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Shoot organogenesis and plant regeneration from stem explants in *Acer truncatum*

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Key words *Acer truncatum*; Shoot organogenesis; *In vitro* regeneration; Histology

Key massage A protocol for *in vitro* regeneration of *Acer truncatum* via de novo shoot organogenesis was firstly presented, and histological analysis was performed to demonstrate the development process.

Abstract

Acer truncatum is an economically important multipurpose tree in China due to its high values in ornamental, ecology, oil-production and medicine. The lack of reliable and stable *in vitro* regeneration system seriously restricts its breeding and industrial development. This study first presented a protocol for *in vitro* regeneration of *A. truncatum* via de novo shoot organogenesis from stem explants. The results showed that sterilization, basal medium, plant growth regulators and light condition significantly influenced shoot regeneration of *A. truncatum*. The best sterilization protocol for stem explants was 75% ethanol (30 s) + 0.1% $HgCl_2$ (10 min), with a lowest contamination rate (4.74%). The highest callus induction rate (97.17%) was achieved when stem segments were cultured on WPM medium with 0.2 mg/L 2,4-D and 0.5 mg/L 6-BA in the dark for 15 days. The optimal shoot differentiation rate (10.51%) was obtained when callus was cultured on NN69 medium containing 0.5 mg/L CPPU. The rooting rate of adventitious shoots was achieved 85% by using NN69 medium with 0.25 mg/L IBA, and the rooted seedlings were transplanted into pots containing a mixture of peat, perlite and vermiculite (2:1:1, v:v:v), the survival rate was more than 80%. Histological analysis demonstrated the origin and development process of indirect shoot organogenesis, including callus formation, meristematic nodule formation and shoot differentiation. This study will be beneficial to the germplasm conservation, mass propagation and genetic improvement of *A. truncatum*.

Abbreviations

AX Auxin

CIM Callus induction medium

CK Cytokinin

CPPU N-(2-chloro-4-pyridyl)-N-phenylurea

DNSO De novo shoot organogenesis

EC Embryogenic callus

IBA Indole-3-butyric acid

MN Meristematic nodule

MS Murashige and Skoog

NAA α-Naphthylacetic acid

NN69 Nitsch & Nitsch Medium

PGRs Plant growth regulators

SIM Shoot induction medium

TDZ Thidiazuron

WPM Woody plant medium

2,4-D 2,4-Dichlorophenxyacetic acid

6-BA 6-Benzyladenine

1 Introduction

Acer truncatum Bunge is an economically important multifunctional tree unique to China in terms of its ornamental, ecological, oil-producing and medicinal values. It is a famous autumn foliage tree and widely used in landscaping and ecological greening due to its remarkable ornamental value, strong adaptability and resistance. The seed oil of *A. truncatum* is rich in unsaturated fatty acids (over 90%), especially the "brain gold"- nervonic acid (5%-6%) , which is used as a novel food resource and has great potential in addressing global brain health issues, such as the rising prevalence of Alzheimer's disease (Li et al. 2019; Qiao et al. 2019; He et al. 2021). In addition, there is a high level of protein (27%) and tannin (60%) in the seed kernel and testa of *A. truncatum* (Wang et al. 2007; Fan et al. 2022), while high concentrations of flavonoids (5-6%) and chlorogenic acid (4%) in the leaves (Su et al. 2004; Yang er al. 2018), which can be utilized as edible, medicinal and animal feed resources. Overall, *A. truncatum* has a broad prospect for comprehensive development.

The bottleneck problems restricting the industrialization of *A. truncatum* mainly include the lack of elite cultivars and the nonuniformity of seedlings. However, the conventional propagation methods such as sowing and grafting are inefficient and time-consuming, which severely constrain its breeding and rapid propagation, thus an reliable and stable *in vitro* regeneration protocol is urgently needed.

Tissue culture serves as a crucial technology for plant rapid propagation and biotechnology breeding. Since the first report on tissue culture in *A. truncatum* was published by Li et al. (2005), studies over the past two decades have mainly focused on micropropagation using axillary bud (Ma et al. 2020; Quan et al. 2024). Howerver, none of these regeneration system can meet the needs for the propagation and genetic manipulation of *A. truncatum* due to various obstacles, including high contamination rate, low proliferation coefficient, and the unsuitability for genetic manipulation.

De novo shoot organogenesis (DNSO) is considered as a vital biotechnological tool to address the above problems due to its high potential in propagation, genetic transformation and as a bioreactor, but no successful DNSO has been achived in *A. truncatum* up to now. Yu et al. (2004) and Yang et al. (2024) investigated the induction of callus from stem, leaf and cotyledon explants of *A. truncatum*, respectively, but both failed to achieve the differentiation of adventitious shoot. Even in *Acer* genus, documented studies of shoot organogenesis are limited to only a few species, such as *Acer ginnala* (Li et al. 2008), *Acer davidii* (Hao 2010), and *Acer negundo* (Zhang et al. 2012), but the differentiation rate and regeneration efficiency in these studies is generally low. In many recalcitrant species include *A. truncatum*, *in vitro* regeneration, especially the differentiation of callus, remains highly challenging. Hence, innovative breakthroughs concerning shoot organogenesis in *A. truncatum* are well-needed, meanwhile, the origin and development process of *in vitro* morphogenesis also needs to be clarified.

The present study aimed to develop a protocol for *in vitro* regeneration of *A. truncatum* via shoot organogenesis. Histological analysis was performed to reveal the origin and development process of *in vitro* morphogenesis. This study will be beneficial to the large-scale propagation, genetic improvement and gene function identification of *A. truncatum*.

2 Materials and methods

2.1 Plant materials and sterilization

Tender stems from annual branches were collected in April from living adult plants of *A. truncatum* grown in Maple Germplasm Resource Nursery of Jiangsu Academy of Agriculture Sciences in Nanjing, China (32°03′N, 118°87′E) and washed with detergent solution for 5 min, followed by rinsing with tap water for 10 min. Then, the stem segments were subjected to surface sterilization under aseptic condition. To screen the best disinfection protocol, the combinations of 75% (v:v) ethanol (20 s, 30 s) and 0.1% $HgCl_2$ (7 min, 10 min) or 3% NaClO (v:v) (7 min, 10 min) were tested (Table 1), then the stem segments were washed thoroughly in sterile distilled water and cut into 7-8 mm segments (nodes and axillary buds removed) as explants (Fig. 3A). Each treatment contained 30 explants and conducted in three replications, the contamination rate and callus induction rate were recorded after 15d of culture.

2.2 Medium and culture conditions

The basal media were Murashige and Skoog medium (MS) (Murashige and Skoog 1962), Nitsch and Nitsch medium (NN69) (Nitsch and Nitsch 1969) and woody plant medium (WPM) (Lloyd and McCown 1980). All media were supplemented with 3% sucrose and 6.5 g/L agar, and the pH was adjusted to 5.80 with 1 M NaOH and 1 M HCl before autoclaving (121 ℃, 118 kPa, 20 min). If not otherwise stated, the cultures were maintained at 25±1 °C, and with a 12 h photoperiod of 116 μmol $m^{-2}s^{-1}$ illumination intensity provided by LED light except callus induction culture, which was conducted under dark. The subculture was conducted every 15 d.

2.3 Callus induction

To screen optimal basal medium for callus induction, three basal media (WPM, MS, and NN69) were examined, and 0.2 mg/L 2,4-Dichlorophenxyacetic acid (2,4-D) and 0.5 mg/L 6-Benzyladenine (6-BA) was added to each treatment.

To screen optimal Plant growth regulators (PGRs) for callus induction, two experiments were carried out in chronological order. Exp 1: Auxin (AX) α-Naphthylacetic acid (NAA) (0.5 mg/L) and 2,4-D (0.2, 0.5, 1.0 mg/L) were used in combination with Cytokinin (CK) 6-BA (0.2, 0.5 mg/L) and N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) (0.5 mg/L) to screen suitable type and concentration of PGRs (Table 3). Exp 2: based on the optimal concentration obtained in Exp 1, a variety of combinations of AXs (0.2 mg/L NAA, 2,4-D, and Indole-3-butyric acid (IBA)) and CKs (0.5 mg/L 6-BA, CPPU, and Thidiazuron (TDZ)) were used to further select the best PGRs combination (Table 4).

To examine the effect of light on callus induction, explants were respectively cultured under two different light regimes (a 12 h photoperiod of 116 μmol $m^{-2}s^{-1}$ illumination intensity provided by LED light or complete darkness) for callus induction (Table 5), the best callus induction medium (CIM) selected through the above process was used.

Each treatment contained 30 explants and three replications, callus induction rate was recorded after 15 d of culture, the growth status of explants and callus during the culture process were also observed.

2.4 Shoot differentiation

The callus induced on optimal CIM for 15 d were transferred to shoot induction medium (SIM) supplemented with 0.5 mg/L CPPU. To screen optimal basal medium for callus differentiation, three basal media (WPM, MS, and NN69) were examined.

To investigate the effect of PGRs on shoot differentiation, two CKs alone (0.25, 0.50 mg/L CPPU,

0.25, 0.50 mg/L TDZ), the combinations of CKs (0.25 mg/L 6-BA+0.25 mg/L CPPU, 0.25 mg/L CPPU+0.25 mg/L TDZ), and the combinations of AXs (0.10 mg/L NAA, 0.1mg/L IBA) and 0.50 mg/L CPPU were used for selecting the optimal PGRs (Table 6).

Each treatment contained 15 explants and three replications. After 120 days of culture, the adventitious shoot differentiation rate (%) was calculated.

2.5 Rooting and acclimatization

Elongated shoots (1-2 cm) were excised from the explants and cultured on rooting medium (NN69 supplemented with 0.25 mg/L IBA) for root induction. The rooting rate (%) was calculated after 30 days of root induction, well-rooted plantlets with 2-3 robust roots (over 2 cm length) were transferred to pots containing a mixture of autoclaved peat, perlite and vermiculite (2:1:1, v/v/v) and acclimatized under controlled conditions in greenhouse (24±1℃, 65% relative humidity). The survival rate (%) was recorded after one month of acclimatization.

2.6 Histological analysis

For histological analysis, explants were sampled at different stages of culture (at 0, 3, 6, 9, 15 d of culture for callus induction, at 30, 60, 90, 120, and 150 d of culture for shoot differentiation). All samples were fixed in FAA fixative (a mixture of formaldehyde, glacial acetic acid and 70% ethanol, 1:1:18 (v/v/v)) for one week, then dehydrated with a graded ethanol series, cleared with xylene, embedded in paraffin wax and cut into 8-10 μm sections using a rotary microtome (LEICA RM2245). The sections were stained with hematoxylin for cell nucleus and eosin for the cytoplasm. Image capture was performed using a light microscope (LEICA DM500).

2.7 Statistical analysis

Statistical analyses was performed with SPSS 27.0 and significant differences among treatments were determined by analysis of variance (ANOVA) followed by Duncan's multiple range test or Student's t-test at $p<0.05$ significance level. Data visualization and graphing were carried out using Microsoft Excel 2013 and GraphPad Prism.

3 Results

3.1 Sterilization and initiation

The contamination rate of stem explants significantly decreased with prolonged sterilization time ($p<0.05$). 0.1% $HgCl_2$ showed better sterilization performance than 3% NaClO. The lowest contamination rate (4.74%) was achieved when 75% ethanol (30 s) + 0.1% $HgCl_2$ (10 min) were used, with 97.17% callus induction rate, but there was no significant difference in callus induction rate

among different treatments (p>0.05) (Table 1).

3.2 Effect of basal medium, PGRs and light condition on callus induction

The callus induction effect of WPM was significantly better than that of NN69 and MS, with the highest callus induction rate of 97.14%, while it was 69.6% and 65.7% on NN69 and MS media respectively (p<0.05) (Table 2). The morphological characteristics of callus varied conspicuously in different media. The callus cultured on WPM and NN69 media have similar morphology, presenting a pale-yellow color and compact texture, with small white fragments on the surface of some explants (Fig. 1A, B), whereas callus cultured on MS medium showed a watery, loose and friable structure (Fig. 1C). Comprehensively considering the callus induction rate and differentiation potential (following in this paper), WPM medium was the optimal basal medium for callus induction of *A. truncatum* stem segments.

In experiment 1 (Table 3), the callus induction rate of 2,4-D treatments were significantly higher than that of NAA when they were used in combination with 6-BA or CPPU, indicating that the effect of 2,4-D on callus induction was superior to that of NAA. Similarly, 6-BA exhibited significantly higher callus induction efficiency than CPPU. The highest callus induction rate (98.00%) was obtained when 1.0 mg/L 2,4-D + 0.5 mg/L 6-BA were used, and the induction rate decreased with the reduction of auxin levels, although no significant difference was found among different AX/CK ratios.

Three distinct types of callus were characterized: type I callus presented a pale yellow color and compact texture, which formed into nodule-like structures during subsequent culture; type II callus showed a more watery and loose structure, and was prone to turn browning during subculture; type III callus were small white fragments formed on the surface of explants, but no morphological changes occurred during subsequent culture. Except for 0.5 mg/L NAA + 0.2 mg/L CPPU treatment which produced type II callus, all the other treatments resulted in type I and III callus, 0.2 mg/L 2,4-D + 0.5 mg/L 6-BA and 0.5 mg/L 2,4-D + 0.2 mg/L CPPU treatments only yielded type I callus. Comprehensively considering the callus induction rate and differentiation potential (following in this paper), 0.2 mg/L 2,4-D + 0.5 mg/L 6-BA was the optimal PGRs combination with 90.28% callus induction rate.

In order to further screen the optimal combination of PGRs for callus induction, experiment 2 was conducted based on the suitable concentrations obtained in experiment 1 (Table 4). The positive effect of 2,4-D on callus induction was significantly higher than that of NAA or IBA (p<0.05), the combination of 2,4-D and 6-BA had the best performance on callus induction with the highest callus induction rate (97.14%), while only 32.41% ~ 51.85% callus induction rate was achieved when NAA or IBA was used. Moreover, callus produced under 2,4-D + 6-BA treatments belong to type I with a large biomass, while the callus under NAA or IBA treatments were type I, III or only type III. 6-BA

was more efficient than CPPU and TDZ in increasing the biomass of callus, and was apt to induce the formation of type I callus. In conclusion, 0.2mg/L 2,4-D + 0.5mg/L 6-BA was the best combination for callus induction.

The callus induction rate under dark treatment was significantly higher than that of 12h photoperiod treatment, with 97.17% and 85.19% callus induction rate, respectively (Table 5). In addition, the callus cultured in the dark was pale yellow with a greater biomass (Fig. 1A), while the callus cultured under light condition was smaller with pale green color due to the light exposure (Fig. 1D). Overall, complete dark culture was more conducive to callus induction.

3.3 Effect of basal medium and PGRs on adventitious shoot differentiation

The highest adventitious shoot differentiation rate (10.51%) was achieved on NN69 medium, followed by WPM medium with 3.93% differentiation rate, however, no differentiation was observed on MS medium (Fig. 2). Therefore, NN69 was considered as the optimal basal medium for shoot differentiation of *A. truncatum*.

The differentiation of adventitious shoots were observed on the media supplemented with 0.25 mg/L CPPU, 0.5 mg/L CPPU and 0.25 mg/L 6-BA + 0.25 mg/L CPPU, respectively (Table 6). The highest differentiation rate (10.51%) was achieved when 0.5 mg/L CPPU was used, indicating that CPPU plays an important role in shoot differentiation of *A. truncatum*. However, no differentiation was observed when CPPU was used in combination with 0.1mg/L NAA or IBA, suggesting that AXs may have an inhibitory effect on callus differentiation. Meanwhile, TDZ was ineffective in shoot differentiation, given that either TDZ alone or in combination with CPPU failed to induce shoot differentiation. Based on above results, NN69 + 0.5 mg/L CPPU was the optimal medium for shoot differentiation.

3.4 Rooting and acclimatization

The single shoots that elongated to 1-2 cm were excised from callus and transferred to rooting medium for root induction. The rooting rate was 85% after 30 d of culture. The vigorous seedlings with well-developed roots were transplanted into pots and acclimatized in greenhouse. Over 80% survival rate was achieved after 30 d of acclimatization.

3.5 Morphological and histological observation of shoot organogenesis

After the stem explants (Fig. 3A) were cultured in CIM under dark for 3 d, the division of parenchyma cells in subepidermal and cortical area were initiated, small protuberances were formed in local epidermis regions (Fig. 4A). After 6 days of culture, swelling was observed at both cut ends of stem segments due to vigorous division of cortical parenchyma cells and meristematic cells around vascular

bundles, indicating the initiation of callus formation (Fig. 4B). In the cortical area close to epidermis, several layers of meristematic cells arranged parallel to epidermis, as well as dome-shaped meristematic cell clusters were observed, showing an outward development trend (Fig. 4B, C). After 15 days of culture, unorganized callus formed at one or both ends of the stem segments, with pale yellow color and compact texture (Fig. 3B, 4D). Callus grew rapidly and turned green gradually after being transferred to SIM and cultured under light for one subculture, with irregular protuberances formed on the surface (Fig. 3C). These protuberances were composed of a central area of parenchymatous cells surrounded by 2-3 layers meristematic cells, which were termed as pre-nodular structures (Fig. 4E). These pre-nodular structures significantly increased in number and diameter, further developed into meristematic nodules (MNs) with different size after 60 days of culture (Fig. 3D). MNs consisted of external layers of small, isodiametric, densely stained meristematic cells and a central area of vascularization, while several rows of rectangular cells longitudinally arranged beneath the MNs and grew outward, resulting in the development of MNs toward the surface (Fig. 4F, G). After 120 d of culture, leaf clusters differentiated on the surface of MNs (Fig. 3E, 4H), some of them progressively developed into shoot clusters with obvious apical meristem, bud primordia, leaf primordia, young leaves and bud axis (Fig. 3F, 4I), then shoots elongated to form new stems with expanded leaves (Fig. 3G). Excised single shoot rooted after rooting induction (Fig. 3H) and acclimatized in greenhouse (Fig. 3I).

4 Discussion

To our knowledge, this is the first report of *in vitro* regeneration in *A. truncatum* via DNSO, shoot differentiation and plant regeneration were successfully achieved from stem explants, opening up new avenues for efficient propagation and genetic transformation of *A. truncatum*.

Components of basal medium have important influence on *in vitro* morphogenesis. In this study, WPM showed the best performance on callus induction, while NN69 was more suitable for shoot differentiation, suggesting the distinct nutrient requirements at different development stages (Phillips et al. 2019). As for callus induction, there were significant differences in callus state on the three tested media. Callus produced on WPM and NN69 media exhibited similar morphological characteristics, with compact texture and smooth appearance, which have been well documented in many woody plants to have great potential for shoot differentiation (Yu et al. 2020; Yang et al. 2023; Wu et al. 2024). However, the callus on MS is more watery and looser, resembling the embryogenic callus described in many studies, which is considered to be prone to form somatic embryo, but further verification is still needed (Du et al. 2020; He et al. 2024). The possible reason is the higher nitrogen and abundant inorganic salts in MS medium, which may contribute to the formation of embryogenic callus (Phillips et al. 2019).

At shoot differentiation stage, NN69 had the best performance, possibly due to its moderate ions content and higher vitamin level. NN69 is commonly used in *in vitro* regeneration of pear species (Wang et al. 2016; Liu et al. 2023), its remarkable effects on initiation and proliferation of axillary bud in micropropagation have also been reported in *A. truncatum* (Ma et al. 2020) and *A. palmatum* (Zhu et al. 2021). However, different results were observed in previous studies of *A. davidii* (Hao 2010), *A. negundo* (Zhang et al. 2012) and *A. ginnala* (Ma 2019), in which shoot differentiation was obtained on MS or WPM. This might be related to genotype differences, physiological state of explants and culture conditions. In addition, only a single type of medium was employed in these studies without comparative analysis.

PGRs play a significant role in *in vitro* plant regeneration. The balance and crosstalk between AXs and CKs are crucial for cell fate determination and cell patterning during DNSO (Cheng et al. 2013). It is well recognized that a high AX/CK ratio stimulates the formation of callus, whereas a high CK/AX ratio is favorable to shoot differentiation. Similar phenomenon were observed in our study, the combination of AX and CK have a excellent performance in callus induction, while shoot differentiation is dominated by CK. This classical two-step shoot regeneration model has been widely recognized, in which the acquisition of organogenic competence starts on AX-rich CIM and the determination of shoot identity occurs after the explants are transferred to CK-rich SIM (Cui et al. 2019; Hao et al. 2020; Elmoreigi et al. 2025). Conformably, studies of transcriptome and gene function indicating that the major regulatory genes in the early stages of DNSO are mainly AX-responsive gene, and the later stages are dominated by CK signaling (Motte et al. 2014; Ikeuchi et al. 2019; Zhou et al. 2025).

Appropriate type and concentration of PGRs is of great important to callus induction. In the present study, 2,4-D displayed the best effect on callus induction than NAA and IBA, possibly due to its higher AX activity and rapid uptake from media (Eyer et al. 2016). Meanwhile, the effect of 6-BA on callus induction was significantly superior to that of CPPU and TDZ, although CPPU played a major role in subsequent differentiation, this probably owing to the high CK activity of CPPU, which led to the imbalanced AX/CK ratio during callus induction. Moreover, the selection of PGRs is significantly depends on the genotype and explant. For instance, in *A. truncatum* 'Lihong', 3.0 mg/L 6-BA + 2.0 mg/L TDZ + 0.5 mg/L IBA + 0.1 mg/L GA_3 had the best effect on callus induction from stem explants (Yang et al. 2024), while 2.0 mg/L 2,4-D + 0.3 mg/L 6-BA + 0.5 mg/L NAA was more efficient in callus induction from leaf explants of *A. truncatum* (Yu et al. 2004), these different responses may be attributed to their intrinsic physiological differences.

High level of endogenous CK promotes the transition of callus cells into shoot regeneration. Consistently, many components of CK biosynthesis and signaling are considered as key drivers of

296 DNSO (Shin et al. 2020), for instance, the activation and localized expression of *WUS* is crucial for this
297 conversion (Meng et al. 2017; Xiao et al. 2018). The appropriate type of CK for shoot differentiation
298 was genotype dependent, for example, CPPU was recommend for shoot differentiation in *Paeonia ostii*
299 'Feng Dan' (Xu et al. 2021) and *Actinidia chinensis* (Caboni et al. 2009), TDZ for coconut (Kong et al.
300 2021), MT for *Coleus forskohlii* (Kumar et al. 2023), 6-BA for *Broussonetia papyrifera* (Zhou et al.
301 2021), 2-iP for *Elaeis guineensis* (Romyanon et al. 2015).

302 CPPU displayed greater efficiency in shoot differentiation than several other CKs, especially in
303 some recalcitrant species (Huang et al. 2015; Du et al. 2020). These kind of species may have a higher
304 requirement for endogenous CKs level to trigger the expression of related gene, thereby starting
305 differentiation, *A. truncatum* might belong to it. In this study, TDZ was ineffective in shoot
306 differentiation, which was inconsistent with the results in previous studies that the use of TDZ alone
307 resulted in shoot differentiation of *A. davidii* (Hao 2010), *A. negundo* (Zhang et al. 2012) and *A.*
308 *ginnala* (Ma 2019), although the differentiation rate were relatively low. This discrepancy may account
309 for genotype, physiological state and endogenous hormone level of explants. Addition of exogenous
310 AX had a negative influence on shoot differentiation of *A. truncatum*, similar results were obtained in
311 *Eucalyptus nitens* (Ayalaet al. 2019) and *Peaonia ostii* (Xu et al. 2022). But in some other studies,
312 proper supplementation of AX had a positive impact on callus differentiation (Kou et al. 2013; Liu et al.
313 2023; Patial et al. 2024), it is speculated that a proper CK/AX ratio is more important for shoot
314 regeneration rather than sole CK and AX levels. The different requirements in PGRs among species
315 might be related to their endogenous hormone levels and development requirements.

316 As we know, there are two plant shoot organogenesis pathways: direct and indirect shoot
317 organogenesis with an intermediate callus formation stage. In this study, shoot organogenesis in *A.*
318 *truncatum* was achieved through the latter way, including three successive development stages: callus
319 formation, MNs formation and shoot differentiation. Callus originated from cortical parenchyma cells
320 and meristematic cells around vascular bundle of stem segments, then MNs formed in the vicinity of
321 epidermis of callus, indicating the origin of shoots is endogenous and exogenous coexist in *A.*
322 *truncatum*, similar phenomenon was observed in *P. ostii* (Xu et al. 2022), and *Humulus lupulus* (Batista
323 et al. 2000; Fortes et al. 2000). This MNs-mediated regeneration pathway has been widely documented
324 in numerous species, such as *Cichorium intybus* (Piéron et al. 1998), *Garcinia mangostana* (Te-chato et
325 al. 2000) and *P. ostii* (Xu et al. 2022). In these studies, the formation of MNs is considered as a
326 prerequisite and sign of DNSO, given that shoot differentiation occurred only in nodular areas.

327 The protocol presented in this study is a significant advance in tissue culture of *A. truncatum*. On
328 the one hand, the long-standing problem in callus differentiation of *A. truncatum* has been overcome
329 for the first time, which shows great potential for large-scale propagation of elite germplasms; on the

other hand, the present protocol allows for the establishment of genetic transformation system, thereby enabling gene function analysis and genetic improvement. But the callus differentiation efficiency is still need to be further improved, which could be ascribed to the recalcitrance of *A. truncatum* and the limitation of culture program, further optimization should take into account the explant selection, medium composition, PGRs and subculture period. Furthermore, the physiological and molecular mechanisms underlying DNSO need to be deeply investigated to guide the scheme optimization of shoot regeneration and created high regenerative capacity germplasm of *A. truncatum* through targeted breeding.

5 Conclusion

In the present study, a reliable *in vitro* regeneration system of *A. truncatum* was developed for the first time via DMSO, successful shoot differentiation and plant regeneration were realized by using stem explants. Histological study revealed the origin and development process of shoot organogenesis. This study will contribute to the germplasm conservation, large-scale propagation and genetic improvement of *A. truncatum*.

CRediT authorship contribution Du Yiming: Writing – review and editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. Zhao Wenqi: Writing – original draft, Investigation, Data curation, Methodology, Visualization. Xin Meng, Li Shushun: Investigation, Resources, Validation. Zhu Lu, Ma Qiuyue, Yan Kunyuan: Software, Methodology, Formal analysis. Zhang Guojun: Writing – review and editing, Supervision, Conceptualization. Li Qianzhong: Writing – review and editing, Supervision, Conceptualization.

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Data availability The authors declare that the data supporting the findings of this study are available within the paper and raw data files will be available from the corresponding author upon request.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interest The authors have no relevant financial or non-financial interests to disclose.

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Tables

Table 1 Effect of sterilization treatments on initiation culture of stem explants

Sterilization treatment	Contamination rate (%)	Callus induction rate (%)
75% Ethanol (20 s) + 3% NaClO (7 min)	15.74±1.61^{b}	98.15±1.61^{a}
75% Ethanol (20 s) + 0.1% $HgCl_2$ (7 min)	20.37±1.61^{c}	94.44±2.78^{bc}
75% Ethanol (30 s) + 3% NaClO (10 min)	8.33±2.78^{a}	93.52±1.60^{c}
75% Ethanol (30 s) + 0.1% $HgCl_2$ (10 min)	4.74±1.70^{a}	97.17±0.09^{ab}

Different letters within a column show significant differences by Duncan's multiple range tests (p<0.05).

Table 2 Effect of basal medium on callus induction in *A. truncatum*

Basal medium	Callus induction rate (%)	Morphological characteristics of callus
WPM	97.14±0.11^{a}	Pale yellow, compact texture, a little small white fragments on the surface of explants
NN69	69.63±1.28^{b}	Pale yellow, compact texture, a minority of explants had small white fragments on the surfaces
MS	65.74±1.61^{c}	Pale yellow, watery and loose texture, with small white fragments on the surface of explants

Different letters within a column show significant differences by Duncan's multiple range tests (p<0.05)

514 **Table 3** Effect of PGRs on callus induction in *A. truncatum*

2,4-D (mg/L)	NAA (mg/L)	6-BA (mg/L)	CPPU (mg/L)	Callus induction rate (%)	Morphological characteristics of callus
1.0		0.5		98.00±2.83[a]	Type I/ III; ++++; slight browning
0.5		0.2		95.06±2.14[a]	Type I/ III; +++
0.2		0.5		90.28±1.97[ab]	Type I; +++
	0.5	0.2		81.48±8.49[b]	Type I/ III; ++++; slight browning
0.5			0.2	88.89±5.23[ab]	Type I; ++/+
	0.5		0.2	70.74±0.52[c]	Type II; ++++/+

515 Different letters within a column show significant differences by Duncan's multiple range tests
516 (p<0.05). Type I callus was pale yellow compact texture, and formed into nodule-like structures during
517 subsequent culture; type II callus was watery, loose structure, and was proned to turn browning during
518 subculture; type III callus was small white fragments formed on the surface of explants, and no
519 morphological changes occurred during subsequent culture. Callus biomass was quantified into four
520 grades and represented by +, ++, +++, ++++.

521 **Table 4** Effect of combination of PGRs on callus induction in *A. truncatum*

2,4-D (mg/L)	NAA (mg/L)	IBA (mg/L)	6-BA (mg/L)	CPPU (mg/L)	TDZ (mg/L)	Callus induction rate (%)	Callus type and biomass
0.2			0.5			97.14±0.11[a]	Type I; +++
0.2				0.5		53.71±1.61[b]	Type III; +
0.2					0.5	30.56±2.78[e]	Type III; +
	0.2		0.5			48.15±1.61[c]	Type I; +, ++
	0.2			0.5		32.41±1.60[e]	Type I/ III; +, ++
	0.2				0.5	51.85±1.61[b]	Type III; +
		0.2	0.5			38.89±1.85[d]	Type III; +
		0.2		0.5		51.85±3.21[b]	Type III; +
		0.2			0.5	40.74±1.28[d]	Type III; +

The type and biomass of callus were as described in Table 3. Different letters within a column show significant differences by Duncan's multiple range tests (p<0.05).

Table 5 Effect of light condition on callus induction in *A. truncatum*

Light conditions	Complete darkness	12h light/12h dark	*t* test	*p* value
Callus induction rate (%)	97.17±0.09	85.19±3.71	5.599	0.005

Table 6 Effect of PGRs on shoot differentiation in *A. truncatum*

NAA (mg/L)	IBA (mg/L)	BA (mg/L)	CPPU (mg/L)	TDZ (mg/L)	Shoot differentiation rate (%)
			0.25		3.34±4.72[b]
			0.5		10.51±0.89[a]
				0.25	0.00±0.00[c]
				0.5	0.00±0.00[c]
		0.25	0.25		4.65±0.16[b]
			0.25	0.25	0.00±0.00[c]
0.1			0.5		0.00±0.00[c]
	0.1		0.5		0.00±0.00[c]

Different letters within a column show significant differences by Duncan's multiple range tests (p<0.05)

Figure captions

Fig. 1 Effects of basal medium and light condition on callus induction in *A. truncatum*

A. Explants and callus cultured on WPM medium in the dark for 15 d; B. Explants and callus cultured on NN69 medium in the dark for 15 d; C. Explants and callus cultured on MS medium in the dark for 15 d; D. Explants and callus cultured on WPM medium under 12h photoperiod for 15 d

Fig. 2 Effect of basal media on shoot differentiation of *A. truncatum*

Different letters show significant differences by Duncan's multiple range tests (p<0.05)

Fig. 3 Morphological observation of shoot organogenesis in *A. truncatum*

A. Stem explants; B. Pale yellow and compact callus formed at cut ends of stem segments after 15 days of culture; C. Callus cultured on SIM for one subculture, pre-nodular structures formed on the surface of callus; D. MNs formed on the surface of callus after 60 days of culture; E. Differentiation of leaf

cluster on the surface of MNs after 120 days of culture; F. Shoot cluster; G. Elongated shoot with neoformed stem and expanded leaves; H. Rooted shoot; I. Survival plantlet after 30 days of acclimatization

Fig. 4 Histological observation of shoot organogenesis in *A. truncatum*

A. Cell division in subepidermal and cortical area resulted in the formation of small protuberances in the local epidermal area after 3 days of culture; B Callus initiation after 6 days of culture due to vigorous division of cortical parenchyma cells (black asterisk) and meristematic cells around vascular bundles (black arrows), several layers of meristematic cells arranged parallel to epidermis, as well as dome-shaped meristematic cell clusters (hollow black arrow); C. Detailed view of meristematic cell clusters (black arrows); D. Unorganized callus after 15 days of culture; E. Pre-nodular structure formed after transferring to SIM for one subculture; F. Callus after 60 days of culture; G. MNs consisted of external layers of meristematic cells and a central area of vascularization, with rectangular cells longitudinally arranged beneath the MNs (black arrows); H. Shoot differentiation on the surface of MNs; I. Shoot clusters; BP bud primordia; BA bud axis; V vascular bundle; LC leaf clusters; LP leaf primordia; MN meristematic nodule; YL young leaves

Figures

Fig. 1

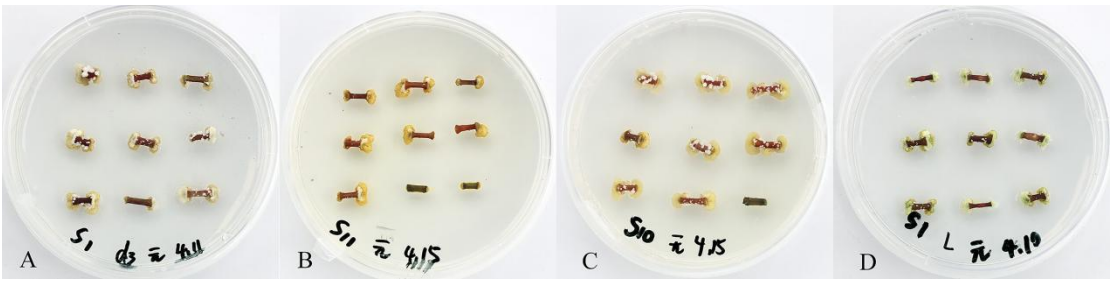
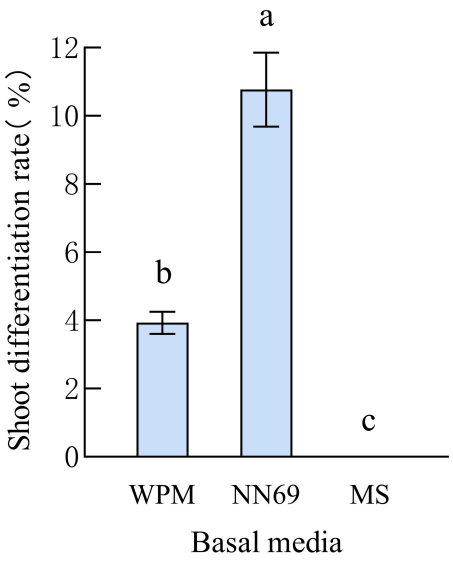


Fig. 2


a
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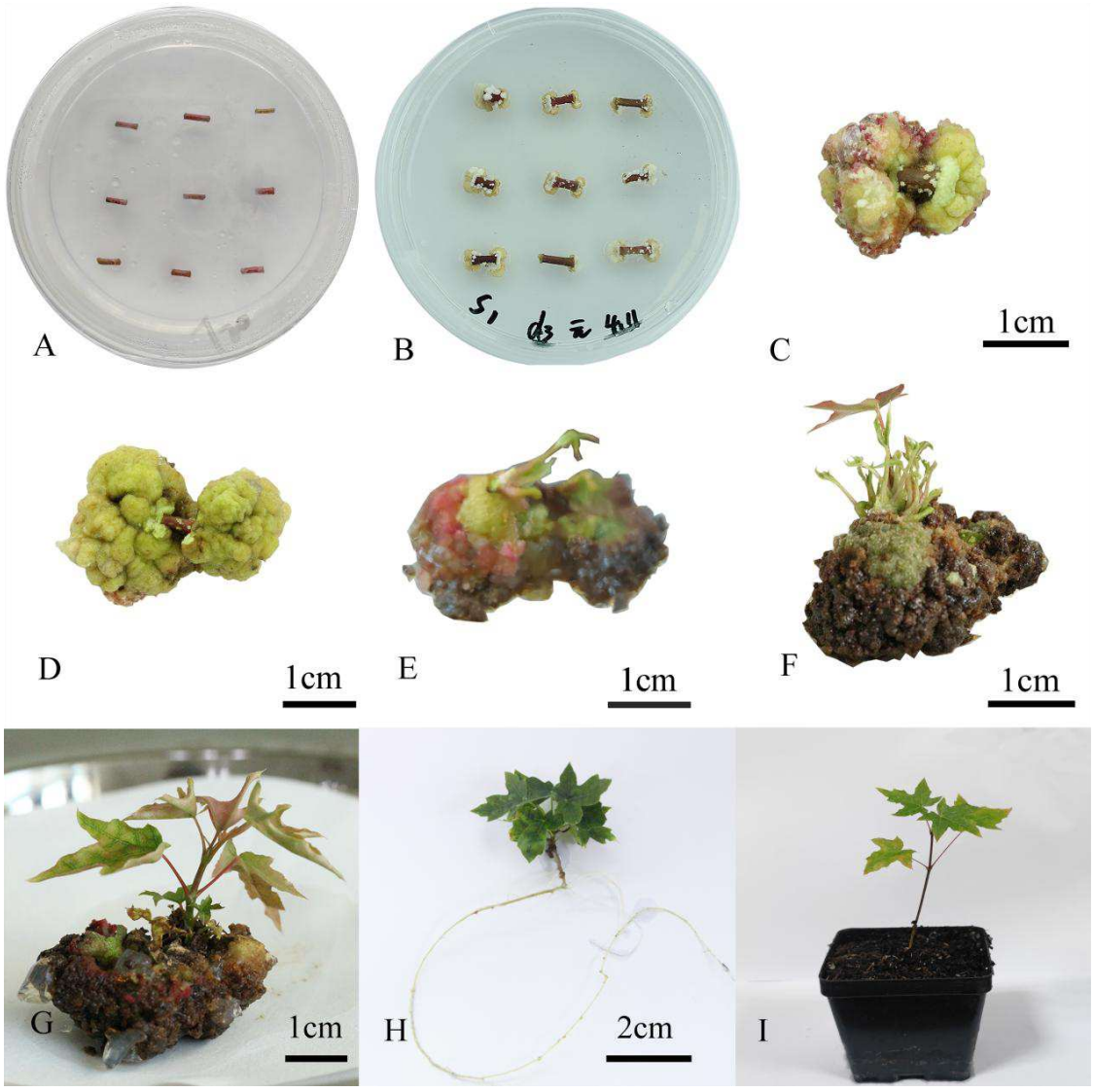
Shoot differentiation rate (%)
WPM
NN69
MS
Basal media

557 **Fig. 3**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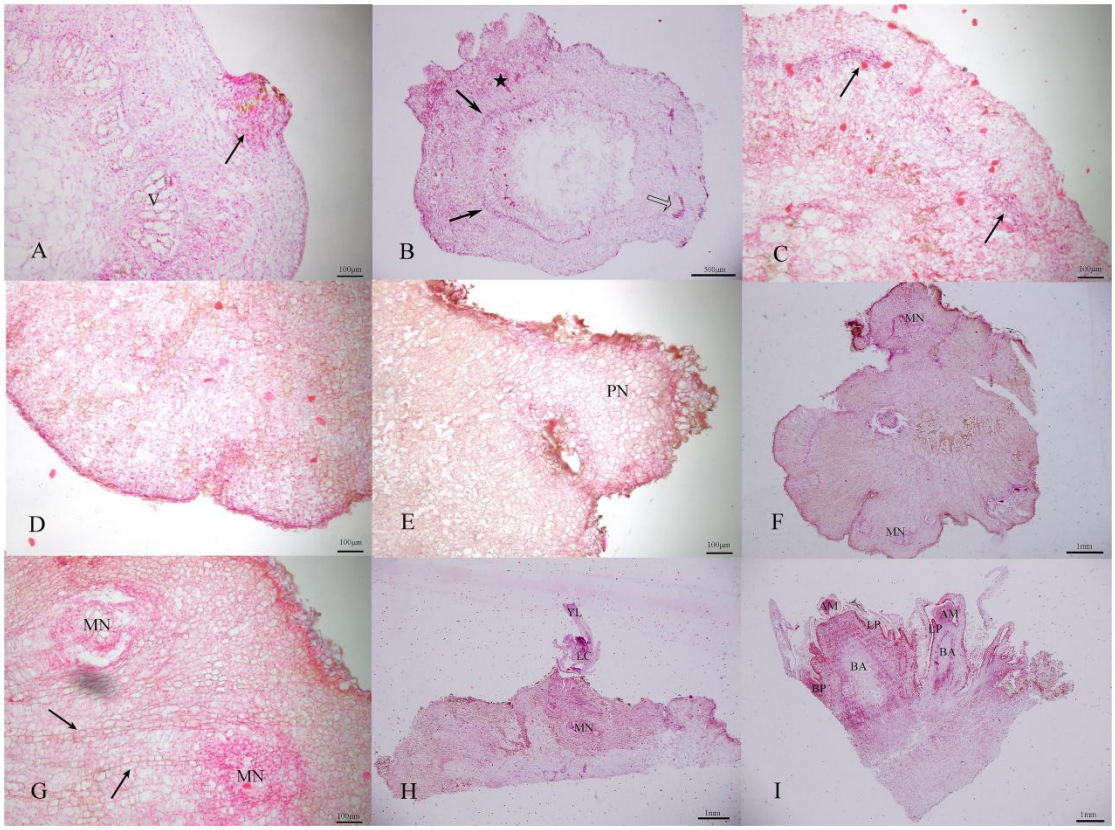
558 **Fig. 4**



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