Micropropagation of Chinquapin (*Castanea henryi*) Using Axillary Shoots and Cotyledonary Nodes

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Abstract. *Castanea henryi* is an important woody grain tree species native to China. The objective of the current study was to find the suitable plant growth regulators (PGRs) and the optimal concentrations for direct organogenesis by using axillary shoots and cotyledonary nodes. Seeds were collected from the field, sterilized, and germinated in vitro. Axillary shoots and cotyledonary nodes of 3-week-old seedlings were used as explants. To find the suitable PGR for adventitious shoot induction, 0.5 mg·L⁻¹ 6-benzylaminopurine (6-BA), 0.1 mg·L⁻¹ indole-3-acetic acid (IAA), 0.1 mg·L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), or 0.1 mg·L⁻¹ 1-naphthaleneacetic acid (NAA) was supplemented to Murashige and Skoog (MS) medium containing 0.65% agar and 3% sucrose. A high induction percentage of adventitious shoots (85.67%) was obtained from cotyledonary nodes supplemented with 0.1 mg·L⁻¹ 2,4-D. The type of explant influenced shoot proliferation rates and quality. Apical explants produced more and longer shoots than nodal segments. For shoot multiplication, 1 mg·L⁻¹ 6-BA + 0.05 mg·L⁻¹ indole-3-butyric acid (IBA) supplemented with MS medium produced 12.33 and 6.25 shoots per explant, respectively, from apical and nodal explants. For shoot elongation and strengthening, 2 mg·L⁻¹ 6-BA + 0.05 mg·L⁻¹ IBA supplemented with MS medium was the best combination, producing shoots with a mean length of 3.50 cm, a diameter of 0.46 cm, and about eight leaves per shoot. The greatest rooting of 76.70% and 11.33 roots per shoot was achieved when cultured in MS medium supplemented with 3.5% perlite + 1.5 mg·L⁻¹ IBA. For acclimatization of the rooted plantlets in the greenhouse, a survival rate of 80% was achieved. This protocol—from multiplication to acclimation—is helpful to realize mass propagation of high-quality trees of chinquapin for increasing production and nut quality.

Chinquapin (*Castanea henryi*) is an economically important species of the *Castanea* genus that produces timber and starch, which are widely distributed in southern China, especially in the Fujian, Zhejiang, and Hunan

Provinces (Fan et al., 2017; Yang et al., 2015). This deciduous tree species has been used increasingly as a woody grain because the nut has several favorable characteristics, including a high starch content of 47.58%–56.94% (Zheng et al., 2002), a high mineral nutrition content, and 18 kinds of amino acids, among which eight are good for health (Fan et al., 2015). What's more, the chinquapin nut is the main economic source for growers in southern China. To meet the demand for chinquapin nuts, in practice, grafting propagation has been used, because cutting is very difficult for *Castanea* species (Tetsumura and Yamashita, 2004). Micropropagation is a way of asexual production that can obtain seedlings with the same

genotype in a short time and ensures genotype stability, which is even helpful for transgenic studies (Corredoira et al., 2004; Li et al., 2017; Mondal et al., 2001).

Micropropagation of *Castanea* species has been investigated on the American chestnut (*C. dentata*) (Oakes et al., 2013, 2016; Xing et al., 1997, 1999), the Chinese chestnut (*C. mollissima*) (Lu et al., 2017; Qi-guang et al., 1986), the European chestnut (*C. sativa*) (Corredoira et al., 2003; Rodriguez, 1982; Sezgin and Dumanoglu, 2014), the Japanese chestnut (*C. crenata*) (Tetsumura and Yamashita, 2004), and their hybrid chestnut (Cuenca et al., 2017; Vidal et al., 2015). Micropropagation of *Castanea* species in most of the reports was successful. To our knowledge, there have been no reports on either in vitro regeneration or successful acclimation of chinquapin.

Producing woody plants from micropropagation is an extremely time- and labor-intensive process, needing many steps and various growing media (Gonçalves et al., 1998; Oakes et al., 2016; Pijut et al., 2010). Axillary shoot explants have been used to achieve regenerated plants in many species, including hybrid chestnuts (Cuenca et al., 2017; Vidal et al., 2015), *Vaccinium* sp. (Litwińczuk, 2013), and *Salix viminalis* (Regueira et al., 2018). Cotyledonary node explants were used mainly in legume crops to regenerate shoots (Abd Aziz et al., 2018), and in the woody plants *C. sativa* and *Camellia oleifera*, cotyledonary node explants were also a success (Li et al., 2016; San-José et al., 2001). However, to date, there has been no efficient system for the micropropagation of chinquapin by using axillary shoots and cotyledonary nodes. Therefore, the objective of this study was to establish a direct organogenesis micropropagation system by optimizing PGRs and growth conditions for explant types of axillary shoots and cotyledonary nodes.

Materials and Methods

Plant materials. Seeds of *Castanea henryi* cv. Huali 1 were collected from the chinquapin experimental field of Central South Forestry University of Science and Technology in Rucheng County, Chenzhou, Hunan Province (lat. 25°33'N, long. 113°45'E). Seeds were surface sterilized with sodium hypochlorite (5%) for 15 min. The tegument was removed and the seeds were disinfected in 75% (v/v) ethanol for 60 s, then rinsed three times with sterile distilled water for at least 30 s each time. Next, the seeds were treated with 0.1% (w/v) mercuric chloride (HgCl₂) solution for 6 min and then rinsed five times with sterile distilled water to remove excess HgCl₂. Excised part of the cotyledons before seed embryos was placed on aseptic MS (Murashige and Skoog, 1962) medium, which contained 0.65% agar and 3% sucrose, in glass tubes for germination. The seedlings were grown under white fluorescent lamps (50 μmol·m⁻²·s⁻¹) for a photoperiod/dark period of 14 h/10 h at a temperature of

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25 ± 2 °C (Fig. 1A). Axillary shoots and cotyledonary nodes from 3-week-old in vitro seedlings were used as explants (Fig. 1B). The culture conditions (light and temperature) were kept the same for the following described experiments.

Adventitious bud induction. The cotyledonary node explants were excised from 3-week-old seedlings cultured in the tube and placed on MS medium containing 0.65% agar and 3% sucrose, supplemented with 6-BA (0.5 mg·L⁻¹), IAA (0.1 mg·L⁻¹), 2,4-D (0.1 mg·L⁻¹), or NAA (0.1 mg·L⁻¹) for direct adventitious shoot induction (Table 1). A total of 162 explants (54 explants per replicate) were cultured. After 30 d, the differentiation rate of adventitious shoots was recorded (Fig. 1C–E).

Multiplication. The axillary shoots excised from 3-week-old seedlings and adventitious shoots from cotyledonary nodes, including apical and nodal explants (with two leaves and >1.0 cm in height), were transferred to MS medium containing 0.65% agar and 3% sucrose, supplemented with 6-BA (1, 2, or 3 mg·L⁻¹), IBA (0.05, 0.2, or 0.5 mg·L⁻¹), or gibberellic acid (GA₃; (0, 0.05, or 3 mg·L⁻¹) for multiplication (Table 2). After 4 weeks, the proliferation coefficient was calculated as follows: the number of

shoots after induction divided by the number of axillary shoots before induction. For each treatment, nine shoots were used and the experiment was conducted three times.

Shoot elongation and strengthening. Multiple shoots were cut apart ≈1.0 cm in length with two leaf segments, which were placed vertically into MS medium containing 0.65% agar and 3% sucrose, supplemented with 6-BA (0, 1, or 2 mg·L⁻¹), IBA (0, 0.05, or 0.2 mg·L⁻¹), or GA₃ (0, 0.3, or 2 mg·L⁻¹) for shoot elongation and strengthening (Table 3). After 4 weeks, diameter, length, and leaves of all shoots were measured. For each treatment, nine shoots were used, and the experiment was conducted three times.

Root induction. Elongated and strengthened shoots reaching at least 3.0 cm in length were inoculated into full-strength MS or half-strength MS medium containing 3.5% perlite, 0.65% agar, and 3% sucrose, supplemented with IBA (0.1, 0.5, or 1.5 mg·L⁻¹) or NAA (0.1, 0.5, or 1.5 mg·L⁻¹) (Table 4). After 4 weeks in culture for root development, the surviving shoots were removed carefully from the medium. Number of roots per plantlet, the length of the longest roots, and the root growth situation were recorded.

Acclimatization. Plantlets with well-developed root systems were chosen, cleaned

by removing the media with tap water, then planted, one plant per pot, into 6- × 7-cm (diameter × height) pots filled with substrate that was a mixture of peat : perlite at 2:1 (v/v), saturated with modified Melin-Norkrans liquid [CaCl₂ · 2H₂O 0.05 g·L⁻¹, MgSO₄ · 7H₂O 0.15 g·L⁻¹, NaCl 0.025 g·L⁻¹, (NH₄)₂HPO₄ 0.25 g·L⁻¹, KH₂PO₄ 0.5 g·L⁻¹, vitamin B1 0.1 mg·L⁻¹, citric acid 0.2 g·L⁻¹, glucose 10 g·L⁻¹, and pH adjusted to 5.5]. All pots were placed in a climate chamber with humidity at 80% for 3 d, and dropped 5% every 3 d thereafter, until reaching to the same humidity as the ambient environment (about 65%). The pots remained in the climate chamber for 1 month. Afterward, the plants were transferred to larger pots 12 × 16 cm (diameter × height) and filled with a potting mix of peat:perlite:loess at 1:1:1 (v/v/v). The pots were then placed in a greenhouse covered with a shade net and watered whenever the substrate surface was dry. Two months later, the surviving plantlets were counted and their height was recorded.

Statistical analysis. Experiments for multiplication and shoot elongation and strengthening followed an orthogonal design, whereas those for adventitious bud induction and rooting were arranged in a completely randomized design. There were three replicates for each treatment for all experiments. All data were expressed as mean value ± SE and were subjected to analysis of variance (ANOVA) (Li et al., 2016) and nonparametric tests using SPSS 19.0 software (Chicago, IL). *P* < 0.05 was considered statistically significant.

Results and Discussion

Cotyledonary node shoot induction. For shoot induction, supplementing 2,4-D at 0.1 mg·L⁻¹ induced adventitious shoots at 85.67% (Table 1), with thick stems and lots of leaves (Fig. 1C–E), whereas 0.1 mg·L⁻¹ 2,4-D combined with 0.5 mg·L⁻¹ 6-BA did not have any adventitious shoots; instead, a large number of compact calluses was formed. It is interesting to note that medium containing IAA induced a lot of roots but no shoots, whereas medium containing NAA induced only a few calluses.

For woody plants, using a cotyledonary node as an explant is relatively easy to promote adventitious shoots (Huang et al., 2014; Li et al., 2016; San-José et al., 2001). Therefore, adventitious shoots were restored from epidermal cells that may retain the characteristics of parenchymal cells. The number and frequency of shoot induction were dependent mainly on the concentration of cytokinin used in the culture medium (Sharma, 2017). However, 6-BA in our study could not induce any shoots but did induce some calluses when combined with NAA and 2,4-D. Instead, 2,4-D, a kind of auxin, did produce adventitious shoots. It is worth investigating whether more adventitious shoots per explant might be induced under the right type of PGR and concentration by using cotyledonary nodes (Abd Aziz et al., 2018; Li et al.,

Fig. 1. Plant regeneration by using axillary shoots and cotyledonary nodes of *Castanea henryi*. (A, B) Aseptic seedling cultured for 3 weeks. Axillary shoots and cotyledonary nodes used as explants. Ap, apical explants; No, nodal explants; Cn, cotyledonary nodes explants. (C–E) Adventitious shoot induction from cotyledonary nodes after 1, 2, and 4 weeks. (F, G) Microshoot multiplication after 1 and 4 weeks from nodal explants. (H–K) Microshoot multiplication after 1, 2, 3, and 4 weeks from apical explants. (L) Shoot elongation and strengthening. (M, N) Rooting. (O) Rooted plantlets transferred to climate chamber after 4 weeks. (P, Q) Acclimatized plants under greenhouse conditions for 2 months.

Table 1. Effects of different plant growth regulator combinations on adventitious shoot induction from cotyledonary nodes of *Castanea henryi*.

Plant growth regulators (mg·L ⁻¹)				Shoot formation rate (%) ^z	Description of shoot formation
6-BA	IAA	2,4-D	NAA		
0.5	0.1			0 ± 0	No adventitious bud but a lot of roots
0.5		0.1		0 ± 0	Many compact calluses were induced
0.5			0.1	0 ± 0	Induced a few calluses
	0.1			0 ± 0	No adventitious bud but a lot of roots
		0.1		85.67 ± 5.05	Healthy adventitious shoots with a lot of leaves
			0.1	0 ± 0	Induced a few calluses

^zValues represent the mean ± SE from three replicates of nine shoots each.

6-BA = 6-benzylaminopurine; IAA = indole-3-acetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; NAA = 1-naphthaleneacetic acid.

Table 2. Effect of different plant growth regulator combinations and type of explant on shoot formation of *Castanea henryi*.

Plant growth regulators (mg·L ⁻¹)			Explant type	Multiplication rate ^{y,z}	Description of proliferation
6-BA	IBA	GA ₃			
1	0.05	0	Apical	12.33 ± 1.70 a	Light-green leaves with a large callus at the base
			Nodal	6.25 ± 0.05 a	Light-green leaves with a large callus at the base
1	0.2	0.05	Apical	11.67 ± 0.74 a	Dark-green leaves with a few calluses at the base
			Nodal	4.50 ± 0.09 b	Light-green leaves with a large callus at the base
1	0.5	3	Apical	5.67 ± 0.47 b	Dark-green leaves with a few calluses at the base
			Nodal	3.40 ± 0.04 c	Dark-green leaves with large callus at the base
2	0.05	0	Apical	1.18 ± 0.15 d	Light-green leaves with large callus at the base
			Nodal	1.08 ± 0.07 g	Light-green leaves with large callus at the base
2	0.2	0.05	Apical	1.82 ± 0.19 d	Dark-green leaves with a few calluses at the base
			Nodal	1.70 ± 0.06 f	Dark-green leaves with large callus at the base
2	0.5	3	Apical	2.21 ± 0.11 cd	Light-green leaves with a few calluses at the base
			Nodal	2.14 ± 0.03 d	Light-green leaves with a few calluses at the base
3	0.05	0	Apical	1.52 ± 0.08 d	Light-green leaves with large callus at the base
			Nodal	1.75 ± 0.04 f	Light-green leaves with a few calluses at the base
3	0.2	0.05	Apical	1.11 ± 0.08 d	Dark-green leaves with no callus at the base
			Nodal	1.10 ± 0.05 g	Light-green leaves with a few calluses at the base
3	0.5	3	Apical	3.52 ± 0.30 c	Dark-green leaves with no callus at the base
			Nodal	1.90 ± 0.04 c	Light-green leaves with a few calluses at the base

^zMultiplication rate = no. of shoots after induction/no. of axillary shoots before induction.^yValues represent the mean ± SE from three replicates of nine shoots each. Different letters followed the mean indicate significance at $P < 0.05$ according to Duncan's multiple range test.6-BA = 6-benzylaminopurine; IBA = indole-3-butyric acid; GA₃ = gibberellic acid.Table 3. Effect of different plant growth regulator combinations on shoot elongation and strengthening of *Castanea henryi*.

Plant growth regulators (mg·L ⁻¹)			Shoot diameter (cm) ^z	Shoot length (cm) ^z	No. of leaves per shoot ^z	Description of shoot
6-BA	IBA	GA ₃				
0	0	0	0.21 ± 0.01 e	1.40 ± 0.01 d	2.80 ± 0.03 e	No visible elongation, thin stem with yellow leaves
0	0.05	0.3	0.27 ± 0.01 cd	1.00 ± 0.07 e	2.68 ± 0.02 e	No visible elongation, small yellowish leaves
0	0.2	2	0.25 ± 0.00 d	1.20 ± 0.03 de	2.79 ± 0.04 e	No visible elongation, thin stem, bright-green leaves
1	0	0.3	0.31 ± 0.02 bc	2.60 ± 0.01 c	5.72 ± 0.04 c	Visible elongation, thin stem, small and light-green leaves
1	0.05	2	0.32 ± 0.03 b	2.30 ± 0.05 c	5.56 ± 0.02 d	Visible elongation, dark-green leaves in various sizes
1	0.2	0	0.27 ± 0.01 cd	2.50 ± 0.03 c	5.64 ± 0.10 cd	Visible elongation, thin stem, leaves in various sizes
2	0	2	0.43 ± 0.04 a	3.67 ± 0.50 b	7.72 ± 0.05 b	Elongated significantly, long internode, large green leaves
2	0.05	0	0.46 ± 0.01 a	3.50 ± 0.03 b	7.60 ± 0.06 b	Elongated significantly, thick stem, large green leaves
2	0.2	0.3	0.32 ± 0.01 b	3.90 ± 0.06 a	7.95 ± 0.10 a	Elongated significantly, thin stem, large green leaves

^zValues represent the mean ± SE from three replicates of nine shoots each. Different letters followed the mean indicate significance at $P < 0.05$ according to Duncan's multiple range test.6-BA = 6-benzylaminopurine; IBA = indole-3-butyric acid; GA₃ = gibberellic acid.

2016), considering the number was low in this study (data not shown).

Apical and nodal explant proliferation rates. Multiplication rates of apical and nodal explants are shown in Table 2 and Fig. 1F–K. Both types of explant, the PGR, and their interaction influenced shoot multiplication rates ($P < 0.0001$). The apical explants produced more and longer shoots than nodal segments (Table 2; Fig. 1G and K). For apical explants, there was no significant difference in multiplication rates between the combination of 1 mg·L⁻¹ 6-BA + 0.05 mg·L⁻¹ IBA and 1 mg·L⁻¹ 6-BA + 0.02 mg·L⁻¹ IBA + 0.05 mg·L⁻¹ GA₃. The multiplication rate was 12.33 and 11.67, respectively. For nodal segments, the greatest multiplication rate (6.25) was

obtained with 1 mg·L⁻¹ 6-BA + 0.05 mg·L⁻¹ IBA. All combinations of PGR in this study produced multiple shoots, with the multiplication rate ranging from 1.08 to 12.33. The multiplication rate decreased when 6-BA concentration was greater than 1 mg·L⁻¹. The multiplication rate decreased when the IBA and GA₃ concentration increased.

A high multiplication rate is an important indicator for assessing a regeneration system. The influence of the position of axillary shoots on shoot quality and multiplication rates has been studied in hybrid chestnut (Vidal et al., 2015). Vidal et al. (2015) found that the apical explants in clone 90025 obtained a greater multiplication rate (3.7) and a longer shoot (3.4 cm) than nodal

explants (2.1 and 1.9 cm, respectively). The current study also showed that apical explants produced more and longer shoots than nodal segments.

A combination of cytokinin and auxin has been proved to be efficient for woody plant shoot proliferation (Li et al., 2016; Mathur et al., 2002; Siwach and Gill, 2011). Moreover, 6-BA has been well documented as an efficient inducer for shoot multiplication in many woody plants, including American and European chestnuts (Asthana et al., 2011; Bunn, 2005; Rodriguez, 1982; Xing et al., 1997). But, it was also reported that a greater concentration of 6-BA was inhibitory in some woody plants, such as *Prunus armenica* cv. Canino (Tornero et al., 2000) and *Pterocarpus*

Table 4. Effect of medium and different plant growth regulator combinations on rooting performance of *Castanea henryi*.

Medium	Plant growth regulators (mg·L ⁻¹)		Rooting rate (%) ^z	No. of roots ^z	Length of roots (cm)	Description of root growth
	IBA	NAA				
MS	0.1		16.70 ± 1.14 e	1.67 ± 0.05 d	0.50	Thin roots
MS	0.5		23.30 ± 0.88 c	2.67 ± 0.17 c	3.00	Thick roots
MS	1.5		76.70 ± 0.42 a	11.33 ± 0.47 a	4.00	White roots with a few calluses at base
1/2MS	0.1		6.70 ± 0.19 f	1.33 ± 0.05 d	0.50	Thin roots
1/2MS	0.5		20.00 ± 0.71 d	1.33 ± 0.03 d	5.00	Thick roots with a lot of calluses at base
1/2MS	1.5		63.30 ± 0.51 b	6.33 ± 0.12 b	3.50	Thick roots with a few calluses at base
MS		0.1	0 ± 0	0 ± 0	0	No roots
MS		0.5	0 ± 0	0 ± 0	0	No roots
MS		1.5	0 ± 0	0 ± 0	0	No roots
1/2MS		0.1	0 ± 0	0 ± 0	0	No roots
1/2MS		0.5	0 ± 0	0 ± 0	0	No roots
1/2MS		1.5	0 ± 0	0 ± 0	0	No roots

^zValues represent the mean ± SE from three replicates of nine shoots each. Different letters followed the mean indicate significance at $P < 0.05$ according to Duncan's multiple range test.

MS = Murashige and Skoog medium; IBA = indole-3-butyric acid; NAA = 1-naphthaleneacetic acid.

santalinus (Balaraju et al., 2011). The current study confirmed the 6-BA effect in multiplication and was in agreement that a greater concentration of 6-BA was inhibitory for *C. henryi*.

Shoot elongation and strengthening. Different PGR combinations influenced shoot elongation and strengthening: nonparametric tests at $P = 0.006$ and $P = 0.002$, respectively, for diameters and lengths; and ANOVA at $P < 0.0001$ for the number of leaves per shoot. There was no visible elongation and strengthening without 6-BA. With increasing 6-BA concentration, shoot elongation and strengthening were more obvious. However, using a constant 6-BA concentration produced no significant difference in shoot elongation and strength with changes in the concentrations of IBA and GA₃ (Table 3). Considering shoot growth, MS medium supplemented with 2 mg·L⁻¹ 6-BA and 0.05 mg·L⁻¹ IBA was the best combination for shoot elongation and strengthening, with a mean length of 3.50 cm, a diameter of 0.46 cm, and about eight leaves per shoot (Fig. 1L).

Our study indicated that 6-BA concentration played an important role in shoot elongation and strengthening. With the appropriate cytokinin and auxin concentration, chinquapin shoots were strong enough for rooting and surviving after potting ex vitro. As the study on the American chestnut showed that in vitro shoots are recommended only if they have more than six leaves before rooting and are greater than 3 cm in height so they would be large enough to survive after potting (Oakes et al., 2016).

Rooting. Both MS medium strength and type of PGR, and their interaction influenced rooting significantly ($P < 0.0001$). Adding IBA to the medium produced roots regardless of concentration, whereas adding NAA only led to calluses at the base of the shoots (Table 4). IBA concentration influenced rooting rate and number of roots significantly. With the increasing concentration of IBA, rooting percentage increased. Compared with half-strength MS, full-strength MS improved rooting rate and the number of roots significantly (Table 4). MS medium combined with 3.5% perlite and 1.5 mg·L⁻¹ IBA led to a rooting rate as much as 76.70%, and strong plantlets for acclimatization.

For *Castanea* species, in vitro rooting is a bottleneck in micropropagation. In our study, visible adventitious roots were induced in 2 weeks and, 4 weeks later, plantlets could be taken from the rooting medium for acclimatization (Fig. 1M and N). However, a previous study on American chestnut showed that plantlet survival in the growth chamber could be improved dramatically by shortening the time in rooting medium from 8 to 10 d to 3 to 4 d, and also by placing plantlets in the dark during this time (Oakes et al., 2013). The results of that study indicated that there is potential for shortening the rooting phase and improving the survival rate during acclimatization for chinquapin micropropagation. Adding perlite to rooting medium improved root induction in *C. oleifera* (Li et al., 2016). Similarly, the rooting rate in our study was acceptable by using perlite in the rooting medium. It has been reported that half-strength MS or quarter-strength MS with the appropriate auxin was more useful than full-strength MS for inducing root growth, as seen in *Llex crenata* and *C. oleifera*, indicating that low-concentration inorganic salt was better for rooting and root development (Dong et al., 2017; Li et al., 2016; Yang et al., 2015). However, in our study, full-strength MS was much better than half-strength 2MS for rooting, which was consistent with the study on red sanders (*Pterocarpus santalinus*) (Balaraju et al., 2011). As our results show, IBA can produce roots regardless of concentration, whereas NAA only led to the development of calluses at the base of shoots. These results are consistent with previous studies that indicated that NAA had a lesser effect on root formation than IBA (Li et al., 2016; Lin et al., 2016).

Acclimatization. Of the 44 plants, 39 survived the transplant from in vitro to the growth chamber (Fig. 1O); five withered after 1 month. Two months after acclimatization in the greenhouse, 80% of plants survived, with a mean plant height of 20 cm (Fig. 1Q).

Conclusion

Adventitious shoots were induced with a high induction rate by supplementing with 0.1 mg·L⁻¹ 2,4-D to MS medium, and apical explants produced more and longer shoots

than nodal segments. For shoot multiplication, a combination of 1 mg·L⁻¹ 6-BA + 0.05 mg·L⁻¹ IBA added to MS medium was recommended using either apical or nodal explants. For shoot elongation and strengthening, 2 mg·L⁻¹ 6-BA + 0.05 mg·L⁻¹ IBA added to MS medium was the best combination. The greatest rooting of 76.70% and 11.33 roots per shoot were achieved by adding 1.5 mg·L⁻¹ IBA to the rooting medium; the rooting percentage increased with IBA concentration. For acclimatization of the rooted plantlets in the greenhouse, a survival rate of 80% was achieved. This protocol will be helpful in realizing mass propagation of high-quality trees of chinquapin for high yield and nut quality.

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