Plant regeneration in *Vernicia fordii* from apical bud

Huijuan Zhang a, b, Shifei Qin a, b, Hui Cao a, b, Qingwei Zhang a, b, *, Keming Luo a, b, **

* Key Laboratory of Plant Resource Conservation and Germplasm Innovation, School of Life Sciences, Southwest University, Chongqing, 400715, China
** Key Laboratory of Eco-environments of Three Gorges Reservoir Region, Ministry of Education, Southwest University, Chongqing, 400715, China

**Corresponding author.

E-mail addresses: qwzhang18@swu.edu.cn (Q. Zhang), kemingl@swu.edu.cn (K. Luo).

Research article

Plant regeneration in *Vernicia fordii* from apical bud

1. Introduction

*Vernicia fordii* Hemsley is a deciduous woody plant in the Euphorbiaceae family, which is an important economic tree species in China and some countries of east and southeast Asia. The seeds of *V. fordii* are rich in oil (up to 50%–70%), thus becoming a commercial source of raw material to produce biodiesel (Chen et al., 2010). Besides, the oil extracted from *V. fordii* has excellent characteristics of dried oil including insulation, acid and alkali resistance and anti-corrosion, it is widely utilized in many industrial applications (Li et al., 2018). Although the cultivation of *V. fordii* is promising because of a large market demand, lack of an efficient breeding technique limits the rapid expansion of *V. fordii* cultivation.

Being a typical cross-pollinated plant, *V. fordii* genotype is highly heterozygous, leading to great individual variation of oil yield and quality (Zhang et al., 2015). However, *V. fordii* cultivation is dependent mostly on seeds and grafts so far, which cannot reach a satisfactory propagation efficiency or guarantee uniform germplasm quality, because 1) the offspring of natural hybridization could not keep excellent traits of parents; 2) vegetative propagation via grafting is laborious and costly. In contrast, plant tissue culture is an efficient approach to keep advantageous trait of specific genotype (Lin et al., 2016). Through the technology of tissue culture, a large number of clonal propagules with the same genetic background can be obtained in a short time. Besides, an efficient regeneration system via tissue culture should be an important part of the genetic transformation of *V. fordii* with the completion of *V. fordii* genome sequencing (Zhang et al., 2020).

To date, the methods of tissue culture of *V. fordii* have been established with leaf and hypocotyl as explants to induce callus formation and subsequent organogenesis (Tan et al., 2013; Lin et al., 2016), which are conducive to genetic transformation in tung tree. However, induction of callus elongates the culture period and might increase the risk of infection. By contrast, direct organogenesis from explants would be more efficient in rapid propagation of *V. fordii*. In this study, apical buds were used as the explants, and a stable regeneration system in *vitro* was established. The regeneration system includes induction, proliferation, rooting, domestication and transplanting process, providing an important approach for rapid propagation of *V. fordii*.

2. Materials and methods

*V. fordii* seeds and shoots were collected from Kaizhou District, Chongqing, China (N30°49', E107°55') which is one of the natural distribution areas of *V. fordii*. The seeds were germinated in a greenhouse. The apical buds in the one-month-old seedlings and in the shoots collected from adult trees in the wild were used as explants. According to...
the studies on *Jatropha curcas* and cassava that also belong to the Euphorbiaceae family, we designed different sterilization treatments. Specifically, apical buds were rinsed with flowing water for 30 min, put in 75% ethanol for 30 s and washed by sterilized water for three times, followed by 10% NaClO or 0.1% HgCl₂ soaking and sterilized water washing (five times) (Hankoua et al., 2005; Misra et al., 2010). Each treatment had 27 explants and was repeated three times. The contamination rate and survival rate were counted 2 weeks after inoculation. The contamination rate was calculated as No. of explants contaminated/total No. of explants, and the survival rate as No. explants survived divided by total No. of explants.

The basic medium used in this study was MS medium (Murashige and Skoog, 1962) with 30 g/L sucrose and 6.5 g/L agar (pH 5.8). Previously it was reported that direct shoot organogenesis occurred when the medium containing 0.5 mg/L 6-BA and 0.1 mg/L IBA was used (Gonzalez-Arnao et al., 2008), so we chose 6-BA and IBA to induce adventitious shoots. The concentrations of 6-BA were set as 1.0, 1.5 and 2.0 mg/L; the concentrations of IBA were set as 0.01, 0.02, 0.05, 0.10 and 0.20 mg/L. The induction rate and bud growth status were recorded 30 days after the inoculation of apical buds. The induction rate was calculated as No. of explants producing adventitious buds or shoots divided by total No. of explants in each treatment.

In the process of subculture, 6-BA and IBA were used according to the report on *J. curcas* (Kaewpoo & Te-chatto 2009; Chauhan and Taylor, 2018). The adventitious shoots produced from apical buds were inoculated on MS medium containing 6-BA (0.5, 1.0, 1.5 mg/L) and IBA (0.01, 0.1, 0.2 mg/L) to proliferate. Each generation of proliferation took 30 days. Sixty days after the first inoculation to proliferate, proliferation rate for each treatment was counted as: total No. of buds produced divided by No. of buds inoculated. Each treatment had 15 buds and had three biological replicates.

To enhance root formation of shoots from subculture, 1/2MS medium and IBA (0.0,0.5,1.0,2.0 mg/L in concentration) were considered according to the report on *J. curcas* (Shui et al., 2010). The shoots longer than one cm were inoculated on medium to root for 15 days, then rooting rate, root number and root length were counted. The rooting rate was calculated as No. of shoots producing roots divided by total No. of shoots inoculated. Each treatment had 15 shoots and were repeated three times.

The condition for adventitious shoot induction, subculture, rooting was: air temperature of 25 ± 2 °C, relative air humidity of 80%, and illumination of 3000 lx (16 h per day). Acclimatization of plantlets with 3–5 cm long roots was conducted: 1) the lids of culture flask were unscrewed and then removed with plantlets intact; 2) the media were washed out and the seedlings were transplanted into sterilized substrate (nutrient soil, vermiculite and perlite, 2:1:1) and covered by plastic membrane. After one week, the plastic membrane was removed.

3. Results

3.1. Explant sterilization

From the results of contamination control and bud survival, it was found that for apical buds from seedlings 75% ethanol treatment for 30 s followed by 0.1% HgCl₂ treatment for 10 min was the optimal among various disinfection treatments (Figure 1a), which produced the highest bud survival rate and simultaneously avoided the occurrence of contamination.

**Figure 1.** Effects of different sterilization treatments on survival rates and contamination rates of apical buds from seedlings (a) and adult trees in the wild (b). Note: after each kind of treatment (75% ethanol, 0.1% HgCl₂ and 10% NaClO), explants were rinsed with sterile water for 5 times (30 s each time); Data presented in the figure are the mean ± standard error (SE) of three replicates; all data were collected two weeks after inoculation; different letters in the figure indicate there is a significant difference tested by the analysis of variance (ANOVA) with p < 0.05.
contamination very well. Considering it is more difficult to do disinfection for apical buds from adult tree outdoor, the treating time of HgCl₂ or NaClO was elongated. Results showed that the contamination rates of apical buds from adult tree were much higher those from seedlings (Figure 1b). Although 0.1% HgCl₂ treatment for 14 min after 75% ethanol treatment for 30 S showed the best effect in contamination control, the survival rate was only 30.9%.

3.2. Primary culture: adventitious bud induction

Firstly, the apical buds from seedlings were used as explants. It was found that the media containing 1.5 mg/L or 2.0 mg/L 6-BA led to higher induction rates than those on media containing 1.0 mg/L 6-BA (Figure 2a), indicating higher 6-BA concentration was superior for inducing adventitious buds or shoots. However, in the experimental process, we observed that glassiness/vitrification occurred when buds grew on media containing 2.0 mg/L 6-BA, which would impede subsequent subculture. Among all media containing 1.5 mg/L 6-BA, the media containing 0.05, 0.10 and 0.20 mg/L IBA had higher induction rates, but the No. of buds produced on these media were lower than that containing 0.01 mg/L IBA (average 91.3 buds each treatment). Meanwhile, explants on the medium containing 1.5 mg/L 6-BA and 0.01 mg/L IBA could form new buds rapidly without callus and the buds or shoots developed normally in terms of morphology. Therefore, in consideration of bud growth status, induction rate and No. of buds produced, the medium containing 1.5 mg/L 6-BA and 0.01 mg/L IBA was optimal for inducing adventitious buds from apical buds. When the apical buds from adult trees in the wild were used as explants, the results were similar (Figure 2b), the medium containing 1.5 mg/L 6-BA and 0.01 mg/L IBA had higher No. of buds and acceptable induction rates, as well as satisfactory growth status. This...
indicates that apical buds from seedlings and adult trees have consistent response to the condition of tissue culture.

3.3. Proliferation by subculture

As there was no difference of adventitious bud induction between the buds of seedlings and those of adult trees, we only used the adventitious buds produced by the former to proliferate by subculture (Figure 3). It was found that the buds or shoots inoculated on the medium containing 1.5 mg/L 6-BA and 0.01 mg/L IBA had the highest proliferation rate (3.85), but glassiness/vitrification occurred in some leaves. Among the other media, the one containing 1.0 mg/L 6-BA and 0.01 mg/L IBA produced a relatively high proliferation rate (3.18) and the explants on it grew well. Based on both proliferation rate and explant growth status, the medium containing 1.0 mg/L 6-BA and 0.01 mg/L IBA was selected.

3.4. Rooting of adventitious buds/shoots

Rooting is important for plant regeneration, which also needs an optimal medium. The results demonstrated that only buds/shoots on the MS medium containing 0.5 mg/L IBA produced roots (Figure 4a), by contrast, 1/2 MS media containing 0.5 mg/L IBA performed better in terms of rooting rate and root number, indicating that 1/2 MS medium was more effective than MS medium for rooting (Figure 4a, b, c). Therefore, 1/2 MS medium containing 0.5 mg/L IBA was selected for rooting. In fact, it led to strong roots, and the survival rate of plantlets after transplanting was 96.4%.

4. Discussion and conclusion

A suitable surface sterilization method is the basis of plant tissue culture. It has been proven that the effect of sterilization depends on plant species, organ type and sterilization approach (George et al., 2008). In this study, 0.1% HgCl₂ showed better disinfection effect than 10% NaClO. Certainly, HgCl₂ treatment over time led to the decrease of survival rate, as HgCl₂ is toxic for plants. Compared with buds from seedlings, the buds from adult trees had significantly higher contamination rates, though the treating time of HgCl₂ or NaClO was elongated. Therefore, seeking for more effective sterilization methods is important when adopting apical buds of adult trees in the wild as explants.

In the process of adventitious bud induction, we observed that the media containing 2.0 mg/L 6-BA resulted in a higher induction rate, but glassiness/vitrification simultaneously occurred. Early in 1988, researchers already found that cytokinin was an inducer of vitrification in plant tissue culture (Leshem et al., 1988), so in this study 1.5 mg/L was seen as the optimal 6-BA concentration. Similarly, the highest 6-BA concentration led to the highest proliferation rate, but they also brought about the problem of vitrification. All these confirm that during plant tissue culture, the positive processes should be enhanced while the negative process avoided or inhibited. Although it has been mentioned that the contamination rate for buds collected from adult trees in the wild was much higher, the results demonstrated that either buds from adult tree or from seedling had similar ability to produce adventitious buds/shoots. This indicates that the system we established is suitable for various apical buds. Besides, in this study we set the concentrations of plant growth regulators in media based on the reports of J. curcas. The results demonstrated that they were effective in some degree, indicating that plants in the same family or genus probably have similar response to the environment of tissue culture.

In summary, the effective plant regeneration of V. fordii from apical buds could be achieved by three steps: 1) adventitious shoot induction with the MS medium containing 1.5 mg/L 6-BA and 0.01 mg/L IBA (30 d, induction rate of 65.1%); 2) subculture to proliferate with the MS medium containing 1.0 mg/L 6-BA and 0.01 mg/L IBA (60 d, proliferation rate of 3.14); 3) rooting process with 1/2 MS medium containing 0.5 mg/L IBA (15d). The growth status of V. fordii material in the whole process of regeneration is depicted in Figure 5.
Declaration

Author contribution statement

Huijuan Zhang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.
Shifei Qin, Hui Cao: Performed the experiments.
Qingwei Zhang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Keming Luo: Conceived and designed the experiments; Wrote the paper.

Funding statement

This work was supported by the National Key R&D Program of China (2017YFD0600703).

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

Chauhan, R.D., Taylor, N.J., 2018. Meta-topolin stimulates de novo shoot organogenesis and plant regeneration in cassava. Plant Cell Tissue Organ Cult. 132, 219–224.

Chen, Y., Chen, Y.-H., Chang, C.-Y., Chang, C.-C., 2010. Biodiesel production from tung (Vernicia montana) oil and its blending properties in different fatty acid compositions. Bioreour. Technol. 101, 9521–9526.

George, E.F., Hall, M.A., Klerk, G.J.D., 2008. Plant Propagation by Tissue Culture. Springer, Netherlands.

Gonzalez-Arnao, M.T., Panta, A., Roca, W.M., Escobar, R.H., Engelmann, F., 2008. Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. Plant Cell Tissue Organ Cult. 92, 1–13.

Hankous, B.B., Ng, S.Y.C., Fawole, I., Puonti-Kaerlas, J., Pillay, M., Dixon, A.G.O., 2005. Regeneration of a wide range of African cassava genotypes via shoot organogenesis from cotyledons of maturing somatic embryos and conformity of the field-established regenerants. Plant Cell Tissue Organ Cult. 82, 221–231.

Kasempoo, Te-chato, 2009. Influence of explant types and plant growth regulators on multiple shoot formation from Jatropha curcas. Sci. Asia 35, 353–357.

Leshem, B., Shaley, D.P., Izhar, S., 1988. Cytokinin as an inducer of vitrification in melon. Ann. Bot. 61, 255–266.

Li, W., Liu, M., Zhang, L., Tan, X., Zhang, F., Wang, Z., 2018. Study of major economic traits in 4 superior families of tung tree. Nonwood For. Res. 26, 29–34 (in Chinese with an abstract in English).

Lin, Q., Li, Z., Zhang, L., Tan, X.-F., Long, H.-X., Wu, L.-L., 2016. High-efficiency regeneration of seedlings from hypocotyl explants of tung tree (Vernicia fordii). Int. J. Agric. Biol. 18, 370–376.

Misra, P., Gupta, N., Toppo, D.D., Pandey, V., Mishra, M.K., Taili, R., 2010. Establishment of long-term proliferating shoot cultures of elite Jatropha curcas L. by controlling endophytic bacterial contamination. Plant Cell Tissue Organ Cult. 100, 189–197.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum 15 (3), 473–497.

Shui, Q.W.J., Song, X.Q., Liu, S.J., 2010. Advance in Jatropha curcas tissue culture. J. Trop. Crops 30, 1227–1231.

Tan, X.F.L.Z., Zhang, L., Long, H.X., Yuan, J., Zeng, Y.L., Lin, Q., 2013. Callus induction from leaves and plant regeneration of tung tree (Vernicia fordii Hemslay). Plant Physiol. J. 49, 1245–1249 (in Chinese with an abstract in English).

Zhang, L., Liu, M., Long, H., Dong, W., Pusha, A., Esteban, E., Li, W., Yang, X., Li, Z., Song, A., Ran, D., Zhao, G., Zeng, Y., Chen, H., Zou, M., Li, J., Liang, F., Xie, M., Hu, J., Wang, D., Cao, H.-P., Provart, N.J., Zhang, L.-S., Tan, X.F., 2020. Tung Tree (Vernicia fordii) Genome provides a resource for understanding genome evolution and improved oil production. Dev. Reprod. Biol. S1672–0229 (18), 30216-X.

Zhang, L., Lu, S., Sun, D., Peng, J., 2015. Genetic variation and geographical differentiation revealed using ISSR markers in tung tree, Vernicia fordii. J. Genet. 94 (1), e5–e9, 20.

Figure 5. The growth status of \textit{V. fordii} material in the whole process of regeneration. a, the primary culture of apical buds; b-c, the subculture of adventitious buds; d-e, the rooting of adventitious buds or shoots; f, the seedlings two months after transplanting.