignin synthesis is the polymerization of monolignols, which is catalysed by POD (Boudet *et al*. 2003). Although many of the physiological changes associated with graft union formation have been established, information concerning this process remains limited.

Pecan [*Carya illinoinensis* (Wangenh.) K. Koch.] is an economically important nut tree originating from Northern America. Normally, pecan seedlings propagated by seeds takes about 10 - 15 years to fructification, due to its long juvenile period. *Via* the technique of grafting, the process of maturation could be accelerated to 5 - 10 years, which promotes the cultivation of pecan (Mo *et al*. 2017). Similar to other members of *Juglandaceae*, the survival rate of grafted pecan is very low due to the high content of phenolic compounds and the large amount of bleeding from the xylem tissue. After years of effort, patch budding performed from July to September in China has achieved over 90 % grafting success for some pecan cultivars, such as Pawnee and Stuart (Zhang *et al*. 2015, Mo *et al*. 2018a,b). However, the survival rate in other pecan cultivars such as Mahan and Jinhua, is still relatively low (Mo *et al*. 2018a,b). A better understanding on mechanisms underlying graft union formation is needed for higher grafting success rate. Previously, studies on anatomical and proteomic changes for pecan graft healing have been conducted (Mo *et al*. 2018a, Yan *et al*. 2018). Mo (2018b) studied the genes and miRNA expressions during the healing process. Since the expressions of genes do not always reflect the physiological and biological indices, studies on physiological and biological changes are necessary, which still have not been sufficiently reported. Therefore, in this research, we investigated the changes in content of nutrients, tannin, and phytohormones as well as enzyme activities during graft union formation in pecan. Our study can not only improve the production efficiency of pecan, but also expand the knowledge necessary for grafting success for plant species that traditionally have poor graft survival, especially woody trees.

## Materials and methods

**Plants and treatments:** The experiment was conducted in a pecan orchard located in Zhangmiao Village, Houbai Town, Jurong City, Jiangsu Province, China (119°9′6″E, 31°52′45″N). We chose 1-year-old seedlings propagated from pecan [*Carya illinoinensis* (Wangenh.) K. Koch.] seeds as the rootstock. Patches containing a single bud were chosen as the scion. The scions were derived from one-year-old shoots that grew on 4 to 5-year-old pecan cv. Pawnee trees. Patch grafting was performed in late August 2014. The rectangular patches had a rough size of 3 cm in length and 1 cm in width. In addition, 1-year-old pecan seedlings propagated without grafting were chosen as controls.

**Physiological determinations:** On 0, 3, 5, 7, 10, 14, 18, 22, 31, and 40 DAG, grafted seedlings were collected. Pruning shears were used to clip the graft union segment from the seedlings. At each sampling time, the stems of ungrafted seedlings were trimmed into short sections of similar length as the graft union (approximately 3.5 cm). The trimming location was around the height of the graft union, and 10 - 12 stems per ungrafted seedling were collected. Samples harvested from grafted and seed-propagated plants were stored in an ultracold freezer (-80 °C) until use. At the beginning of the physiological determinations, phloem and cambium in the budding zone (the patch scion and a range of approximately 0.5 cm on each side of the rootstock-scion interface) were scraped by a box cutter into small pieces. The same tissues from untreated stems (no region limit) were also scraped into small pieces.

POD activity was determined by spectrophotometry. Approximately 0.2 g of powdered sample (in liquid nitrogen) was homogenized with 0.1 M phosphate buffered saline (PBS, pH 6.0). The homogenate was centrifuged at 1 359 *g* and 4 °C for 15 min, and the resultant supernatant was used as the POD extract. The reaction mixture contained 200 $cm^3$ of 0.1 M PBS (pH 6.0), 112 $mm^3$ of guaiacol, and 76 $mm^3$ of 30 % (v/v) hydrogen peroxide. After homogenizing 3 $cm^3$ of reaction mixture with 1 $cm^3$ of enzyme extract, the absorption of the homogenate at 470 nm was immediately quantified, and the absorption was recorded every 2 min (six times in total). One unit of POD (U) was defined as the amount of enzyme necessary to cause an increase of 0.01 absorbance units per minute of reaction time.

For PPO activity determination, approximately 0.2 g of fresh sample was weighed and ground into powder in liquid

nitrogen. To avoid the influence of endogenous phenols, we added approximately 0.1 g of polyvinylpyrrolidone during the process. The powdered sample was homogenized with 5 $cm^3$ of 0.05 M PBS, pH 6.5. The homogenate was centrifuged at 764 *g* and 4 °C for 10 min. PPO activity was determined by incubating 1 $cm^3$ of supernatant in a reaction mixture containing 3 $cm^3$ of PBS (0.05 M, pH 6.5) and 1 $cm^3$ of 0.1 M catechol at 37 °C for 10 min before absorbance was recorded at 525 nm. One unit of PPO activity was defined as the amount of enzyme that caused a change of 0.01 absorbance units per min.

For extraction of soluble sugars, approximately 0.2 g of powdered tissue (in liquid nitrogen) in a conical flask was immersed in approximately 20 $cm^3$ of distilled water. Then, the conical flask was closed with film and treated with boiling water for 60 min. After cooling to room temperature, the soluble sugar extract was transferred to a 50 $cm^3$ volumetric flask by filtration. Two times, a small quantity of distilled water was added to the residue, the mixture was shaken for a moment and filtered to the same volumetric flask, and then the volume was adjusted to 50 $cm^3$ with distilled water. The 2 % anthrone (m/v, in concentrated sulfuric acid; 5 $cm^3$) was fully mixed with 1 $cm^3$ of soluble sugar extract. The mixture was immediately incubated in boiling water for 1 min. The soluble sugar content was determined spectrophotometrically at 630 nm after cooling to room temperature.

The residue mentioned above was used to determine the starch content. Distilled water was added, and after treatment with boiling water for 15 min, 2 $cm^3$ of 9.2 M perchloric acid was added and boiled for another 15 min to hydrolyse the starch to soluble sugar. After cooling to room temperature, starch hydrolysate was filtered to a 50 $cm^3$ volumetric flask. Two times, a small quantity of distilled water was added to the residue, the mixture was shaken for a moment and filtered to the same volumetric flask, and then the volume was adjusted to 50 $cm^3$ with distilled water. The hydrolysate (2 $cm^3$) was used for starch content quantification. The method for subsequent experiments was the same as for the soluble sugar content.

To assay the soluble protein content, approximately 0.2 g of fresh sample was ground into powder by liquid nitrogen, homogenized with 5 $cm^3$ of distilled water, and then centrifuged at 1 359 *g* for 10 min. The supernatant (1 $cm^3$) was added to 5 $cm^3$ of Coomassie brilliant blue G-250. After 2 min of reaction, the soluble protein content was spectrophotometrically determined by measuring the absorbance at 595 nm.

The POD and PPO activities and content of soluble sugars, starch, and soluble proteins were determined in accordance with the method described by Zhang (2008), Kong (2008), and Li (2000), with small modifications.

The tannin content was determined according to a modified method described by Yang (1989). Approximately 0.5 g of fresh sample was placed into a conical flask and approximately 30 $cm^3$ of distilled water was added. The conical flask was sealed with film, and the tannin was extracted in a water bath at 90 °C for 90 min. After rapid cooling, the tannin extract was filtered into a 100 $cm^3$ volumetric flask. Distilled water of (approximately 30 $cm^3$) was added to the residue twice, the mixture was shaken for a moment, filtered to the same volumetric flask, and diluted with distilled water to a volume of 100 $cm^3$. Subsequently, 1 $cm^3$ of extract was mixed with 1 $cm^3$ of 0.1 M $FeCl_3$ and approximately 20 $cm^3$ of distilled water. After 5 min, 1 $cm^3$ of 0.008 M $K_3Fe(CN)_6$ was added, and then the tannin extract was brought to a 50 $cm^3$ volume with distilled water. The solution was left for 30 min, and then the absorbance was measured at 760 nm.

ABA, zeatin riboside (ZR), and IAA were extracted, purified and analysed according to a modified method described by Yang (2001a, b) In summary, approximately 0.5 g of fresh sample was ground into powder *via* liquid nitrogen and homogenized in 2 $cm^3$ of 80 % methanol for extraction. The mixture was left at 4 °C for 240 min, and centrifuged at 1 000 *g* (4 °C) for 8 min. The residue was added to an additional 1 $cm^3$ of the same solution, re-extracted at 4 °C for 60 min, and then centrifuged under the same conditions. Supernatants were collected, filtered through a C-18 cartridge (*Sep-Pak*, *Waters*, Milford, MA, USA), dried in nitrogen, and then placed into tubes, and then the volume was adjusted to 10 $cm^3$ with diluent [500 $cm^3$ of 0.15 M (pH 7.5) PBS + 0.5 $cm^3$ of *Tween-20* + 0.5 g of gelatine].

Analysis of ABA, ZR, and IAA content was carried out with enzyme-linked immunosorbent assay (ELISA). Before injection, a series of ABA, ZR, and IAA standard solutions with known concentrations were prepared. The standard solutions and hormone extract were injected into ELISA plates in sequence, with 50 $mm^3$ in each well (holes containing standard solution were named 'standard holes'). Then, 50 $mm^3$ of diluent (containing antibody) was added to each of these wells. The ELISA plate was incubated in a humidified incubator at 37 °C for 30 min. Subsequently, 0.1 % *Tween-20* (v/v, in 0.15 M of PBS) was used to clean the ELISA plate four times. 100 $mm^3$ of diluent (containing horseradish peroxidase labelled goat anti-rabbit immunoglobulin) was injected into each well. After incubating at 37 °C for 30 min, the reaction mixture was washed from the ELISA plate by the method mentioned above. Finally, 100 $mm^3$ of o-phenylenediamine (OPD) solution [10 - 20 mg of OPD + 10 $cm^3$ of 0.076 M citrate-PBS (pH 5.0) + 40 $mm^3$ of 30 % hydrogen peroxide (v/v)] was added, and the plate was placed in the dark and kept moist at 37 °C for colour change. The reaction in each well was terminated using 50 $mm^3$ of 2 M concentrated sulfuric acid when two phenomena were observed: differences in colour among standard holes, and the mixture in the standard hole of the largest concentration showing a relatively light colour. The absorbance for each treatment was recorded at 490 nm.

**Statistical analysis:** Data are presented as the mean and standard error (SE) of three replicates. Statistical analysis was carried out using *SPSS v. 19.0* software. The activities of POD and PPO, and the content of tannins, soluble sugars, soluble protein, starch, IAA, ZR and ABA were submitted to one-way analysis of variance (*ANOVA*). For each index, the significance of differences was determined from two aspects: between mean values of grafted and

control plants in each sampling period, and among mean values of different periods in grafted plants, both by Tukey test at $P \leq 0.05$.

## Results

The POD activity in both the grafted plants and the controls increased from 0 - 5 DAG, but it was lower in grafted plants than in control plants (Fig. 1*A*). The POD activity in the graft union had a sharp increase from 7 to 10 DAG and was the highest [18.1 U $g^{-1}$(f.m.) $min^{-1}$] on 10 DAG, which was 73.7 % greater than that for the control [10.4 U $g^{-1}$(f.m.) $min^{-1}$]. Although the POD activity in treated plants subsequently showed a decreasing trend from 10 DAG to end of the grafting formation, it remained significantly higher than that of the control, except at 31 DAG. The PPO activity in grafted plants had a smaller variation during graft development than that in untreated plants (Fig. 1*B*). The activity in the graft union fluctuated around the value of 25 U $g^{-1}$(f.m.) $min^{-1}$, and the highest value was observed at 3 DAG [26.3 U $g^{-1}$(f.m.) $min^{-1}$]. The PPO activity in the grafted plants was significantly higher at 22 DAG than that in the control.

Except at 0 and 40 DAG, the soluble sugar content in the treated plant was significantly lower than that in the control plants (Fig. 2*A*). The highest value in treated plants was found on the day of grafting [0.034 μg μ$g^{-1}$(f.m.)]. From 0 - 3 DAG, the value in the treated plants sharply declined. At the end of graft development, the grafted plants had similar content of soluble sugar as the control plants. Except at 31 and 40 DAG, the starch contents in the graft union were lower than those in the control during graft healing (Fig. 2*B*). For grafted plants, the starch content at the beginning of the experiments was [0.039 μg μ$g^{-1}$(f.m.)], which was similar to that of the control plants. Subsequently, there was an obvious decrease in the starch content of grafted plants from 0 to10 DAG. In contrast to the control, the starch content in grafted plants was significantly enhanced from 18 to 40 DAG, reaching the highest content at the end of the graft formation, which was 109 % higher than that in the control [0.041 μg μ$g^{-1}$(f.m.)]. In the control plants, the soluble protein content varied slightly during graft union formation (Fig. 2*C*). The soluble protein content in the treated plants was higher than that in the control only at 7, 14, and 31 DAG. On 22 DAG, the soluble protein content in the treated plants decreased to the lowest value [1.3 μg $mg^{-1}$(f.m.)], being 32.7 % lower than that in the control [1.9 μg $mg^{-1}$(f.m.)]. At the end of graft development, grafted plants showed similar soluble protein content as control plants.

In general, the variation in the tannin content on the graft interface showed an inverse trend during the experiment compared to that of the control (Fig. 3). On the day of grafting, the tannin content in the treated plants was higher [0.032 μg μ$g^{-1}$(f.m.)] than that in the control plants [0.018 μg μ$g^{-1}$(f.m.)]. From 0 to 7 DAG, the tannin content in the graft showed a significant decrease, and was about 0.02 μg μ$g^{-1}$(f.m.) until the end of graft union formation. Only at 0, 3, and 5 DAG, the tannin content in the graft union was significantly higher than that in the control plants. At the end of the graft development, the grafted plants showed the second lowest tannin content [0.017 μg μ$g^{-1}$(f.m.)], while the control plants had the highest tannin content [0.034 μg μ$g^{-1}$(f.m.)].

There was a large fluctuation in the IAA content of the control plants from 0 to 10 DAG (Fig. 4*A*), and the highest value was observed at 3 DAG [88.8 ng $g^{-1}$(f.m.)]. The fluctuation in the control decreased during the later stages of the experiment. The highest value of treated plants

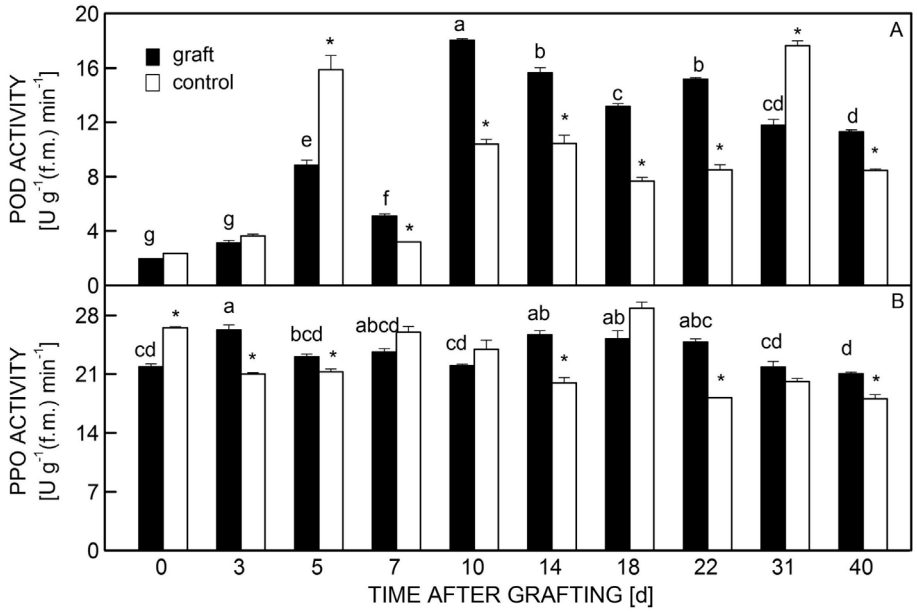


Fig. 1. Changes in peroxidase (POD; *A*) and polyphenol oxidase (PPO; *B*) activities during graft development. Means ± SEs, $n = 3$, * indicates significant differences between graft and control tissues at each DAG ($P \leq 0.05$) and different letters indicate significant differences among periods in the grafted plants ($P \leq 0.05$).

was observed on the day of grafting [85.5 ng $g^{-1}$(f.m.)], and the value was 81 % higher than that of the control [47.2 ng $g^{-1}$(f.m.)]. Subsequently, the IAA content in treated plants sharply decreased to the lowest value [35.8 ng $g^{-1}$(f.m.)] at 5 DAG. Only at 0, 10, 18, and 31 DAG, the IAA content was in treated group significantly higher than in the control group, and the second highest content appeared at 18 DAG [82.7 ng $g^{-1}$(f.m.)]. Generally, the ZR content in treated plants varied conversely during the trial when compared to the control (Fig. 4*B*). On the day of grafting, the ZR content in treated plants was the highest [18.7 ng $g^{-1}$(f.m.)], which was almost 6 times that for the control plants [3.2 ng $g^{-1}$(f.m.)]. The maximal ZR content significantly declined from 0 to 7 DAG in

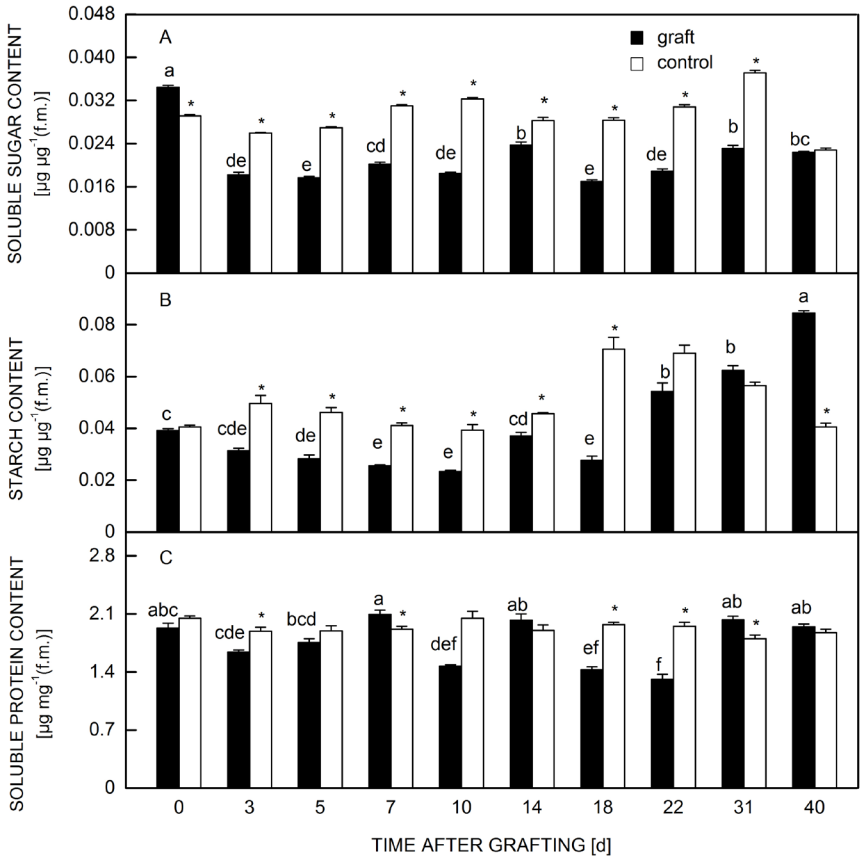


Fig. 2. Changes in content of soluble sugars (*A*), starch (*B*), and soluble proteins (*C*) during graft development. Means ± SEs, $n = 3$, * indicates significant differences between graft and control tissues at each DAG ($P \leq 0.05$) and different letters indicate significant differences among periods in grafted plants ($P \leq 0.05$).

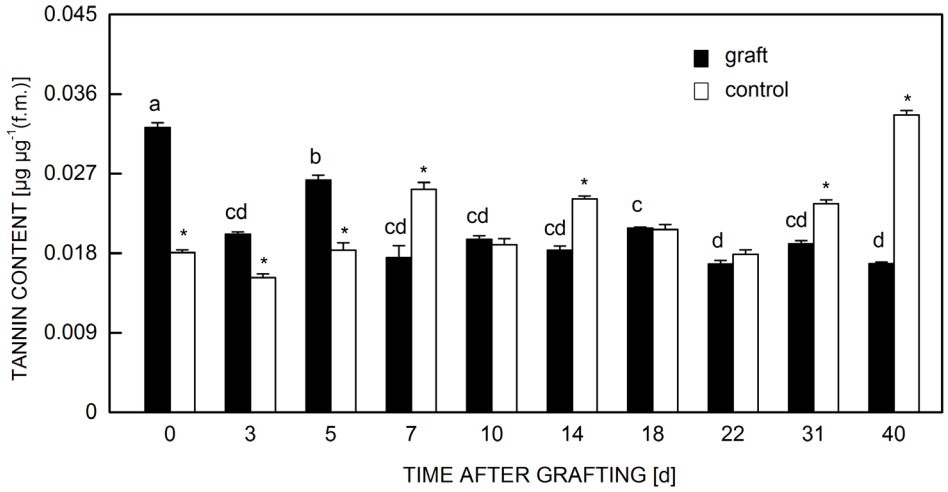


Fig. 3. Changes of tannin content during graft development. Means ± SEs, $n = 3$, * indicates significant differences between graft and control tissues at each DAG ($P \leq 0.05$) and different letters indicate significant differences among periods in grafted plants ($P \leq 0.05$).

treated plants, reaching to its lowest value. Aside from the content at 0 DAG, the ZR content in the grafted plants was significantly higher than that of control on 18 and 22 DAG. Except at 14 DAG, the ABA content of grafted plants was significantly lower than that of control plants during the whole experiment (Fig. 4*C*). The highest value of ABA in the grafted plants was shown at 14 DAG [100.9 ng $g^{-1}$(f.m.)], which was 3.3 % lower than that in untreated plants [104.4 ng $g^{-1}$(f.m.)].

## Discussion

Changes in the metabolic system occur during graft union formation. ROS and singlet oxygen are produced following wounding stress caused by grafting (Prasad *et al*. 2020). POD and SOD are normally used to effectively maintain ROS at a moderate level. In melon/*Cucurbita* grafting, there were higher POD activities and lower content of ROS on compatible graft interfaces than on incompatible interfaces (Aloni *et al*. 2008). However, Zarrouk (2010) found a lower POD activity in compatible peach/plum graft unions than in incompatible unions, which supports our results. The POD activity in the pecan graft union and controls showed a simultaneously increase at 0 - 3 DAG, but that of the former was lower than that of the latter. The POD activity increased probably in order to reduce the cellular damage from ROS. And the lower oxidative activity might avoid the negative impacts of POD: the production of amorphous dark pigments as melanin that inhibit the growth of calli (He *et al*. 2009), which was adverse for graft healing. Similarly, PPO activity in grafted plants significantly increased at 0 - 3 DAG relative to that in the control. This improved the oxidation of phenolic compounds and then produced toxic quinine which is defensive against wound invasions from pathogenic microbes (Hartmann *et al*. 2011, Mohammadi *et al*. 2002). At 7 - 10 DAG, PPO activity in graft was lower because of its inhibitory effect on graft tissue development (He *et al*. 2009; Xu *et al*. 2015). Tannin is polyphenolic compound, involved in the response to wounding as one of the substrates of PPO. An upregulation of genes involved in tannin synthesis is observed when plants are under mechanical injury, which consequently increases the tannin content, as a part of plant defence system (Tsai *et al*. 2006, Häring *et al*. 2008). In our results, the tannin content in the graft union was almost twice that in the control at 0 DAG. However, the tannin content in grafted plants showed a decreasing trend during graft union development, which contradicted the content in the control. Previous studies indicate that a high tannin content can limit the proliferation of calli, as well as the formation of vascular tissue; thus, tannin has negative effects on healing in the long term (Xu *et al*. 2015, Kuriyama *et al*. 2019).

The healing of grafting wounds involves the process of tissue regeneration, which is accompanied by vigorous

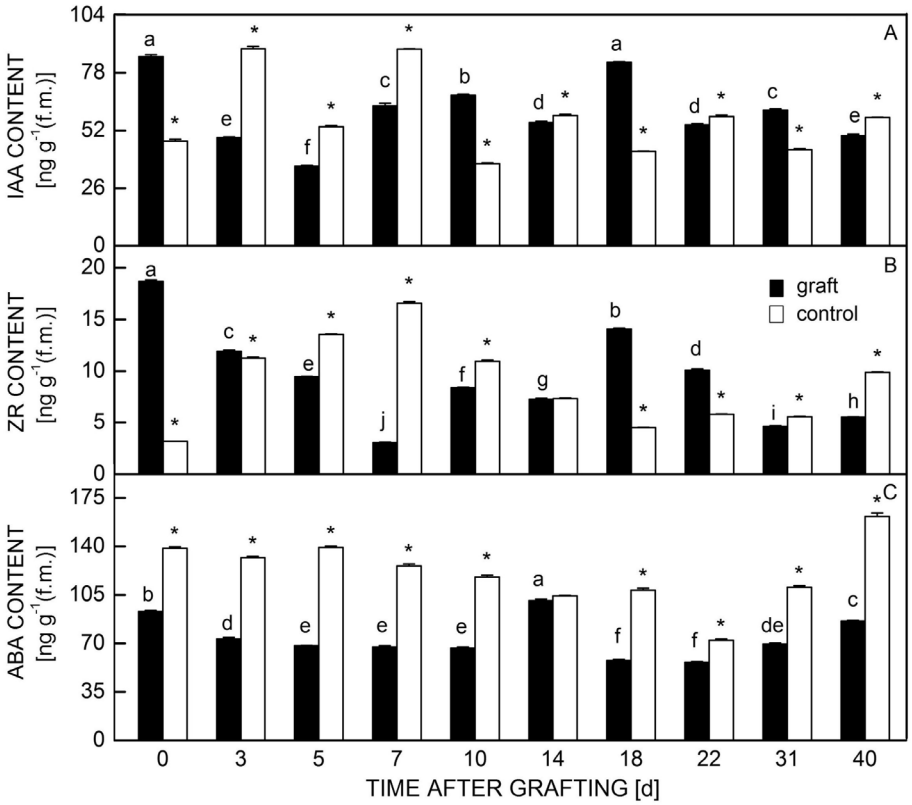


Fig. 4. Changes in content of indole-3-acetic acid (IAA; *A*), zeatin riboside (ZR; *B*), and abscisic acid (ABA; *C*) during graft development. Means ± SEs, $n = 3$, * indicates significant differences between graft and control tissues at each DAG ($P \leq 0.05$) and different letters indicate significant differences among periods in grafted plants ($P \leq 0.05$).

cell divisions and differentiation. At the early stage of graft development, calli between the rootstock and scion grew to form a bridge (Baron *et al*. 2019), which partly re-established the water supply. Studies have shown a positive relationship between callus growth and POD activity (Arnaldos *et al*. 2002, Alhasnawi *et al*. 2016). Our previous anatomical observations showed that, at 7 - 10 DAG, calli bridge was formed on the pecan graft interface (Mo *et al*. 2017). In this study, POD activity in both the graft union and control increased during the same period, but the former had a faster change, which might have improved the connection of the calli. Deeper studies have revealed that POD facilitates the deposition and covalent linkage of cellular extension, with consequences for plant cell growth (Jackson *et al*. 2001, Pereira *et al*. 2011). Plant hormones are one of the main endogenous factors that regulate the vegetative and reproductive development of plants. IAA and cytokinin content is positively correlated with the callus formation rate in grape graft unions (Zhou *et al*. 2020). In our results, the IAA content increased during 5 - 10 DAG, similar to the ZR content increasing from 7 - 10 DAG in grafted plants, which is consistent with our anatomical observation that calli proliferated and filled the gap between the rootstock and scion in this period (Mo *et al*. 2017). Soluble sugars, starch, and soluble proteins are involved in histogenesis since this is an energy-consuming process (Peng *et al*. 2020). A positive relationship between the sugar content and scion viability has been observed in grapevines (Phillips *et al*. 2015). In pomegranate, a higher grafting success rate is related to a higher soluble sugar content in scions because of the role of sugars in cell division (Karimi *et al*. 2017). For pecan, grafted plants had lower soluble sugar, soluble protein, and starch content than the control plants for most of the times during graft union formation, probably due to the consumption of sugars as the main energy source. Additionally, we speculated that there was an upper limit for the accumulation of soluble protein in the graft union, because of the three similar peaks in the soluble protein content.

For scions, vascular reconnection can recover the water and nutrient supplies from the rootstock, which plays a critical role for grafting survival. In this study, POD and PPO activities in pecan graft unions were significantly higher than that in the controls at 22 DAG, consistent with the differentiation of new vessels (Mo *et al*. 2017). The results suggested that, POD and PPO are not only protective enzymes, but also participated in this process. During the reestablishment of the vascular system, lignin deposits in the cell walls of the vascular bundle sheaths and vessels (Zhao *et al*. 2020). POD and PPO are positively related to the biosynthesis of lignin *via* playing different roles, the former provides precursors for lignification by oxidizing phenolic compounds, while the latter catalyses the oxidative polymerization of monolignols (coniferyl alcohol, sinapyl alcohol, paracoumaryl alcohol) to form lignin (Boudet *et al*. 2003, Yang *et al*. 2007, Barros *et al*. 2015, Sabella *et al*. 2018, Sharma *et al*. 2019, Zhao *et al*. 2020). Hormonal effects on plant vascular tissue formation have been widely studied (Baron *et al*. 2019). In *Arabidopsis*, IAA and cytokinin responsive genes were increasingly expressed in graft junctions at the stage of vascular reconnection, while ungrafted plants showed no such expression (Melnyk *et al*. 2018). As expected, the average content of IAA and ZR on the graft interface of pecan was significantly higher than in the control at 14 - 31 DAG, which was consistent with cambial growth and vascular differentiation (Mo *et al*. 2017). The IAA response mainly occurred in the pericycle, while cytokinin acts both in the pericycle and vascular cambium, and it is the major hormonal factor in regulating cambial activity (Nieminen *et al*. 2008, Melnyk *et al*. 2018). Moreover, IAA and ZR content in the graft union sharply increased relative to those in the control at 14 - 18 DAG, thus it seems that the two hormones have distinguishing but synergetic effects on improving cambial activity, which has been demonstrated by Immanen (2016). Although researchers have found that ABA is responsible for plant dwarfing by modifying vascular development, a full understandings of the effect of ABA on vascular reconnection is still lacking (Tworkoski and Fazio 2011, 2015, Baron *et al*. 2019). Additionally, ABA can inhibit callus growth and meristem activity (Sujittra *et al*. 2017, Takatsuka *et al*. 2019, Zhou *et al*. 2020). The ABA content in the pecan graft union was lower than that in the control during graft formation, which might imply that ABA had adverse effects on various aspects of the healing process, not just vascular reconnection.

To sum up, this paper suggested that various physiological and biochemical compounds took part in the graft union formation in pecan. At early stage, PPO and POD played defensive roles against wounding stress. Furthermore, POD might be required for callus proliferation and for vascular reconnection, similar to the probable effects of IAA and ZR. Graft development is an energy-consuming process, therefore it required the provision of sugars. Adversely, ABA and tannin seemed to have inhibitory effects on graft tissue formation. However, further in-depth analyses of the histochemical and molecular mechanisms underlying graft union formation are essential, because of its high complexity.

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