

## Communication

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# *In vitro* plant regeneration of *Zenia insignis* Chun

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**Abstract:** *Zenia insignis* Chun is a large, fast-growing deciduous tree. In this study, we successfully developed a reliable and efficient protocol for the regeneration of fertile plants via callus induction from leaf segments of young *Z. insignis* seedlings. The best results were obtained with a medium containing 11.00  $\mu\text{M}$  6-benzyladenine (6-BA), 1.20  $\mu\text{M}$  indole-3-butyric acid (IBA), and 0.45  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid (2,4-D), which yielded morphogenic callus within 2 weeks at a frequency of 62.23%. We tested the effect of IBA alone and in combination with 6-BA on the bud differentiation response of *Z. insignis* callus. Shoots differentiated normally when cultured on differentiation medium containing 6.00  $\mu\text{M}$  6-BA and 1.20  $\mu\text{M}$  IBA. Regenerated buds elongated successfully in medium containing 1.20  $\mu\text{M}$  gibberellic acid (GA<sub>3</sub>). The elongated shoots were then transferred to Murashige and Skoog basal medium supplemented with various combinations of naphthalene acetic acid (NAA) for root induction; well-developed roots were achieved on MS basal medium supplemented with 0.01  $\mu\text{M}$  NAA at a rooting rate of 89.23%. Rooted plantlets were successfully acclimatised to a greenhouse at a survival rate exceeding 90.00%.

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**Keywords:** callus induction; regeneration; root induction; *Zenia insignis* Chun

## 1 Introduction

*Zenia insignis* Chun is a large, fast-growing deciduous tree. It is a rare monotypic species, and one of only two protected wild tree species in China. *Zenia insignis* Chun is mainly distributed in tropical Asia, India, Malaysia, the Indochinese Peninsula, and southern and southwestern China [1, 2]. *Zenia insignis* is an ideal raw material for manmade construction boards, with cellulose content approaching 50.11% and lignin content approaching 22.01%. Its products are widely used in construction, interior decoration, furniture manufacturing, and other industries [3, 4]. In addition, *Z. insignis* have been considered as one of the few potential tree species that can replace herbaceous plants used to remediate metal contamination [5–8]. This species has ecological value because of its root nodules and high metal accumulation potential, particularly for Zn and Cd [9, 10]. Thus, *Z. insignis* is a multipurpose tree species, which may become important in soil–water conservation. *Z. insignis* cuttings do not root easily. Previous studies on seeding, introduction of afforestation technology, and comprehensive utilization of wood and tissue culture of *Z. insignis* have been reported [11–14]. However, molecular biology research on *Z. insignis* like genetic assays of progeny and genetic engineering has not yet been reported. Therefore, it is important to establish an efficient system for *Z. insignis* regeneration by tissue culture.

Studies of *Z. insignis* tissue culture have mainly concentrated on the use of seedling leaves, petioles, and stems. The best material for callus induction is the stem, which approaches 98% inductivity in MS medium supplemented with 1.00 mg/L 6-benzyladenine (6-BA) and 0.50mg/L indole-3-butyric acid (IBA). However, this callus induction process requires at least 30 days [15]. Although the hypocotyl, seedling stems, annual shoots, and leaves have been used as explant materials, a complete system for the regeneration of *Z. insignis* has

not yet been established. Researchers have been able to produce callus only by supplementing culture media with 6-BA and naphthalene acetic acid (NAA) and to proliferate buds on a medium containing IBA, kinetin (KT), and/or 6-BA [16,17].

This study was to establish a highly efficient and sustainable regeneration system from *Z. insignis* explant leaves, in order to provide the basis for the later establishment of a genetic transformation system. Compared to the hypocotyl and stem as explants, leaves have the following advantages as genetic transformation material: a shorter root induction process, higher induction rate, convenient procurement, ease of use, and suitability for repeated trials [18]. The success of transformation methodology using leaves as explant material has been reported several times, for example, in oat [19], Valencia sweet orange [20], and ramie [21].

## 2 Materials and methods

### 2.1 Plant materials and seed germination

*Zenia insignis* seeds were collected from a tree aged  $\geq 2$  years in Debao, Guangxi, China. Seeds were immersed in water at 85°C for 12 min and incubated at 25°C for 24 h on a culture dish fitted with filter paper to retain moisture. Seeds that were clearly enlarged were selected for germination, then surface-sterilised by washing with 75% ethyl alcohol for 28 s, and soaked in 0.1%  $\text{HgCl}_2$  for 8 min under sterile conditions. We then rinsed the seeds with distilled water five times and germinated them on Murashige and Skoog (MS) [23] basal medium without growth regulators. After culturing for 10 days, the main leaf that developed from each *Z. insignis* seed was used as an explant.

### 2.2 Culture medium and growth conditions

MS medium was used as the basal medium for all cultures; it contained 3% sucrose and was gelled with 0.7% agar. We dispensed the medium into sterile 250 ml glass culture vessels (25 ml per vessel). The pH of the media was adjusted to 5.80 prior to autoclaving at 121°C for 20 min. Cultures were maintained in cool white fluorescent tubes ( $90 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) with a 12 h photoperiod and relative humidity of 70% at  $25 \pm 1^\circ\text{C}$ . The same culture conditions were used for all experiments, unless otherwise indicated.

### 2.3 Callus induction

Leaves were excised from the seedlings, cut into pieces measuring approximately  $5 \times 5$  mm, and placed with the abaxial side on the surface of the medium. Explants were cultured on MS medium supplemented with 6-BA (0.00, 5.50, 11.00, and 16.50  $\mu\text{M}$ ) and either IBA (0.00 and 0.08  $\mu\text{M}$ ) or 2,4-D (0.00 and 0.45  $\mu\text{M}$ ) for callus induction. The callus induction rate was observed weekly over a total of 4 weeks.

### 2.4 Shoot regeneration and elongation

To study the effect of plant growth regulators (PGRs) on shoot regeneration, the callus (compact, green, with shoot bud-like structures) was subcultured in MS basic medium supplemented with IBA (1.80 and 3.60  $\mu\text{M}$ ), 6-BA (0.00, 4.50, and 8.00  $\mu\text{M}$ ), and gibberellic acid ( $\text{GA}_3$ ) (0.00, 0.03, 0.06, 0.12, and 0.24  $\mu\text{M}$ ). Cultures were observed monthly and the regeneration rate was calculated as the percentage of explants with adventitious shoots. The number of adventitious shoots and effective shoots ( $> 1$  cm) per planted explant was recorded after 4 weeks.

### 2.5 Root formation

Shoots approximately 3–4 cm in length were subcultured in hormone-free MS basal medium for 2 weeks to eliminate any carry-over effects due to endogenous hormones [23]. Shoots were then transferred to MS basal medium supplemented with various concentrations of NAA (0.00, 0.10, and 0.15  $\mu\text{M}$ ). After 10 days of culture, we recorded root formation frequency, number of roots, and number of lateral roots.

### 2.6 Transplantation of *in vitro* culture plantlets

Plantlets in the culture vessels were transferred from the culture room to a greenhouse. After 7 days of culture in the described media, plantlets 5–7 cm in height with 4–6 leaves were removed from the jars and the agar was gently removed by rinsing with water. We transplanted 30 plantlets into pots (diameter: 10 cm; height: 14 cm) with a mixture of peat moss and loam at a 2:1 volumetric ratio. The pots were covered with polythene films to maintain high relative humidity (approximately 85%), and the films were gradually opened after 5 days in the greenhouse.

to allow plantlets to acclimatise to ambient conditions. Plants were watered every 2–3 days until the films were fully opened and then watered as required.

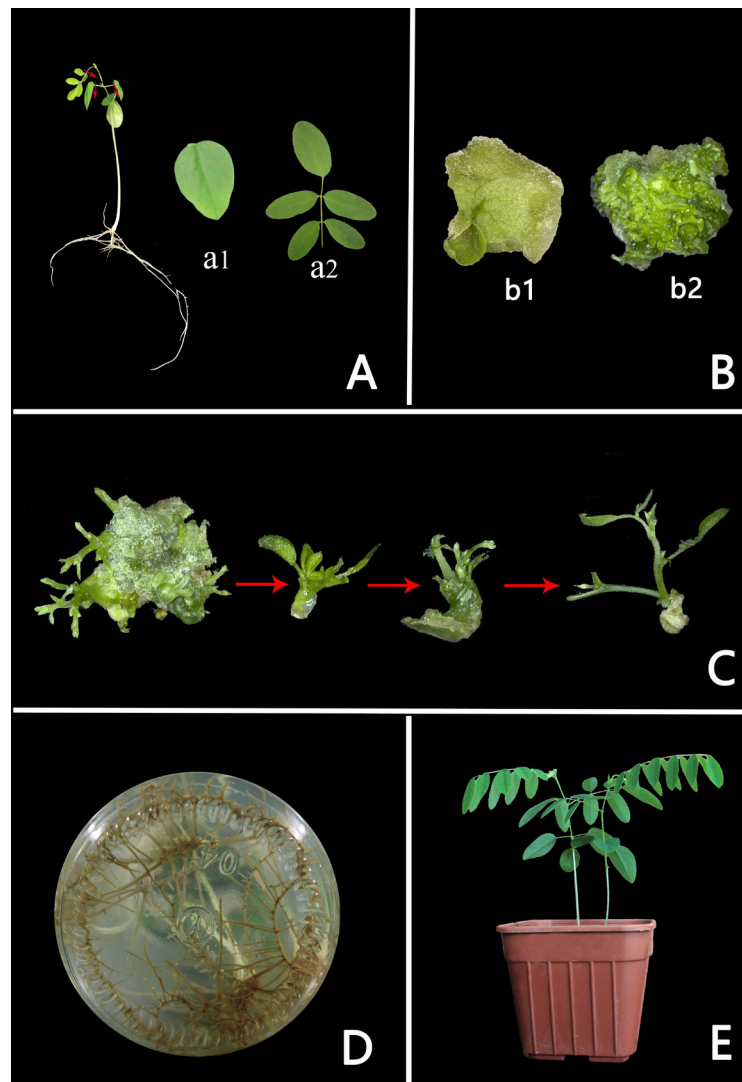
## 2.7 Statistical analysis

All experiments were repeated three times, with 10 explants per treatment. Data were analysed using one-way analysis of variance (ANOVA). Duncan's multiple range test was applied at the 0.05 probability level to compare individuals within a treatment [24] using the SPSS ver. 19.0 software.

## 3 Results

### 3.1 The effects of 6-BA, 2,4-D, and IBA on callus induction

Leaves from *in vitro*-germinated plants were used as explants (Fig. 1 A), placed on MS media containing 6-BA and 2,4-D, either alone or in combination with IBA, for callus induction. At 7 days of culture, leaves gradually began to expand; 1 week later, the edges of swollen leaves was induced on 12 different regeneration MS media (IBA, 6-BA, and 2,4-D) listed in Table 1.



**Figure 1.** In vitro plant regeneration of *Zenia insignis* Chun from leaves. (A) From a1 to a2, all leaves completely matured from *in vitro*-germinated plants were used as explants; (B) b1: callus developed on a leaf explant in MS medium with 11.00  $\mu\text{M}$  6-BA, 1.20  $\mu\text{M}$  IBA and 0.45  $\mu\text{M}$  2,4-D for 4 weeks; b2: the shoots were regenerated in MS medium with 6  $\mu\text{M}$  6-BA and 1.2  $\mu\text{M}$  IBA for 2 weeks; (C) The regeneration shoots reached the greatest elongation at 3.1 cm in MS medium with 6.00  $\mu\text{M}$  6-BA, 1.20  $\mu\text{M}$  IBA, 1.20  $\mu\text{M}$  GA3 for 15 days (D) Root systems of regenerated shoot-buds in MS medium supplemented with 1.86  $\mu\text{M}$  NAA; (E) An intact regenerated plantlet (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

**Table 1.** Effects of 6-BA, 2,4D and IBA concentration on callus induction of *Z. insignis*

Number	PGRs ( $\mu\text{M}$ )			Callus induction rate (%)
	6-BA	2,4D	IBA	
1	0.00	0.00	0.00	0i±0
2	5.50	0.00	1.20	27.78±0.66e
3	11.00	0.00	1.20	28.89±0.65e
4	16.50	0.00	1.20	16.67±0.45e
5	5.50	0.45	0.00	37.78±0.89d
6	11.00	0.45	0.00	50.22±0.44b
7	16.50	0.45	0.00	46.20g±0.65c
8	5.50	0.45	1.20	56.67±0.23ab
9	11.00	0.45	1.20	62.23±0.22a
10	16.50	0.45	1.20	51.12±0.87b
11	11.00	0.90	1.20	35.26±0.25d
12	11.00	1.80	1.20	26.97±0.53e

Each value represents the mean  $\pm$  standard error (SE) of three replicates, each with 10 explants. Means followed by the same letter in the same column are not significantly different from each other at  $P \leq 0.05$  level, according to Duncan's multiple range test.

PGRs, particularly cytokinins and auxins, both alone and in combination, are known to play an important role in callus induction and proliferation [25]. There were significant differences in callus formation among the various media in this study. The fastest morphogenesis from the different types occurred in compact, golden-yellow, and nodular callus, with few particle masses.

In treatments supplemented with a combination of 6-BA and 1.20  $\mu\text{M}$  IBA, the highest rate of callus induction was 28.89%, induced by 11.00  $\mu\text{M}$  6-BA. However, a combination of 0.45  $\mu\text{M}$  2,4-D and 11.00  $\mu\text{M}$  6-BA was clearly more efficient, exhibiting a 50.22% increase in callus induction frequency (Tab. 1). As concentrations of 6-BA increased (11.00–16.50  $\mu\text{M}$ ), the frequency of callus induction showed a downward trend in two different medium supplement combinations (50.22%–46.20%). Highest regeneration rates (62.23%) were obtained at moderate concentrations of 6-BA (11.00  $\mu\text{M}$ ) in combination with 1.20  $\mu\text{M}$  IBA and 0.45  $\mu\text{M}$  2,4-D (Tab. 1). Most calli were light green and healthy (Fig. 1 b1). When 6-BA concentration increased from 11.00 to 16.50  $\mu\text{M}$ , there was a decrease in callus induction.

However, as can be seen in Table 1, 0.90  $\mu\text{M}$  and 1.80  $\mu\text{M}$  2,4-D show a sharp decrease in the rate of callus induction compared to 0.45  $\mu\text{M}$  2,4-D (35.26% and 26.97% respectively). At the same time, high concentrations (1.80  $\mu\text{M}$ ) of 2,4-D produced browning of the callus during subsequent culture, affecting the regeneration process (Fig. 2).

### 3.2 The effect of 6-BA and IBA on shoot induction

Morphogenic callus (robust, healthy, and green) growing on Number 9 medium (Tab. 1) was transferred to regeneration medium for bud differentiation. The medium for callus differentiation was selected after testing 9 different combinations of growth regulators (Tab. 2).

Medium Number 1 (Tab. 2), containing no growth regulators, produced an average of 2.22 shoots; regeneration percentage was 9.03%. Media containing



**Figure 2.** Callus developed on a leaf explant in MS medium with 11.00  $\mu\text{M}$  6-BA, 1.20  $\mu\text{M}$  IBA and 1.80  $\mu\text{M}$  2,4-D, which appeared the browning of the callus during subsequent culture.

only 1.20  $\mu\text{M}$  and 2.40  $\mu\text{M}$  IBA yielded shoot numbers and regeneration rates that were not significantly different (10.93% and 15.52%). To improve the shoot numbers further, the medium was supplemented with 6-BA. The greatest proliferation occurred in MS medium containing 6.00  $\mu\text{M}$  6-BA and 1.20  $\mu\text{M}$  IBA (Fig. 1 b2), which was the most effective combination to stimulate differentiation. The number of available shoots per explant peaked at 7.50, and 51.22% was the maximum regeneration rate. We found that the higher concentration IBA (2.4  $\mu\text{M}$ ) plus the 6-BA didn't show obvious advantages, and the highest regeneration percentage among the three treatments was 23.02%. As the concentration of 6-BA increased, there was a decrease in the shoot regeneration rate, with a distinct turning point between 1.20  $\mu\text{M}$  and 2.40  $\mu\text{M}$  IBA. When the IBA was 1.20  $\mu\text{M}$ , the regeneration percentage rose with the concentration of 6-BA, but as 6-BA reached 6.00  $\mu\text{M}$  it went down from 51.22% to 30.53%. At 2.4  $\mu\text{M}$  IBA, regeneration percentage decreased from 3  $\mu\text{M}$  6-BA (23.02%) to 12  $\mu\text{M}$  6-BA (10.98%). Additionally, when the concentration of 6-BA was higher than 12.00  $\mu\text{M}$ , no adventitious buds were obviously vitrified in this study.

### 3.3 The effect of GA<sub>3</sub> on shoot elongation

In this context, we studied the effect of GA<sub>3</sub> on *Z. insignis* shoot elongation, in MS medium supplemented with 3.50  $\mu\text{M}$  6-BA, 1.20 IBA, and various concentrations of GA<sub>3</sub> (0.00, 1.20, 2.40, and 4.80  $\mu\text{M}$ ). The greatest regeneration rate was observed in regeneration medium Number 2 (Tab. 3), which contained 1.20  $\mu\text{M}$  GA<sub>3</sub>, with an average of 5.47 effective shoots per explant, with the greatest elongation at 3.1 cm (Fig. 1 C). As GA<sub>3</sub> concentration increased, the opposite of the desired effect was observed: the average regeneration rate decreased to 21.58%, with an average of 1.91 shoots produced. Overall, the number of effective buds was greater in this treatment than in the PGR combination containing no GA<sub>3</sub> (Number 1).

### 3.4 The effect of NAA on root induction

Regenerated shoots (2–4 cm in length) were transferred to MS medium with 0.03% acticarbon for 2 weeks to absorb endogenous plant hormones. We then transferred

**Table 2.** The effects of 6-BA and IBA concentration on shoot proliferation of *Z. insignis*

Number	PGRs ( $\mu\text{M}$ )		Regeneration percentage (%)	Shoot number
	6-BA	IBA		
1	0.00	0.00	9.03 $\pm$ 0.20d	2.22 $\pm$ 0.41e
2	0.00	1.20	10.93 $\pm$ 0.25b	2.87 $\pm$ 0.90e
3	0.00	2.40	15.52 $\pm$ 0.09cd	4.20 $\pm$ 0.52d
4	3.00	1.20	18.94 $\pm$ 0.24c	5.60 $\pm$ 2.44c
5	6.00	1.20	51.22 $\pm$ 0.38a	7.50 $\pm$ 0.50a
6	12.00	1.20	30.53 $\pm$ 0.18b	4.80 $\pm$ 2.57cd
7	3.00	2.40	23.02 $\pm$ 1.09bc	6.20 $\pm$ 1.93b
8	6.00	2.40	11.87 $\pm$ 0.98d	6.94 $\pm$ 2.22ab
9	12.00	2.40	10.98 $\pm$ 0.33d	4.35 $\pm$ 3.17d

Each value represents the mean  $\pm$  standard error (SE) of three replicates, each with 10 explants. Means followed by the same letter in the same column are not significantly different from each other at  $P \leq 0.05$  level, according to Duncan's multiple range test.

**Table 3.** The effects of GA<sub>3</sub> on shoot elongation of *Z. insignis*

Number	GA <sub>3</sub> / $\mu\text{M}$	Regeneration percentage (%)	Number of effective shoots per explant
1	0.00	30.33 $\pm$ 1.22b	2.55 $\pm$ 0.79bc
2	1.20	50.26 $\pm$ 0.98a	5.47 $\pm$ 1.11a
3	2.40	29.08 $\pm$ 1.30b	3.20 $\pm$ 1.28b
4	4.80	21.58 $\pm$ 1.02c	1.91 $\pm$ 1.56c

Each value represents the mean  $\pm$  standard error (SE) of three replicates, each with 10 explants. All the media contained 3.5  $\mu\text{M}$  6-BA, 1.2 IBA. Means followed by the same letter in the same column are not significantly different from each other at  $P \leq 0.05$  level, according to Duncan's multiple range test.

the shoots to MS medium containing 2.0% sucrose and supplemented with different concentrations of NAA (some without hormone) to observe root growth. Functional roots became evident on the shoots after 7 days of culture in all root induction media. The medium containing 1.86  $\mu\text{M}$  NAA had the greatest effect, inducing the highest rooting rate (89.23%; Figure 1 D). But the increase in NAA did not promote the rooting rate: there was a downward trend of 89.23% to 50.33%, and the roots were more slender. It is clear that 1.86  $\mu\text{M}$  NAA had a significant effect on the rooting rate, especially the formation of adventitious roots. However, roots induced on MS medium containing no PGR performed slightly differently compared to those containing 1.86  $\mu\text{M}$  NAA. The rooting rate was 85.53%, but with an average of only 1.55 roots, and the roots were thick. Besides, it appeared that yellowing and shedding from the leaves in the subsequent of plant growth.

## 4 Discussion

It is known that 2,4-D is beneficial for callus induction and shoot regeneration. For example, barley seedlings have been shown to achieve callus induction only when grown in the presence of high concentrations of 2,4-D [26]. In our study, 2,4-D also produced a significant callus response, but at higher concentrations of 2,4-D (0.90  $\mu\text{M}$ ) there was a sharp slump in callus induction rate. This result is consistent with those found in a study on *Gossypium hirsutum* [27] and two studies on *Capsella bursa-pastoris* [28, 29]. It may be that 2,4-D is a powerful mutagen that can induce and inhibit the generation of somatic embryos, leading to cell variation during callus induction, thus affecting regeneration frequency [30, 31]. 6-BA also plays an important role in callus induction, but there was a downward trend in the callus induction rate from 11.00 to 16.50  $\mu\text{M}$ . This finding is consistent with other reports [32].

It has been shown that 6-BA promotes bud and shoot differentiation at high concentrations. 6-BA was beneficial to cell division and affected organ differentiation, as

reported by Chen and Gao [33], who found that the mixed components of 6-BA and auxins could increase the rate of induced buds in *Plumbago auriculata*. Higher 6-BA concentrations strongly inhibited shoot elongation, leading to the production of few or no large shoots. This finding is consistent with the cotyledonary explants of *Brassica napus* [34]. It may be that the plants have reached the specific balance between internal and external hormones and the presence of hormone receptors in a saturated state [35]. Leshem et al. [36], Miedema [37], and Pasqualetto et al. [38] all found that high levels of cytokinin could produce hyperhydricity in in vitro-cultured melon, *Beta vulgaris*, and apple respectively. However, no adventitious buds were obviously vitrified in this study. The specific reasons for this problem are worth further research.

$\text{GA}_3$  plays an important role in shoot elongation: it has been shown to enhance shoot elongation through stem internode extension [39]. Deore and Johnson [40] showed that induced shoot buds of *Jatropha curcas* were multiplied and elongated in the MS medium supplemented with 6-BA (4.44  $\mu\text{M}$ ), kinetin (Kn) (2.33  $\mu\text{M}$ ), IAA (1.43  $\mu\text{M}$ ), and  $\text{GA}_3$  (0.72  $\mu\text{M}$ ). Ying Liu et al. [41] also indicated that  $\text{GA}_3$  plays an important role in promoting elongation in regenerated *Jatropha curcas* buds irrespective of the regeneration methods. Other studies have confirmed this finding. *Xanthium* plants treated with  $\text{GA}_3$  have been shown to have more than double the growth rates of controls [42]. Adventitious buds of *Pinus* experienced inhibition of growth as the concentration of  $\text{GA}_3$  reached 2  $\text{mg L}^{-1}$  [43]. In our study,  $\text{GA}_3$  shortened the time of bud elongation and there was normality found in the growth of buds in the late stage.

Roots were also induced on MS medium containing no PGR. This may have resulted from the presence of endogenous auxin in *in vitro* shoots [44]. It seems that the addition of NAA can promote the formation of adventitious roots, and the observed root thickness increase is similar that seen in *Neolamarckia cadamba* [45]. But NAA also has a saturation point for its promotion. Similar observations

**Table 4.** The effects of various concentrations of NAA on root induction in *Z. insignis*

Number	NAA/ $\mu\text{M}$	Rooting rate (%)	Root number	Root status
1	0.00	85.53 $\pm$ 0.12a	1.55 $\pm$ 0.74c	Thick
2	1.86	89.23 $\pm$ 0.15a	5.13 $\pm$ 0.28a	Thick, a few adventitious roots
3	5.58	70.25 $\pm$ 0.87b	3.28 $\pm$ 0.89b	Thick
4	16.74	50.33 $\pm$ 0.44c	1.25 $\pm$ 0.57c	Slender

Each value represents the mean  $\pm$  standard error (SE) of three replicates, each with 10 explants. Means followed by the same letter in the same column are not significantly different from each other at  $P \leq 0.05$  level, according to Duncan's multiple range test.

have been reported in *Jasminum nudiflorum* [46]. It is necessary to study the optimum concentration for rooting. Besides, the addition of NAA also promotes the growth of adventitious roots to some extent. This may be due to an appropriate concentration of auxin acting on cells, which is beneficial for binding with ATP enzyme on the plasma membrane. This acidifies the cell wall environment, and some unstable hydrogen bonds are easy to break, so that the molecular structure of cell wall polysaccharides is intertwined. The cell wall tends to relax, making the cells that form the adventitious roots easily broken through [47].

## 5 Conclusion

In this study, we established a high-frequency plant regeneration system for *Z. insignis* using leaf cultures. We achieved the highest induction frequency (62.23%) and the highest number of differentiated shoots per explant (7.50). When the plantlets were 5-7 cm in height with 4-6 leaves, they were transplanted into the field for continued growth and development with a survival rate of 56.67%. This leaf-based regeneration technique is an efficient and rapid method for the large-scale production of *Z. insignis* plants over a relatively short period at a high regeneration rate, which we expect will contribute to the propagation of commercial cultivars, benefit germplasm preservation and propagation, and promote the rapid propagation of seedlings for genetic engineering research.

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**Conflict of interest:** Authors state no conflict of interest

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