Visualization of water transport pathways in various organs on the fruit-bearing shoot of walnut trees

Visualización de vías de transporte de agua en varios órganos del brote frutal de nogales

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SUMMARY

To reveal the regular patterns of water transport in fruit-bearing shoots of walnut (*Juglans regia*), water transport pathways for various organs were observed using dye-tracing technique; water potential, water status and water transport rate were determined for each of these organs. Water potential in the pedicel, petiole, lateral shoot and main shoot was -2.59, -2.85, -1.68 and -0.37 MPa, respectively. Significant differences were noted among various organs in terms of water status. The ratio of bound water to free water in the petiole was the highest (1.93 RBC), followed by the main shoot, lateral shoot and pedicel. Water transport speed in the petiole was the fastest (3.13 cm/min), followed by the lateral shoot, pedicel and main shoot. However, the water transport rate of the main shoot was the highest, and higher than the sum of the other three organs. The xylem in the petiole was separated from the main shoot; at the top of the main shoot, the xylem was divided into two parts, one of which was connected to the xylem of the pedicel and the other to the lateral shoot. During the shell-hardening period of fruits, the dye was found at the edge of the fruit vascular bundle, although, it was not at the central vascular bundle. No dye was found in the main and accessory buds, instead, only a small part of the xylem was dyed between the accessory bud and the main shoot. Water transport pathways were different for various organs. Thus, the transport and distribution of water in various organs was affected by their water potential, tissue structure and water requirement.

Key words: Juglans regia, water, dye-tracing, water potential, xylem.

RESUMEN

Para revelar pautas regulares de transporte de agua en brotes frutales de nogal (*Juglans regia*), se observaron vías de transporte de agua de diversos órganos mediante rastreo de tintes; se determinó: potencial hídrico, estado del agua y tasa de transporte de agua de cada órgano. El potencial hídrico en el pedicelo, peciolo, brote lateral y brote principal fue de -2,59, -2,85, -1,68 y -0,37 MPa, respectivamente. El estado del agua mostró diferencias significativas entre los órganos. La relación entre el agua ligada y el agua libre en el pecíolo fue la mayor (1,93 RBC), seguida por: brote principal, brote lateral y pedicelo. La velocidad de transporte de agua en el pecíolo fue la mayor (3,13 cm/min), seguida del brote lateral, pedicelo y brote principal. Pero la tasa de transporte de agua del brote principal fue la mayor y más alta que la suma de los otros tres órganos. El xilema del peciolo estuvo separado del brote principal; en su parte superior estuvo conectado al xilema del pedicelo y al brote lateral. Durante el período de endurecimiento de la cáscara del fruto, el colorante estuvo en el borde del haz vascular del fruto, pero no en el haz vascular central. No se encontró colorante en yemas principales ni accesorias, en cambio, solo se tiñó una pequeña parte del xilema entre la yema accesoria y el brote principal. Las vías de transporte de agua fueron diferentes para los distintos órganos. El transporte y distribución del agua en varios órganos fueron afectados por su potencial hídrico, la estructura de los tejidos y las necesidades de agua.

Palabras clave: Juglans regia, agua, rastreo de colorante, potencial hídrico, xilema.

INTRODUCTION

Water is the carrier of plant nutrients during plant functioning, and a variety of physiological and metabolic reactions take place in water, directly or indirectly. Therefore, water is essential for the material circulation and energy metabolism in plant operation. Water plays a decisive role in the activities of plants, and the study of plant water physiology remains a key issue in plant physiology (Johnson *et al.* 1965, Blackman *et al.* 1985, Cochard *et al.*

2002, Javaux *et al.* 2016). The process of water transport in plants is driven not only by the negative pressure generated by leaf transpiration, and the growth and metabolic activities of tissues and organs, but also by the external environment, the tissue structure and water requirement of organs (Markhart *et al.* 1979, Ford *et al.* 2004, Martorell *et al.* 2013). Much of the current research is primarily focused on studying the influence of external environmental factors on plant water transport. In contrast, there are very few reports on the structure of water transport pathways for various plant organs (Xie *et al.* 2016).

With the developments in the study of transport physiology, it has been established that the long-distance transport of water from the roots to the leaves is carried out mainly through the xylem, a process in which xylem vessels play an important role (Fricke 2017). The driving force for conducting both water and dissolved mineral ions from the roots to the leaves is primarily composed of two forces: the transpiration pull from leaves and root pressure (Tyree *et al.* 2002). Vessels show a great variety of structural features that could affect water transport in xylem of plants (Taneda *et al.* 2007). Drought as well as freeze-thaw events can induce embolism in the plant xylem. Embolisms can impede water transport in plants, in severe cases leading to the death of roots, branches and even whole plants (Schenk 2014). Hydraulic conductivity, number of vessels, average diameter of vessels and vessel length, all show a gradual exponential decrease from the base of the plant to higher up the stem (Nijsse 2001). Sperry (1991) suggested that in rapidly growing *Populus tremuloides* Michx. branches, the vessels of the outer growth ring were functional, whereas vessels in older xylem were mostly embolized. Water transport in plants is not just a simple flow through xylem vessels and sieve tubes, but also includes xylem-phloem exchange during transport (Patrick *et al.* 2001). A study by Ohya (2008) showed that the net volume of water escaping from xylem vessels was not dependent on the transpiration rate of the plant. The self-diffusion effect of water was strong for lateral water movement, although another driving force besides thermal motion was included in the process, and the process was also affected by the water permeability of the plasma membrane. Transport across the cell-to-cell pathway can involve water crossing plasma membranes, and thus, the rate of water uptake can be influenced by the abundance and activity of aquaporins (Gambetta 2013).

Walnut (*Juglans regia* L.) is a deciduous tree of the Juglandaceae, and is one of the famous "four nuts" in the world. Walnut forests provide several ecological and economic benefits (Gauthier *et al.* 2011). Walnut cultivation has become an important pillar industry for mountain farmers to improve their economic situation, and it has been listed as a national strategic economic forest tree by the State Forestry Administration in China. It has been shown that the yield and quality of the walnut depend directly on the status of water supply (Cristofori *et al.* 2009). Shoots are the main parts of walnut trees during the growing season,

and fruit-bearing shoots support many other organs such as leaves and buds. Shoots are not only a part of tree structure, but also the main organ of water consumption through the leaves growing on shoots. These organs represent key avenues of water use in tree bodies, even though there are some differences in their water use patterns and water needs, due to the fact that the differences would be structural in terms of wood anatomy. Understanding the xylem structure and water utilization patterns of various organs on the fruit-bearing shoot can provide a theoretical basis for rational irrigation and efficient water use in walnut trees.

In this study, the water transport pathways for each organ were observed using the dye-tracer method for elucidating the fruit-bearing shoot water utilization patterns in walnut. In addition, we investigated water potential in different organs across time and developmental stages by obtaining data on water potential, water status and water transport rate. We combined different approaches to comprehensively map out the potential routes for water transport and the physiology behind them. Results would be especially relevant in an ecological context, primarily in cases where there is limited water availability.

METHODS

Site description. The study was conducted in the trial orchard of Hebei Agricultural University, which is located in the Lianchi District of Baoding City, Hebei Province (38°49′–38°56′N, 115°21′–115°34′E). This area belongs to the warm temperate continental monsoon climate, with four distinct seasons. Annual average temperature is 12.7 °C, the highest temperature is 43.3 °C, and the lowest temperature is -26.8 °C. Annual sunshine time is 2,447–2,871 hours, the frost-free period lasts 165–210 days, average annual rainfall is 575.4 mm, and average annual evaporation is 1,758.3 mm. The soil type is sandy loam.

Experimental materials and methods. The walnut trees were seedlings of the precocious walnut cultivar Juglans regia "lvling" which were sowed in the spring of 2015. The Walnut trees were about 1.0 cm in diameter and 1.5 m in height. The row spacing was 20 cm × 40 cm, and the direction of planting was east-west.

At the shell-hardening period, 18 fruit-bearing shoots with 1–2 fruits and one lateral shoot were selected to carry out the experiments from 120 total samples. Six of them were used to determine physiological indexes (plant water potential, plant bound water and free water), and the other 12 were used for dye-tracer observation. Dye-tracer observation was carried out at about 9:00 am on June 20, 2017 (temperature is 29 ºC), and related physiological indexes were measured at the same time. Specific test contents are as follows:

1. Water potential: Water potential was determined using a Psypro dew point water potential measurement system (Wescor, Logan, America).

2. Plant water contents: Total, free, and bound water were determined by Abbe refractometer according to the method described by Slavik (1974).

3. Water transport rate: Cut the organs off the walnut tree and place the cut end immediately inserted into a plastic bottle with the dye solution. After 5 min, the transport distance of the dye in the organs could be observed. Through the distribution of dyes in different organs, the cross-sectional area of water passing through can be calculated. Transport rate = transport distance / time×the cross-sectional area of water passing.

4. Observation of water transport pathway: The water transport pathway of organs was observed by the dye-tracing technique, following Bhaska *et al.* (2005). The dye used in the experiment was 0.2 % (m/V) basic fuchsin solution. The cutting end of the fruit branch of walnut was put into the dye and removed after 2 hours. The distribution of dye in various organs or tissues were observed un-

der an Olympus SZ61 stereo microscope (Olympus, Tokyo, Japan) and a Motic BA210 basic biological microscope (Motic, Amoy, China).

Statistical analyses. The differences among organs were detected using one-way analysis of variance (ANOVA). The statistical evaluation of data was performed by the DPS 7.05 program (P ≤ 0.05).

RESULTS

Water transport between the main shoot and the leaf. At the node between the petiole and the shoot, the water transport pathway in the petiole was associated with the main shoot by the xylem that separated from the main shoot, and the xylem of petiole came from the inner layer of the main shoot xylem (figure 1A–E). Radial distribution of the dye indicated that the xylem of the petiole came from the
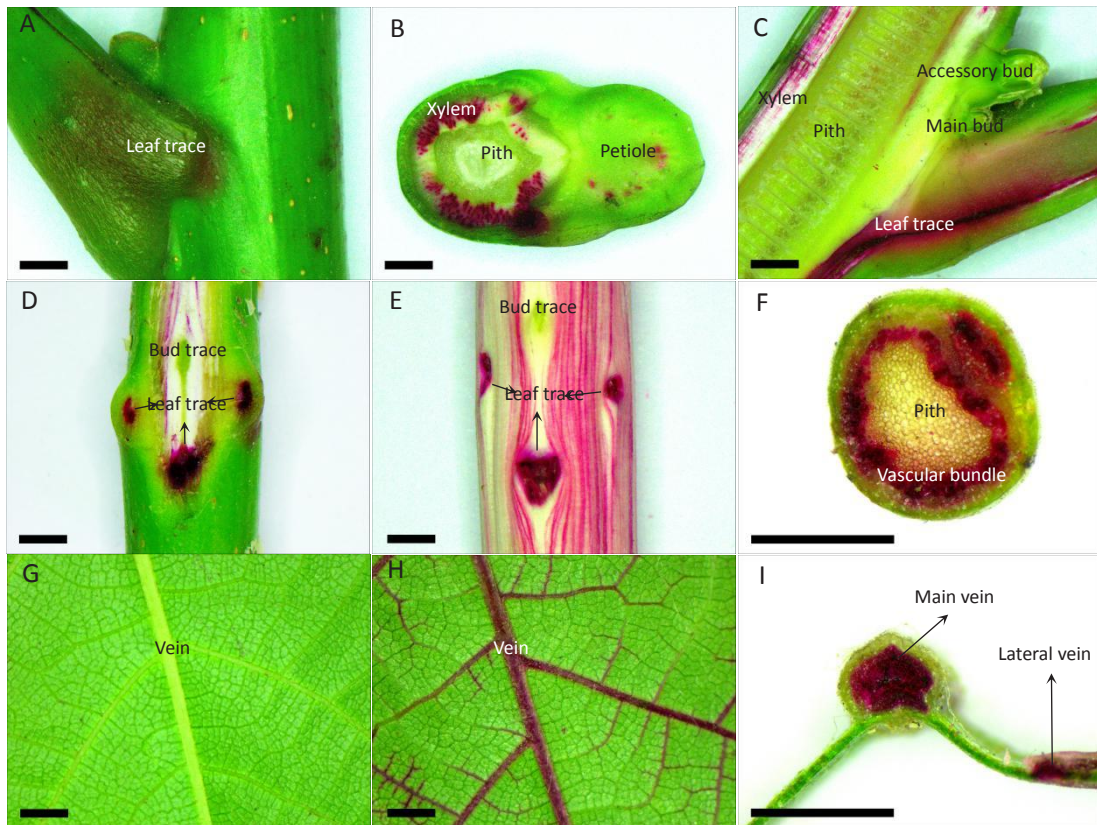

Figure 1. Distribution of the dye in the main shoot and the leaf. A: The junction of the main shoot and the leaf; B: The cross-section of the junction of the main shoot and the leaf; C: The Longitudinal section of the junction of the main shoot and the leaf; D: The Lateral section of the junction of the main shoot and the leaf; E: The xylem of the junction of the main shoot and the leaf; F: The cross-section of the petiole; G: The leaf before and after the entering of dye; H: The cross-section of the vein. The length of the scale in the graph is 2.5 mm.

Distribución del colorante en el brote principal y en la hoja. A: Unión del brote principal y la hoja. B: Sección transversal de la unión del brote principal y la hoja. C: Sección longitudinal de la unión del brote principal y la hoja. D: Sección lateral de la unión del brote principal y la hoja. E: Xilema de la unión del brote principal y la hoja. F: Sección transversal del peciolo. G: Hoja antes y después de la entrada del tinte. H: Sección transversal de la vena. La longitud de la escala del gráfico es de 2,5 mm.

primary xylem at the base of the internode of the main shoot (figure 1C). From the distribution of the dye in the leaves, it was clear that the regular pattern of water entering the leaves is: the petiole → main leaf veins → lateral leaf veins → mesophyll cells (figure 1G and H). In the vascular bundles of the petiole, xylem and a part of the phloem were dyed red (figure 1F), the vascular bundles and pith of the veins were also dyed red (figure 1I), while the dye could not be seen clearly in the phloem of the main shoot. This phenomenon indicated that the rate of lateral diffusion of water in various organs was different.

Water transport between the main shoot and the lateral shoot, buds and fruit. The lateral shoot was developed from the bud on the main shoot, and the connection between the main shoot and the lateral shoot was similar to that between the main shoot and the pedicel (figure 2A and B). The dye color of the longitudinal section at the junction of the main shoot and the pedicel suggested that the water transport rate in the lateral shoot was similar to that in the petiole, whereas both of them were clearly faster compared with the pedicel.

In the dye-tracer experiment, the dye was not seen in the main bud (figure 2D–F), which indicated that the main bud did not establish a direct contact with the xylem of the main shoot during the shell-hardening period, which retained a relatively independent status. Although no dye was found in the accessory bud either, a small portion of the xylem between the accessory bud and the main shoot was dyed (figure 2E and F). The accessory bud had just sprouted, and the main bud was in dormancy at this time. It can be concluded that the xylem in the bud was formed after the sprouting.

From the longitudinal section of the junction of the main shoot and other organs, it was found that the pith of the main shoot was curved towards the pedicel (figure 2B). The xylem of the pedicel was connected directly with that of the main shoot, and water flowed in sideways into the fruit at the junction of the main shoot and pedicel (figure 2G–I). From the distribution of the dye in the pedicel, only the

Figure 2. Distribution of the dye in the main shoot, lateral shoot, fruit and buds. A-B: Distribution of the dye at the junction of the main shoot and the pedicel, lateral shoot and leaf; C: Longitudinal section of the lateral shoot; D-F: Distribution of the dye at the junction of the main shoot and the bud; G: Longitudinal section of the pedicel; H-I: Distribution of the dye in the fruit. The length of the scale in the graph is 2.5 mm.

Distribución del colorante en el brote principal, el brote lateral, el fruto y las yemas. A-B: Distribución del colorante en la unión del brote principal y el pedicelo, el brote lateral y la hoja. C: Sección longitudinal del brote lateral. D-F: Distribución del colorante en la unión del brote principal y la yema. G: Sección longitudinal del pedicelo. H-I: Distribución del colorante en el fruto. La longitud de la escala del gráfico es de 2,5 mm.

xylem in the underside of the pedicel was dyed (figure 2B and G), which indicated that the xylem at the underside of the pedicel was the main water transport pathway to the fruit. The dye was found at the dorsal vascular bundles of the fruit (figure 2H and I) nevertheless, it was not found in the central vascular bundle of the fruit (figure 2I). The result clearly indicated that the water requirement for kernel development was low, and the kernel maintained its internal water balance through water diffusion with the husk.

Structural characteristics of water transport channel. Structural characteristics of the water transport channel of each part of the fruit-bearing shoot are presented in figure 3. The diameter of vessels in the pedicel was the least, about 0.02 mm, and was significantly lower compared with the other parts. The density of vessels in the petiole was the highest, which was about the same as that in the pedicel, whereas lateral shoot had lowest density of vessels. From the distribution of the dye in the cross-section of various organs, it could be seen that the dye color of various xylem vessels in the same organ was different and no dye was found in some of the xylem vessels. The result indicated that the water transport rate among various xylem vessels was also different, and it was not necessarily associated with the diameter and density of the catheter.

Water potential in organs. The water potential of the various organs on the fruit-bearing shoot exhibited significant differences (figure 4). The water potential of the main shoot was the highest, followed by the lateral shoot and the pedicel, whereas the petiole showed the lowest value. There was a large difference in the water potential between the main shoot and the pedicel and petiole, although the difference between the main shoot and the lateral shoot was relatively much lower.

Water status in organs. Water status in various organs on the fruit-bearing shoot is presented in figures 5 and 6. The free water content in the lateral shoot was the highest, which was significantly higher than that of the main shoot and the petiole. The free water content in the pedicel was not significantly different from that in the lateral
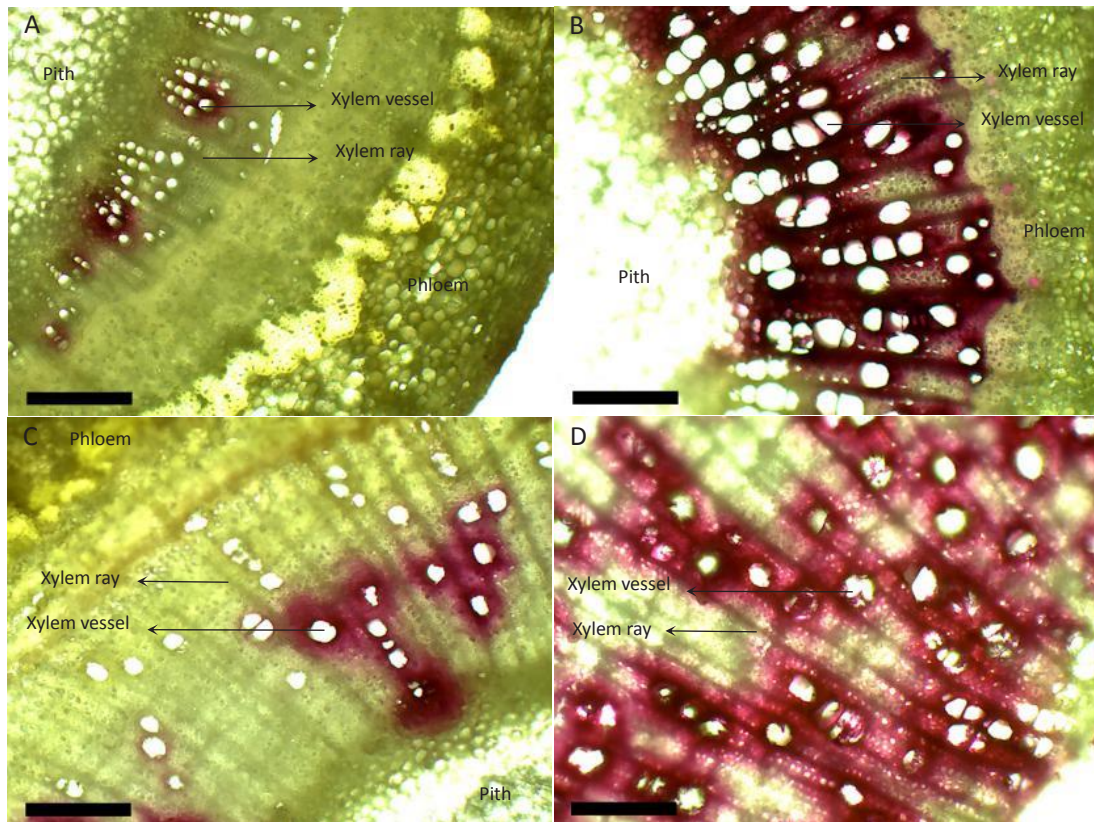

Figure 3. Distribution of the dye in xylem of the pedicel, petiole, lateral shoot and main shoot. A: Distribution of dye in the xylem of pedicel; B: Distribution of dye in the xylem of petiole; C: Distribution of dye in the xylem of lateral shoot; D: Distribution of dye at the xylem of shoot. The length of the scale in the graph is 0.25 mm.

Distribución del colorante en el xilema del pedicelo, el peciolo, el brote lateral y el brote principal. A: Distribución del colorante en el xilema del pedicelo. B: Distribución del colorante en el xilema del peciolo. C: Distribución del colorante en el xilema del brote lateral. D: Distribución del colorante en el xilema del brote. La longitud de la escala del gráfico es de 0,25 mm.
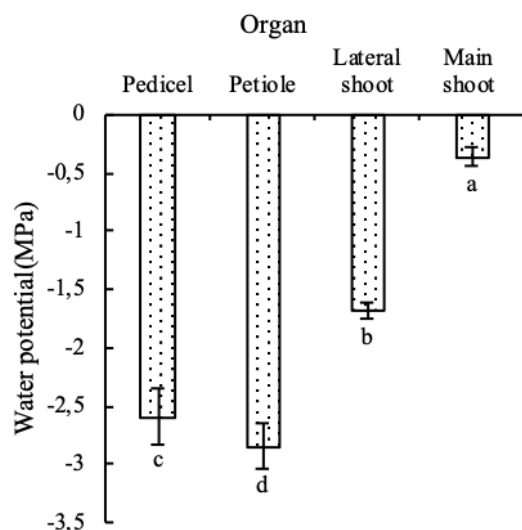
Figure 4. The water potential of various organs on the fruit-bearing shoot. Different lowercase letters indicate that there are significant differences among the treatments ($P < 0.05$).

Potencial hídrico de varios órganos en el brote del fruto. Diferentes letras minúsculas indican que hay diferencias significativas entre los tratamientos ($P < 0,05$).
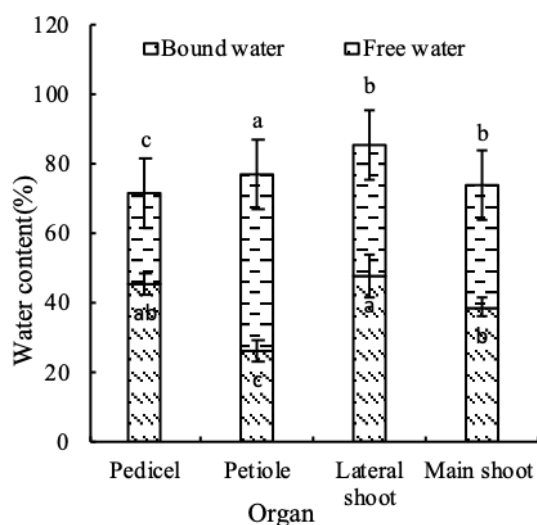

Figure 5. The water status in various organs on the fruit-bearing shoot.

Estado hídrico de varios órganos en el brote frutal.

shoot. However, the highest bound water content was observed in the petiole, which was significantly higher than the others, whereas the bound water content in the pedicel was the lowest. The total water content in the lateral shoot was the highest, which was significantly higher than the others. There was no significant difference among the pedicel, the lateral shoot and the main shoot regarding the ratio of bound water to free water, and these were signi-
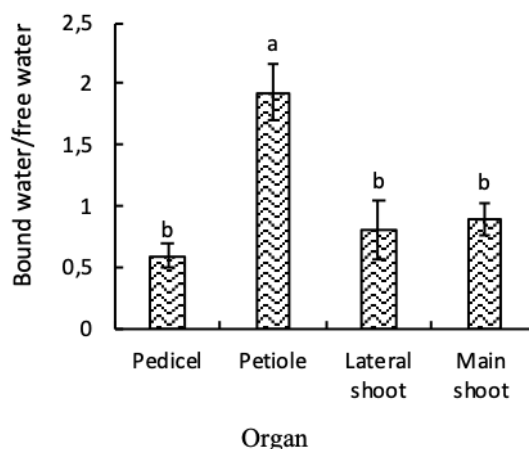
Figure 6. The ratio of bound water to free water in various organs on the fruit-bearing shoot.

Relación entre el agua ligada y el agua libre en varios órganos del brote frutal.

ficantly higher than the ratio of bound water to free water of the petiole.

Water transport rate in organs. The leaf, bud, fruit and lateral shoot were growing on the main shoot, which is the last passageway of water into these organs through long distances. The water in the leaf, bud, fruit and lateral shoot was directly derived from the main shoot, and therefore, these organs form a competitive relationship with each other, such that the water transport rate in one part would directly affect the distribution of water in others. The water transport speed in various organs exhibited significant differences ($P < 0.05$) (figure 7). The water transport speed in the petiole was the highest, and it was significantly higher compared with that in the main shoot and the pedicel. The water transport speed in the lateral shoot showed no significant difference with that in the petiole. It was found that the higher the degree of lignification, the slower was the water transport rate. However, the water transport rate of the main shoot was the highest, and higher than the sum of the other three organs.

DISCUSSION

There was a significant difference in the water transport rate for the various organs on the fruit-bearing shoot due to the difference in their anatomic structure and water requirement. In this experiment, the water transport pathway from the main shoot to the other organs of the fruit-bearing shoot could be clearly seen using the dye-tracing technique. During the longitudinal transport of water, the water transport pathways from the main shoot to the other parts were independent from each other, and the corresponding xylem was not connected with each other. The position of the lateral shoot, leaf and fruit was close to the
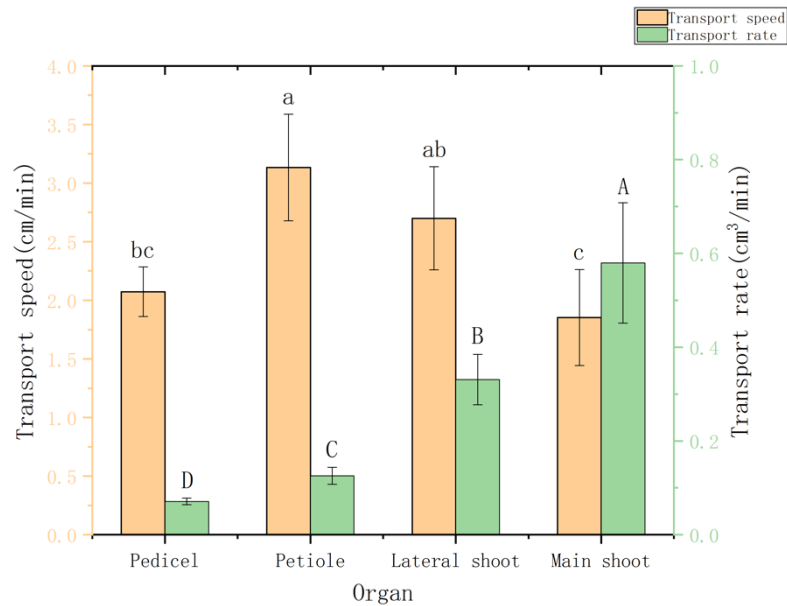
Figure 7. The water transfer speed and transfer rate in various organs on the fruit-bearing shoot.

Velocidad de transferencia de agua y la tasa de transferencia en varios órganos del brote frutal.

main shoot, and the water competition relationship among them was mainly related to their water potential (Tyree and Zimmermann 1983) and structure (Jožica *et al.* 2017). The traditional cohesion-tension theory held that the low water potential produced by transpiration was the main driving force for water transport in the xylem (Tyree 1997). Zhang *et al.* (2006) found that there are stomata on different green organs, though the number of stomas exhibited significant differences. The difference in the number of stomata caused differential transpiration pull, which may be the main reason for the difference in water transport rate between different organs on the fruit-bearing shoot, although it is also limited by the diameter and structure of the conduit. If the transport rate was determined only by the potential difference, the water transport rate should have been higher in the pedicel than in the lateral shoot, as the water potential in the former was lower than that in the latter, though the result was the opposite. The differences in the structure of the two organs (the water transport pathway at the junction between the main shoot and the lateral shoot was straight, while the water transport pathway between the main shoot and the pedicel was curved) were suspected to make the difference in terms of resistance to water. Zimmermann *et al.* (2004) also mentioned that in addition to transpiration pull, other factors play an important role in the long-distance transport of water. It was found that the pith of the petiole was all dyed, a part of the pith of the lateral shoot was dyed, and the pith of the pedicel and the main shoot were not dyed (figure 6B). This indicated that there was a horizontal transport of water in plants, nonetheless there were differences in the horizontal transport

rate of water in different organs. Using hydrogen isotope tracer technique, Liu *et al.* (1998) confirmed that water can be transported laterally in plants. The difference in the rate of lateral diffusion of water may be related to water loss in the stomata of the epidermis and metabolic processes in the cell.

Water is an important factor in the growth and development of organs or tissues. At the beginning of sprouting, the accessory buds had no xylem, and there was a small part of the xylem found between the accessory bud and the main shoot (figure 2E). However, there was no xylem between the accessory bud and the main shoot before sprouting (figure 1C). Result indicated that the connecting xylem between the shoot and buds was formed after sprouting, and the water requirement for the organs was different in different developmental stages. When Drazeta *et al.* (2004) and Dichio *et al.* (2003) studied the water transport pathway in apple fruit, it was found that the water requirement of fruits was different at different developmental stages. The water transport efficiency in the xylem of the fruit decreased with the growth and development of the fruit. This could explain why the dye was found only in the edge vascular bundle of the fruit though no dye was found in the central vascular bundle of the fruit (figure 2H and I). Choat *et al.* (2009) and Wang *et al.* (2015) extensively discussed the reasons for the decline in xylem water transport rate in the fruit, and found that, during the development of fruits, the expansion of parenchyma cells in the xylem caused the main vascular bundle to be extruded and deformed, which resulted in the dispersion of some vascular bundles and the dislocation between the vessels that lose the collaboration

function, such that the water transport rate in the xylem decreased. Understanding the water requirement of the fruit at different developmental stages could help make the irrigation strategies more scientific.

Not only the width increments but also the anatomy of the xylem and phloem differed in the stem and branches. The xylem vessel is the main channel for longitudinal water transport, and vessel features strongly influence the amount of water that can be transported in a living tree. There is a strong vessel size–conductivity relationship because the hydraulic efficiency of the vessel increases proportionally to the fourth power of its radius (Hagen–Poiseuille law); therefore, even small differences in vessel size would drastically change water transport efficiency and security (Lynch *et al.* 2014). Jožica *et al.* (2017) found an inverse correlation between vessel density and diameter in all sampling parts, which can be explained by hormonal regulation (Aloni 2015). In addition to the auxin radial distribution pattern, steep concentration gradients of soluble carbohydrates (particularly sucrose) across developing vascular tissues in plants suggest a role for sugar signaling in vascular development (Uggla *et al.* 2001).

CONCLUSIONS

During the longitudinal transport of water, the water transport pathways from the main shoot to the other parts were independent of each other, and no connection was observed among their xylem tissues. Water was transported vertically and horizontally in plants, and there was a significant difference in the transport rates for different organs. The longitudinal water transport rate was determined mainly by water potential, and the organizational structure of organs also showed a significant effect. Moreover, the water requirement of the organs at different developmental stages was different. However, the water transport rate in organs was determined in vitro using the dye-tracer method, and the dye also had some effect on the water transport rate. We need to look for better technical means to determine the real water transport rate in organs under normal growth. In addition, we also need to further study the structure of various organs to better explain the mechanism of water transport, for example, the proportion of different types of vessels and the water transport rate in various types of vessels.

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