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Own-rooted Walnut Propagule of Four Walnut (*Juglans*) Rootstocks and Main Cultivated Cultivar Liaoning 1 Acquirement through Layering under Field Conditions

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Abstract. **Walnut, a woody plant, is regarded as having difficulty rooting when propagated by vegetative methods, such as cutting and layering. A layering experiment was conducted in 2018 and 2022. In 2018, some *Juglans* species, including *J. regia* L. seedling (JR), *J. regia* cv. Liaoning 1 (JR LN1), *J. hopeinesis* Hu seedling (JH), *J. mandshurica* Maxim seedling (JM), and *J. nigra* L. seedling (JN), were the mother plants. The specific research hypotheses were that own-rooted walnut propagule could be obtained through layering. the rooting capacity of different *Juglans* species would be different, and the rooting ability of JN would be the highest among the samplings. The results indicated that all of these species in the experiment could be rooted by etiolation and indole-3-butyric acid (IBA) treatment and that root occurrence was found 6 to 7 weeks after IBA treatment. The layers (shoots from the mother plant) on the seedlings of JR, JH, and JM obtained rooting percentages (RP) of 75.55%, 84.45%, and 86.67%, respectively, and root numbers (RNs) of 21.8, 42.8, and 38.8, respectively, after 20 days of etiolation and 1% IBA treatment. JR LN1 had difficulty rooting in equal conditions and had a RP of 31.11%. In 2022, JR LN1 was the only mother plant and the IBA concentration was increased to obtain satisfactory RP and RN. With the 4% and 8% IBA treatments, RPs of 88.9% and 93.3% and RNs of 40.3 and 27.7, respectively, were achieved. During the experiment, the RP, RN, root length (RL), and root diameter (RD), as well as the layer height (LH) and layer diameter (LD), were investigated and evaluated. Layers with low vigor were more likely to root, as shown by a nonparametric test conducted for the height and diameter of the layers of the rooting and nonrooting groups. A significantly negative correlation ($r = -0.548$) was observed between RN and LH. Moreover, the quality of the best results of JR LN1 layering propagule and that with 'liaoning 1' 1-year-old seedling were compared. Our results provide more support for the possibility of vegetative propagation of walnut by layering and more information regarding the clonal cultivation of walnut trees and the own-rooted seedling establishment of walnut cultivars.**

Walnut is the common name given to 25 species of deciduous trees belonging to the genus *Juglans* (order Fagales, family *Juglandaceae*), which are distributed across the temperate and subtropical regions of the northern hemisphere (Bernard et al. 2017). In China, walnut has been listed as one of the national strategic tree species and has become a pillar industry in many rural areas. It plays an increasingly important role in adjusting the agricultural planting structure, thus increasing farmers' income, earning foreign exchange from export, and guaranteeing national grain and oil security and ecological security (Tian et al. 2010). At the end of 2021, China has 7,454,935 ha of cultivated walnut area, with 5,403,500 tons of nut production and 59,472 tons of oil production (Guan 2021). However, the walnut industry in China has entered a bottleneck period mainly because of the large plantation in shallow hilly areas with poor soil and long-term use of seedling-plants and traditional rootstocks that resulted in low yield and poor quality (Liu et al. 2020). Therefore, the selection of suitable walnut rootstocks and its effective vegetative propagative methodology are critical steps in the walnut industry that not only guarantee the longevity of a planted tree but also promote vegetative growth in the scion, shorten the time of fruiting, and enhance stress resistance (Gregory et al. 2013; Rouphael et al. 2018; Warschefsky et al. 2016).

The importance and merit of rootstocks were demonstrated in agriculture, horticulture, and silviculture (Gregory et al. 2013; Lee et al. 2010; Nawaz et al. 2016; Rouphael et al. 2018; Wang et al. 2017; Warschefsky et al. 2016). In terms of walnut rootstocks and cultivars, researchers have focused more attention for both scion cultivar breeding (Hassani et al. 2014; Leslie and Mcgranahn 2014; Wang et al. 2014; Zhang et al. 2014) and propagation techniques (Leslie et al. 2010; Vahdati and Hassankhah 2014; Vahdati et al. 2021; Yu et al. 2014).

The traditional method of walnut propagation involves grafting the scion cultivar onto the seed-derived rootstock. Because the seed-derived rootstock is a sexually reproducing individual, this would have a great impact on scion cultivars (Cañas-Gutiérrez et al. 2022; Gautier et al. 2019; Nimbolkar et al. 2016). Therefore, from a strict point of view, grafting the scion cultivar on the seed-derived rootstock could not produce genetically uniform propagules. Since 1972, softwood cutting and hardwood cutting for paradox (*J. hindsii* × *J. regia*) have been performed by University of California Davis researchers (Hartmann and Martin 1972). Whereas walnut trees are recognized as difficult to root (Pei and Gu 2002), the results of cutting have not been satisfactory and there has been no breakthrough in this area to apply it to commercial production (Vahdati et al. 2022). With the maturity and development of tissue culture technology, walnut micropropagation technology has been gained more attention and achieved tremendous progress in rootstock and cultivar propagation (Davis and Sankhla 1988; Gotea et al. 2012; Leslie and McGranahan 1992; Liu et al. 2018; McGranahan et al. 1987; McKenna and Sutter 1996; Payghamzadeh and Kazemitabar 2011; Pei et al. 2007; Yegizbayeva et al. 2021). Rootstock micropropagation for paradox cutting has been successful and used in commercial rootstock production (Ribeiro et al. 2022).

However, the phased research results showed that the performance of own-rooted walnut tree of a cultivar was better than that of grafted ones. Own-rooted walnut trees provide a means of establishing genetically uniform orchards without the risk of blackline disease caused by cherry leafroll virus in trees grafted to black or hybrid walnut rootstocks (Mircetich and Rowhani 1984). Own-rooted walnut plants may be more precocious and productive than grafted trees (López 2001; McGranahan et al. 1988). Five years of field experimental results indicated that own-rooted 'Chandler' had significantly greater trunk circumference and yield than 'Chandler' on Paradox rootstock (Hasey et al. 2001). It has been suggested that the development of size-controlling walnut rootstocks or own-rooted cultivars can maintain consistent vigor or precocity on scions (Rezaee et al. 2009).

Layering is a method of asexual reproduction in trees in which the shoot of a tree is pressed into the matrix when it is not separated from its mother to promote the rooting of the pressed part and then cut from the mother plant to form a new independent plant (Roman and Tadeusz 2003; Webster 1995). To the best of our knowledge, only a few studies have addressed the fact that the rootstock or cultivar was commercially propagated through layering. Gutenev and Bogoroditskii (1974) obtained a root percentage (RP) of 21.6% in vertical layering trials. Poissonnier (1978) reported that 55% of the layers were rooted during an experiment of stooling and air layering of a hybrid walnut. Gutenev (1980) conducted layering on 3-, 6-, and 12-year-old walnut seedlings and adopted ringing and treated layers with incinerator bottom ash (IBA). Although an RP of 88% has been achieved, the number and quality of roots were not good. Pandey et al. (1982, 1983) and Pandey and Sinha (1984) rooted the stump sprouts with a combination of IBA plus indole-3-acetic acid (IAA) plus α-naphthaleneacetic acid (NAA) at 7500 ppm, and the RP was approximately 80%. Vahdati and Khalighi (2001) conducted a stool layering experiment and discovered that the favorable conditions for layer rooting were 5000 or 7500 ppm IBA+IAA+NAA, mounding with a mixture of perlite and sand (2:1), and wiring the base of the layers. Vahdati et al. (2008) also reported that the rooted rates of low-vigor, semi-vigorous, and high-vigor seedlings were 40%, 31.42%, and 17.14%, respectively. During the experiment, the plant growth regulators prepared in lanolin were smeared at the base of the shoots, and longitudinal incisions were made.

In China, *J. mandshurica* Maxim seedling (JM), *J. hopeinesis* Hu seedling (JH), and *J. nigra* L. seedling (JN) can be used as a substitute stock for Persian walnut or English walnut (*Juglans regia* L.) to adapt to different ecological environments (Liu et al. 2018, 2019). Liaoning 1 has been one of the main cultivated walnut cultivars in China (Zhao et al. 2020) for more than 30 years.

The objective of the present research was to evaluate the adventitious rooting capacity and root quality in layering from four *Juglans* rootstocks, including *J. regia* L. seedling (JR), JH, JM, JN, and, particularly, walnut cultivar *J. regia* cv. Liaoning 1 (JR LN1) and to establish methodology for application in the production of commercial rootstock and own-rooted varieties under the field condition.

Materials and Methods

Plant material. The experiment was conducted in 2018 and 2022. In 2018, annual walnut seedlings of four *Juglans* rootstock, including JR, JR LN1, *JH, JM*, and *JN*, were the mother plants for layering. JR LN1 was propagated by budding in 2017, and rootstocks were propagated by seeds (*J. regia* L.). The other species were all derived from the seeds (Table 1). In 2022, 106 annual JR LN1 was used as the mother plant, which was propagated by budding in 2021. Also, JR LN1 seeds were sown in mid-April for their 1-year-old offspring quality comparison with that of the JR LN1 layering propagule. The seeds were collected in 2021 and stored in a cool house; they were soaked for 7 d before sowing in the cold water (reservoir).

Experimental site. The experiments were performed at Songmudao Village, Paotai town, Jinpu new district, Dalian City, Liaoning province, northeastern China (121°45′E, 39°24′N), where the average annual air temperature is 9.3 °C and the extremely low temperature can reach −21.4 °C. The number of annual frost-free days was 169 and the annual precipitation was 623 mm during the study plot. The number of sunshine hours was 2851 h. The nursery soil texture is a brown loam with a pH of 7.5.

Mother plant planting. The mother plants that were prepared during the last year were planted at the angle of 30 ° in relation the level of the ground in late March during the experimental year (Fig. 1). The stem of the mother plants was fixed with "L"-type number 14 iron hooks to keep the stems flat with the ground. The mother plants were planted with a width of 50 cm, planting bed depth of 40 cm, and spacing of approximately 20 cm. In the planting bed, 5 kg/m^2 manure was evenly applied in advance to ensure the mother plants grew well and to promote as many layers as possible. Next to the long side of the planting bed (Fig. 1), a 1.5-m-wide rooting bed was made. The rooting bed was dug to a depth of approximately 10 cm and flattened. After planting, the total stems of the mother plants were buried in 3 to 4 cm of soil.

Etiolating and layering. In June, when the new layers (shoots) on the stem of the mother plant grew to approximately 20 cm, the bottom of the new shoots was covered with 10- to 15-cm-thick river sand, and the etiolation treatment was performed for 20 d. After 20 d of etiolation, the river sand was spread at the base of the layers, and leaves 10 cm above the ground were removed in individual layers. The bases of the individual layers were circled with a binding line. The core of the binding line was a 0.5-mm galvanized line, and the outer coating was PVC material. The 10 cm above the base of all layers was treated individually with a thin layer of IBA-lanolin paste using a small hairbrush. IBA (Beijing Xuedong Chemical Factory) was thoroughly mixed with lanolin (exclusion of control) to form a homogenous paste in advance. To prepare the IBA-lanolin paste, the lanolin was placed in a small steel basin, which was heated by an induction cooker. When the lanolin reached the liquid state, the IBA was added to the basin; then, the mixture was stirred thoroughly with a glass rod. Finally, the basin was moved from the induction cooker and allowed to cool before application. At the end of the layering treatment, a covering of 10 to 15 cm of river sand was applied again at the base of the layers. When the water content of the sand decreased in the field, spray irrigation was performed.

Data recording and statistical analyses. A complete randomized block design with three replicates per treatment was used during the experiment. Each replicate included 15 layers (samplings). After layering, to investigate the shortest rooting time, the sand at the base of some of the layers was removed and recovered every 7 d to observe the rooting status. At the end of October, sand was removed to investigate the rooting and development of the layers. All root numbers (RNs) in each layer were counted. All of root diameters (RDs) near the root growing point and root lengths (RLs) of the rooted layers were measured with a digital caliper and meter ruler. The layer diameter (LD) at the base of the layer and layer height (LH) of all layers were measured. A one-way analysis of variance (ANOVA) including the RP, RN, RL, and RD determined during the experiments was performed after a test of homogeneity was conducted. If $P > 0.05$, then the means for

Table 1. Experimental materials and indole-3-butyric acid (IBA) concentration.

			IBA-lanolin paste preparation		
Yr	Mother plants	Abbreviation	IBA (g)	Lanolin (g)	IBA concn (%)
2018	*Juglans regia* cv. Liaoning 1	JR LN1	1	99	1
	Juglans regia L.	JR			
	Juglans hopeinesis Hu	JH			
	Juglans mandshurica Maxim	JM			
	Juglans nigra L.	JN			
2022	*Juglans regia* cv. Liaoning 1	JR LN1	0	100	0 (ck)
			0.5	99.5	0.5
			1	99	1
			2	98	2
			4	96	4
			8	92	8

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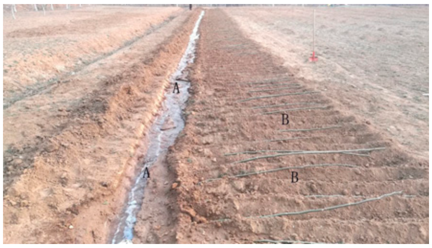

Fig. 1. Mother plant. (**A**) Planting bed. (**B**) Rooting bed.

Fig. 2. Layering stage and rooting status. (**A**) The day of indole-3-butyric acid (IBA) treatment. (**B**) The day after IBA treatment. (**C**) At 3 weeks after IBA treatment. (**D**) At 6 weeks after IBA treatment. (**E**) At 10 weeks after IBA treatment. (**F**) At 15 weeks after IBA treatment.

Fig. 3. The mother plant with rooted layers of *J. hopeinesis* Hu seedling (JH) at 15 weeks after the 1% incinerator bottom ash (IBA) treatment.

groups in homogeneous subsets were displayed directly. If $P < 0.05$, then data were transformed by ln(x + 3) before performing the ANOVA and Duncan's multiple range test. For the experimental data of 2022, without considering untreated control, a nonparametric test of the LH and LD of the rooting and nonrooting groups was conducted. During the Pearson correlation analysis, a two-tailed test was conducted. Quality of JR LN1 propagule propagated through layering (4% IBA treatment) and that of JR LN1 1-year-old plants were evaluated and compared using Student's *t* test.

Results

Rooting status of layering. The main experiment procedure is described in Fig. 2. Figure 2A shows the status of the layer before IBA treatment. After IBA treatment (Fig. 2B), the layer continued to grow (Fig. 2C). The callus began to form at the base of the new layers at almost 3 weeks after IBA treatment, and root occurrence was found 6 to 7 weeks after IBA treatment (Fig. 2D). In 2018 and in 2022, roots on new shoots were discovered at 42 d and 49 d, respectively, after IBA treatment. In 2022, adventitious roots (ARs) originated from the bark and grew continually (Fig. 2E). The ARs of the rooted layers had good lignification conditions when investigated during fall (Fig. 2F). The ARs on the layers were concentrated in the rooting zone treated with IBA (Fig. 3); these were similar in the different rooted layers. The height and rooting status of the layers on the same mother plant differed. The mother plant and the layers of JH are shown as an example (Fig. 3).

Comparison of the RP, RN, RL, RD, LH, and LD of different species in 2018. The RPs of layers from JR, JR LN1, JH, JM, and JN were 75.55%, 31.11%, 84.45%, 86.67%, and 8.89%, respectively (Table 2). The data from the experiment indicate that the factor between samplings was not significant ($P = 0.866$). However, the factor between species was significant ($P < 0.0001$), indicating that the differential response to the treatments depended on the species and was not influenced by the sampling. As observed in Table 2, the survey showed varying abilities of different walnut species. The RP of *J. mandshurica* Maxim and *J. hopeinesis* Hu were significantly higher than those of *J. regia* L, *J. regia* cv. Liaoning 1, and *J. nigra* L. The RP of *J. nigra* L. was the lowest. *Juglans regia* cv. Liaoning 1 had more difficulty rooting than *J. regia* L. seedlings. The RN, RL, RD, LH, and LD were not significantly different among different species (Table 2).

The root growth and development of the different species are shown in Fig. 3. Linear regression between the root diameter and root length ($P < 0.01$) was significant (y = 0.0775, x = −0.3514), and the determinant coefficient was $R^2 = 0.7417$. JM was used as an example.

Comparison of the RPs of JR LN1 under different IBA concentration treatments in 2022. The RPs of 0, 0.5, 1, 2, 4, and 8 IBA treatment were 0.0%, 20.0%, 42.2%, 64.5%, 88.9%, and 93.3%, respectively (Fig. 4 and Table 3). The RPs of seedlings under all IBA treatments were significantly different from that of the untreated control. The 4% and 8% IBA treatments had the highest RPs, but their RPs were not significantly different.

The RNs on rooted layers of JR LN1 under 0.5%, 1%, 2%, 4%, and 8% IBA treatments were 2.5, 6.9, 10.2, 40.3, and 27.7, respectively (Table 3). The RN was significantly different among JR LN1 layers under different IBA treatments, and the 4% IBA treatment had the highest root number.

The RL of rooted layers under 0.5%, 1%, 2%, 4%, and 8% IBA treatments were 6.9 cm, 6.6 cm, 5.4 cm, 8.0 cm, and 7.0 cm, respectively (Table 3). As observed in Table 3, the RLs were not significantly different between the different IBA treatments. These results indicated that different IBA concentrations had no effect on the RLs of the rooted layers.

The RDs of rooted layers under 0.5%, 1%, 2%, 4%, and 8% IBA treatments were 2.0 mm, 1.2 mm, 0.9 mm, 0.8 mm, and 0.5 mm, respectively (Table 3). As shown in Table 3, the RD of the 0.5% IBA treatment was significantly thicker than that of the other IBA treatments.

The LH in rooted layers under 0.5%, 1%, 2%, 4%, and 8% IBA treatments were 71.9 cm, 61.3 cm, 65.6 cm, 55.1 cm, and 63.1 cm, respectively (Table 3). Whereas the LH in rooted layers was not significantly different between the different IBA treatments. The LD in rooted layers under 0.5%, 1%, 2%, 4%, and 8% IBA treatments were 13.4 mm, 11.1 mm, 11.8 mm, 11.0 mm, and 11.6 mm, respectively (Table 3). No significant difference was found between the different IBA treatments.

The height and diameter of the layers of the rooted and nonrooted groups showed a significant difference when the nonparametric test was conducted ($P < 0.05$). The LHs of the rooted and nonrooted groups were 57.05

Table 2. Rooting percentage (RP), root number (RN), root length (RL), root diameter (RD), layer height (LH), and layer diameter (LD) in different species.

Species	RP	RN	RL (cm)	RD (mm)	LH (cm)	LD (cm)
JR	75.33 ± 2.22[i] b[ii]	21.8 ± 4.0 a	13.2 ± 1.5 a	1.20 ± 0.16 a	22.4 ± 4.1 a	2.22 ± 0.82 a
JR LN1	31.11 ± 2.22 c	14.7 ± 3.7 a	11.2 ± 2.3 a	1.87 ± 0.31 a	33.3 ± 7.5 a	1.07 ± 0.24 a
JH	84.45 ± 2.22 a	42.8 ± 13.6 a	22.7 ± 3.1 a	1.45 ± 0.26 a	19.4 ± 3.6 a	0.98 ± 0.15 a
JM	86.67 ± 3.85 a	38.8 ± 10.3 a	34.9 ± 3.0 a	1.76 ± 0.14 a	39.8 ± 6.1 a	1.26 ± 0.15 a
JN	8.99 ± 2.22 d	22.0 ± 3.0 a	15.7 ± 5.8 a	1.39 ± 0.38 a	28.3 ± 20.7 a	0.96 ± 0.47 a

[i] Mean ± *SE*.

[ii] Values followed by the same letter are not significantly different at $P < 0.05$ using Duncan's multiple range test.

JH = *Juglans hopeinesis* Hu; JM = *Juglans mandshurica* Maxim; JN = *Juglans nigra* L.; JR = *Juglans regia* L.; JR LN1 = *Juglans regia* cv. Liaoning 1.

Fig. 4. Comparison of the rooting status of different species under the 1% incinerator bottom ash (IBA) treatment.

cm and 71.91 cm, respectively. The LDs of the rooted and nonrooted groups were 11.20 mm and 12.73 mm, respectively. This indicated that the layers with low vigor were more likely to root.

A significantly negative correlation (r = −0.548) was observed between RN and LH. Significantly positive correlations were observed (r = 0.533 and r = 0.553) between RD and LH and RD and LD, respectively; however, RD was not significantly correlated with RN (r = −0.670) (Table 4).

The quality of the best result of JR LN1 with the layering propagule and that of JR LN1 with 'liaoning1' 1-year-old offspring were compared (Table 5, Fig. 5). The height of the layering propagule with the 4% IBA treatment was 55.1 cm, which was significantly higher than that of the 1-year-old seedling (21.5 cm). The diameter of the 1-year-old seedling was better than that of the layering propagule. The RN with the 4% IBA treatment propagule was 40.3, which was significantly higher than that of 1-year-old seedling (30.8). The RL and RD of the seedling were better than those of the layering propagule. Without exception, the layering propagule has no main root; however, the main root length of the seedling was 30.5 cm.

During the 2022 experiment, 106 mother plants produced 360 layers. On average, each mother plant produced 3.4 layers. With the 4% and 8% IBA treatments, each mother plant produced 3.3 and 2.9 rooted layers, respectively.

Discussion

The results showed that the walnut species presented here could be propagated through layering. This is very valuable for the clonal propagation of walnut rootstocks and cultivars. The consistency of the rootstock is the key to ensuring the consistency of the performance of the scion cultivars grafted onto it (Vahdati et al. 2021). The novel perspectives of this study can be applied not only for walnut but also for other tree species. Paula et al. 2020 reported that trait differentiation of avocado (*Persea americana* Mill.) could not be assessed during their study because genotyping of clonal-derived rootstocks was not performed. Emerging genomic technologies might unlock woody plant trait diversity beyond the model tree species poplar, eucalyptus, willow, oak, chestnut, and pecan (Andrés et al. 2020). Clonal propagation will be mostly used for horticultural tree improvement programs. Moreover, it was proven that a peach cultivar Granada on layering-based rootstocks resulted in similar or superior yield and the same attributes of fruit quality, which were better than those achieved using rootstocks derived from seeds (Luciano et al. 2010). For cultivars, own-rooted plants can be more consistent and stable than seed-derived rootstocks. During this study, the propagation coefficient (1:3.4) with the 4% IBA treatment was not high. Compared with cutting or tissue culture, the layering method presented here is more practical and easier to perform (Leslie and Mcgranahn 2014).

During the layering experiment involving different walnut species, JN had a very low RP. This was not consistent with our expected results. At the very least, JN should root more easily than JR. Regarding JH and JM, which can be used as hardy rootstock for JR in China, a higher rooting effect than expected was obtained.

During our experiment, we observed a direct relationship between the vigor of the layers and rooting ability. This is consistent with the observations of Vahdati et al. (2008). Root occurrence was observed 6 to 7 weeks after layering during our experiment, and a significantly negative correlation (r = −0.29) was observed between the LH and RN. This is consistent with the observations of Vahdati et al. (2008).

Table 3. Rooting percentage (RP), root number (RN), root length (RL), root diameter (RD), layer height (LH), and layer diameter (LD) of JR LN1 under different incinerator bottom ash (IBA) concentration treatments.

IBA (%)	RP	RN	RL (cm)	RD (mm)	LH (cm)	LD (cm)
0 (ck)	0.0 ± 0.0[i] e[ii]					
0.5	20.0 ± 3.9 d	2.5 ± 0.7 e	6.9 ± 0.8 ab	2.0 ± 0.4 a	71.9 ± 4.7 a	13.4 ± 0.9 a
1	42.2 ± 2.2 c	6.9 ± 0.4 d	6.6 ± 0.5 ab	1.2 ± 0.1 b	61.3 ± 1.1 ab	11.1 ± 0.8 a
2	64.5 ± 2.2 b	10.2 ± 0.7 c	5.4 ± 0.8 b	0.9 ± 0.1 bc	65.6 ± 6.8 ab	11.8 ± 0.9 a
4	88.9 ± 5.9 a	40.3 ± 0.9 a	8.0 ± 0.9 a	0.8 ± 0.0 bc	55.1 ± 6.2 b	11.0 ± 0.8 a
8	93.3 ± 6.7 a	27.7 ± 3.7 b	7.0 ± 0.6 ab	0.5 ± 0.1 c	63.1 ± 2.3 ab	11.6 ± 03 a

[i] Mean value ± *SE*.

[ii] Values followed by the same letter are not significantly different at $P < 0.05$ using Duncan's multiple range test.

Table 4. Pearson correlations between layer height (LH), layer diameter (LD), root number (RN), root length (RL), and root diameter (RD) among rooted layers evaluated in JRLN1 regardless of the incinerator bottom ash (IBA) treatment in 2022.

Parameter	LH	LD	RN	RL
LD	0.899**			
RN	−0.548*	−0.381		
RL	−0.008	0.117	0.463	
RD	0.533*	0.553*	−0.670**	−0.203

*, ** Pearson correlations are significant at the 0.05 and 0.01 levels, respectively.

Fig. 5. Comparison of *J. regia* cv. Liaoning 1 (JR LN1) layering propagule with 'liaoning1' 1-year-old seedling. (**A**) JR LN1 layering propagule. (**B**) The 'liaoning1' 1-year-old seedling.

Based on 2 years of experiments, a high IBA concentration was beneficial to rooting in layering. However, 20 d of the etiolation treatment (Pei et al. 2004) could not be ignored during the experiment. Although it was not the research objective during this experiment, etiolating is very helpful in the asexual propagation of woody plants (Lu et al. 2014; Vieitez et al. 1987). The anatomical and physiological traits and molecular mechanisms of plants changed after etiolation (Hartmann et al. 2011).

The treatment of JR LN 1 with 4% and 8% IBA resulted in RPs of 88.9% and 93.3%, respectively. These RPs were much higher than expected. Considering the RN, the 4% IBA treatment was better than the 8% IBA treatment. Additionally, it was observed that the 8% IBA treatment produced more callus at the base of the layer than the 4% IBA treatment, and that the quality of roots directly from internal tissues was better than that of those from the callus. Vahdati et al. (2008) reported that more than 80% of the layers in the high-vigor seedlings showed heavy callus formation and swelling, without any rooting or with only a few roots of poor quality.

Table 5. Quality comparison of ‘Liaoning 1’ propagule propagated through layering [4% incinerator bottom ash (IBA) treatment] and the ‘liaoning1’ 1-year-old seedling.

	H (cm)	D (mm)	RN	RL (cm)	RD (mm)	MRL (cm)
Layering	55.1 ± 6.2[i]	11.0 ± 0.8	40.3 ± 0.9	8.0 ± 0.9	0.8 ± 0.0	
Seedling	21.5 ± 2.8	13.5 ± 0.8	30.8 ± 4.6	15.6 ± 1.0	1.5 ± 0.2	30.5 ± 3.6
Significance	S[ii]	S	S	S	S	

[i] Mean value ± *SE*.

[ii] Significance at $P < 0.05$ according to the *t* test (n = 15).

D = diameter; H = height; MRL = main root length; RD = root diameter; RL = root length; RN = root number; S = significance.

During the experiment, lanolin was used as a dispersal agent for IBA because lanolin fixed IBA to the desired rooting segment of the layer so that hormones were less affected by rainfall and had the greatest effect on rooting under the field condition. We did not consider individual seedling variations of a same species. In other words, the same rooting ability of different seedlings of a species was not considered. The samplings in the experiment were very juvenile because aging shoots or cuts are difficult to root.

It is undeniable that walnut micropropagation has the advantages of a large reproduction coefficient and short reproduction cycle. However, walnut layering has the advantages of low cost, practicability, and easy mastery by nursery owners. The methodology of layering presented in our research can be an alternative for walnut clonal propagation. The scientific research gap of this study was that we could not obtain a satisfactory RP among the sampling species.

Conclusion

In conclusion, *Juglans* has difficulty rooting when propagated by the clonal method. We explored the method of propagating the walnut rootstocks, especially the walnut cultivars, by performing layering. The height and RN of the obtained walnut propagule through layering somehow exceeded the seed-derived propagule. To acquire an own-rooted walnut propagule of a targeted genotype, it is necessary to first prepare its grafted plant. Subsequently, the grafted plant is used as the mother plant to implement layering propagation. This methodology could provide us with subsequent clonal walnut rootstock propagation, clonal cultivation of the whole walnut tree, and research of the own-rooted propagule establishment of walnut cultivars. We will perform more studies of improving quality and acclimatizing the propagule through layering to reveal the mechanism and controlling genes involved in walnut layering propagation.

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