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Thidiazuron induced high frequency shoot organogenesis in callus from *Kigelia pinnata* L.

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Abstract. An efficient regeneration system was developed for *Kigelia pinnata* L., a multipurpose tree belonging to the family Bignoniaceae. The nodal segments were cultured in vitro, and the optimum concentrations of plant growth regulators for callus induction were determined. The friable organogenic calli were derived from the basal cut end of the nodal segments. The highest yield of morphogenic callus (100%) was observed when nodal segments were cultured on Murashige and Skoog (MS) medium supplemented with 3 µM 2,4 dichlorophenoxyacetic acid (2, 4-D). The morphogenic callus maintained high regeneration during the first four subcultures in the callus induction medium. The maximum shoots (28/culture) were regenerated at the highest frequency of 100% when 3 µM thidiazuron (N-phenyl N' 1,2,3-thidiazol-5-yl urea) (TDZ) and 0.5 µM naphthaleneacetic acid (NAA) were added to MS medium. The emergence of multiple shoots from the calli was histologically documented. The regenerated shoots showed maximum rooting on ½ MS medium containing 4 µM indole-3- butyric acid (IBA). The effect of vesicular arbuscular mycorrhizae (VAM) association in averting the transplantation shock was tested and proved to be highly beneficial, giving a 100% survival rate after 60 d of transplantation. This efficient plant regeneration system provides a foundation for generating transgenic plants of this multipurpose tree.

Keywords: *Kigelia pinnata* L.; Medicinal plant; Plant regeneration; Thidiazuron; Tissue culture; Vesicular arbuscular mycorrhizae.

Abbreviations: 2, 4-D, 2,4 dichlorophenoxyacetic acid; **IBA**, Indole-3- butyric acid; **NAA**, Naphthaleneacetic acid; **TDZ**, thidiazuron (N-phenyl N' 1,2,3-thidiazol-5-yl urea); **VAM**, vesicular arbuscular mycorrhizae.

Introduction

Forest species predominate in temperate and equatorial zones, and the wood produced by the trees is the most abundant material on the earth's surface (Gammie, 1981). Wood provides fuel for most of the population of the world, particularly in developing countries, and is a leading industrial raw material. Despite its economic importance, the production of wood is threatened by population growth, desertification, industrial development, and attack by numerous parasites. The classical conservation techniques such as crossing, sexual and somatic hybridization, and breeding give a genetic blind mixture. These techniques are limited by the sterility of the descents, the genetic barrier between species, and the long life cycle of certain trees (Sederoff, 1995). Plant tissue culture offers many unconventional techniques for plant improvement. While tissue culture technology has been developed for the mass propagation of several fruit tree species, several other tree species are lagging behind due to a recalcitrant nature to in vitro techniques.

Kigelia pinnata, a fast growing, multipurpose tree used for ornamental and roadside planting, belongs to the family Bignoniaceae. Several parts of the plant are employed for medicinal purposes by certain aboriginal people. Traditional healers in India have used various parts of this plant to treat a wide range of skin ailments, from relatively mild complaints, such as fungal infections, boils, and psoriasis, to the more serious diseases like leprosy, syphilis, and skin cancer. Other medicinal applications include the treatment of dysentery, ringworm, tapeworm, post-partum haemorrhaging, malaria, diabetes, pneumonia, and toothache. An antimalarial compound known as lapachol has been extracted from the root of *K. pinnata* (Binutu et al., 1996). Another compound obtained from the wood, quinone, shows antimalarial activity against drug resistant strains of *Plasmodium falciparum* superior to chloroquine and quinine (Carvalho et al., 1988). Conventionally, *K. pinnata* reproduces via viable seeds, but the low percentage of seed viability limits its natural propagation. Hence alternative methods like in vitro techniques could be used to propagate this plant and thereby multiply elite genotypes. Micropropagation has many advantages over conventional propagation of fruit trees (Stushnoff and Fear, 1985) and is important for the regeneration following transformation (Ainsley et al., 2000) and cryopreservation (Channuntapipat et al., 2000). Thidiazuron (TDZ), a urea-derived cytokinin, is a potent cytokinin for woody plant tissue culture (Huetteman and

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Preece, 1993) and is extensively used for the induction of shoot regeneration in several plant species (Li et al., 2000; Mohan and Krishnamurthy, 2002; Liu et al., 2003). The present study was carried out to standardize an efficient regeneration system for *K. pinnata* L. using thidiazuron.

Materials and Methods

Plant Material for Callus Initiation

Callus cultures of *K. pinnata* were initiated from nodal segments of healthy disease free plants growing near our college campus. The nodal segments were washed in 5% Teepol solution for 10-15 min followed by disinfecting in 0.1% mercuric chloride ($HgCl_2$) solution for 5 min in sterilized autoclaved bottles and finally washed three times with sterile-distilled water. Since the use of sodium hypochlorite and bromine water did not prevent contamination, mercuric chloride was used as sterilizing agent throughout the experiment. Utmost care was taken while using mercuric chloride, and autoclaved hand gloves were employed while sterilizing the explant. The surface sterilized nodal segments were cultured on MS (Murashige and Skoog, 1962) medium supplemented with various concentrations (1-7 μM) of 2, 4-D (Sigma-Aldrich, U.S.A.) in a test for callus induction. Callus was subcultured onto fresh medium (MS + 3 μM 2, 4-D) every 45 d.

Induction and Development of Multiple Shoots

For multiple shoot induction, calli (about 8 mm × 8 mm in size) were transferred to MS medium supplemented with various concentrations (0.5 - 9 μM) of TDZ (Sigma-Aldrich, USA) alone or in combination with NAA (Sigma-Aldrich, USA) (0.5 and 1 μM). The % cultures responding, mean shoot length, and the number of shoots per tube were calculated 45 d after callus culture.

Rooting and Transplantation

Shoot clusters with a shoot 1.5 cm or longer were transferred to ½ MS solid medium supplemented with IBA (Sigma-Aldrich, USA). The rooted plants were taken out of the tubes, and the agar was gently removed under running tap water. The shoots were transferred to glass bottles containing a mixture of autoclaved vermiculate and sand (2:1) moistened with MS basal liquid medium and were grown at 25±2°C under a 16-h photoperiod. This is referred to in the Results and Discussion as primary transplantation. After four weeks the individual plantlets were transferred to plastic pots (25 cm diameter) containing potting mixture and acclimatized under greenhouse conditions for 60 d. Four different potting mixtures were used for acclimatization. They were autoclaved garden soil; autoclaved garden soil and VAM; autoclaved garden soil and soil rite (1:2); autoclaved garden soil, soil rite (1:2) and VAM. These plantlets were transferred to greenhouse with 85% humidity and a temperature of 28±20°C. This is referred to in the Results and Discussion as secondary transplantation.

VAM Inoculation

Spore of *Glomus fasciculatum* and *Glomus macrocarpum* were isolated from the soil obtained from the rhizosphere *of Leucaena leucocephala* growing in the college campus. Sudan grass (*Sorghum halepense*) grown in flat earthen pots was inoculated with these spores. Both species of *Glomus* were multiplied and maintained independently. The rhizosphere soil of the 6-month-old pot culture of Sudan grass was used as inoculum of these VAM fungal species. Each gram of this inoculum consisted of 30-40 spores plus hyphae, arbuscules, and vesicles of fungus, identified using the standard staining techniques of Phillips and Hayman (1970), plus root segments of Sudan grass and soil particles. For establishment of mycorrhizal association with plantlets of *K. pinnata*, about 1 g of inoculum of either *G. fasciculatum* or *G. macrocarpum* was applied below the roots, during secondary transplantation (Puthur et al., 1998; Sharmila et al., 2000)

Culture Conditions

All the media were fortified with 20 g/l sucrose (Sigma-Aldrich, USA) and gelled with 0.8% agar (Sigma-Aldrich, USA), and the pH was adjusted to 5.8 after adding the growth regulators. The media were steam sterilized in an autoclave under 1.5 kg/cm^2 and 121°C for 15 min. All the cultures were grown at 25±2°C under a 16-h photoperiod supplied by two Philips TL 40W fluorescent tubes. At least twenty-four cultures were raised for each treatment, and all experiments were performed three times. Analysis of variance and Duncan's multiple range test were used for comparison among treatment means.

Histological Studies

The calli with multiple shoots were fixed for 24 h in FAA (formalin + glacial acetic acid + 70% ethanol in a 1:1:18 ratio by volume). Following dehydration in tertiary butanol the material was infiltrated and embedded in paraffin wax (E-Merck) (Johansen, 1940). The paraffin blocks were mounted on wooden stubs, and 8 mm thick sections were cut using a Spencer microtome (USA) attached with a steel knife. The sections were mounted on microslides, dewaxed, and double stained with safranin (1%) and astra blue (1%) (Johansen, 1940).

Results and Discussion

Callus Induction

When nodal segments were cultured on callus induction medium (i.e. 2, 4-D) callus growth was initiated from the basal cut end of the nodal segments in about four d, and rapid growth followed for the majority of the explants. Pale yellow friable morphogenic calli were well developed 45 d after nodal segments culture (Figure 1a). The rate of callus induction and the percentage of cultures responding varied depending up on the 2, 4-D concentrations applied. 2, 4-D in the range of 1-7 μM alone produced pale yellow calli for each concentration tested, but based on

the rate of callus induction and the percentage of cultures responding, 3 µM 2, 4-D was optimum. Above or below this concentration the rate of callus induction or the percentage of cultures responding decreased (data not shown). Hence further experiments were carried out with 3 µM 2, 4-D.

For detailed experiments, maintaining callus with a high regeneration ability for a prolonged period is essential. Moreover, most of the transformation procedures have relied on inducing long term morphogenic potential after repeated subculture, and this is critical for successful transformation. With this goal in mind the callus was multiplied on 3 µM 2, 4-D and maintained a high regeneration rate from 1-8 subcultures on this medium. The multiplication rate was high with an average 16.3 fold in the first four subcultures and significantly higher than that in subsequent subcultures (Table 1). The multiplication rate was maintained at approximately an 11 fold level for subsequent subcultures. No significant differences in regeneration rates or the number of plants generated per callus piece appeared among the first four subcultures. From the fifth subculture onwards regeneration rates and the number of plants generated per callus piece showed significant differences. Although the callus maintains its regeneration capacity for a longer period, the available literature suggests that prolonged subculturing may lead to a higher frequency of mutants especially in higher concentrations of 2, 4-D (Choi et al., 2001).

Table 1. Effect of repeated subculturing on rates of callus multiplication and plant regeneration from morphogenic callus. Medium for callus multiplication - MS + 2, 4-D (3 µM), Medium for callus regeneration - MS + TDZ (3 µM) + NAA (0.5 µM). Culture period: 45 d. The values represent the mean (±S. E.) of three independent experiments. At least 24 cultures were raised for each experiment. Means within a column followed by the same letter are not significantly different by Duncans's multiple range test (P> 0.05).

Subculture	Rate of callus multiplication (fold)	Regeneration rate (%)	Number of shoots /callus piece
1	16.5a	100a	28±0.4a
2	16.3a	98a	27±0.1a
3	16.3a	96a	27±0.6a
4	16.1a	96a	26±0.2a
5	11.1b	86b	22±0.7b
6	10.8b	84b	20±0.4b
7	11.0b	84b	19±0.5b
8	11.1b	80b	19±0.1b

Table 2. Effect of different concentrations of TDZ and NAA on shoot regeneration from morphogenic callus of *Kigelia pinnata* after 45 d of culture. The values represent the mean (±S. E.) of three independent experiments. At least 24 cultures were raised for each experiment. Means within a column followed by the same letter are not significantly different by Duncans's multiple range test (P> 0.05).

Growth regulator (µM)		% Explants producing shoots	No. of shoots per callus explant	Average shoot length (cm)
NAA	TDZ			
0	0	0	0	0
	0.5	85b	11±0.3a	0.9±0.1a
	1.0	87b	14±0.4a	1.3±0.2a
	3.0	89b	17±0.2a	1.5±0.1b
	5.0	83b	13±0.5a	1.1±0.2a
	7.0	81b	11±0.2a	1.2±0.1a
	9.0	78b	12±0.3a	1.0±0.1a
0.5	0	0	0	0
	0.5	100d	21±0.3b	1.4±0.1b
	1.0	100d	23±0.2b	1.6±0.2b
	3.0	100d	28±0.4c	2.1±0.1c
	5.0	100d	18±0.1b	1.9±0.1c
	7.0	93c	14±0.1a	1.5±0.3b
	9.0	91c	17±0.3a	1.4±0.1b
1.0	0	0	0	0
	0.5	77a	13±0.4a	0.8±0.3a
	1.0	71a	12±0.2a	0.7±0.2a
	3.0	70a	15±0.1a	0.9±0.3a
	5.0	72a	13±0.1a	0.9±0.2a
	7.0	70a	12±0.3a	0.8±0.1a
	9.0	68a	14±0.2a	0.7±0.2a

Shoot Regeneration

The morphogenic callus generated from the nodal segments was transferred to various concentrations of TDZ alone or in combination with NAA (Table 2). Shoot initiation was observed one week after callus culture. Often several shoots developed from one callus mass (Figure 1b). The optimum response in terms of percentage of explants producing shoots and the highest number of shoot buds per explant was recorded on MS medium supplemented with TDZ (3 μM) and NAA (0.5 μM). On this medium 100% cultures responded with an average 28 shoots per culture. The regenerated shoots attained a height of about 2 cm in about 45 d of callus culture. TDZ, a synthetic phenylurea, is considered to be one of the most active cytokinins for shoot induction in plant tissue culture (Huetteman and Preece, 1993; Murthy et al., 1998). TDZ-induced shoot regeneration from different explants of many recalcitrant species as well as from medicinal plants has been reported (Bhagwat et al., 1996; Seneviratne and Flagmann, 1996; Murthy et al., 1998; Pelah et al., 2002; Schween and Schwenkel, 2002; Liu et al., 2003; Mithila et al., 2003; Thomas, 2003). Several reports suggest that TDZ results in shoot regeneration better than other cytokinins (Barna and Wakhlu, 1995; Thomas, 2003). TDZ-induced morphogenesis probably depends on the levels of endogenous growth regulators, and TDZ modulates the endogenous auxin levels (Murthy et al., 1995; Hutchinson and Saxena, 1996). In the present study TDZ in combination with NAA gave better results than TDZ alone. Hence, the synergistic combination of auxin and cytokinin promoted shoot regeneration. The promoting effect of auxin and cytokinin combinations on organogenic differentiation has been well established in several systems (Lisowska and Wysonkinska, 2000; Pereira et al., 2000; Pretto and Santarém, 2000; Xie and Hong, 2001; Koroch et al., 2002).

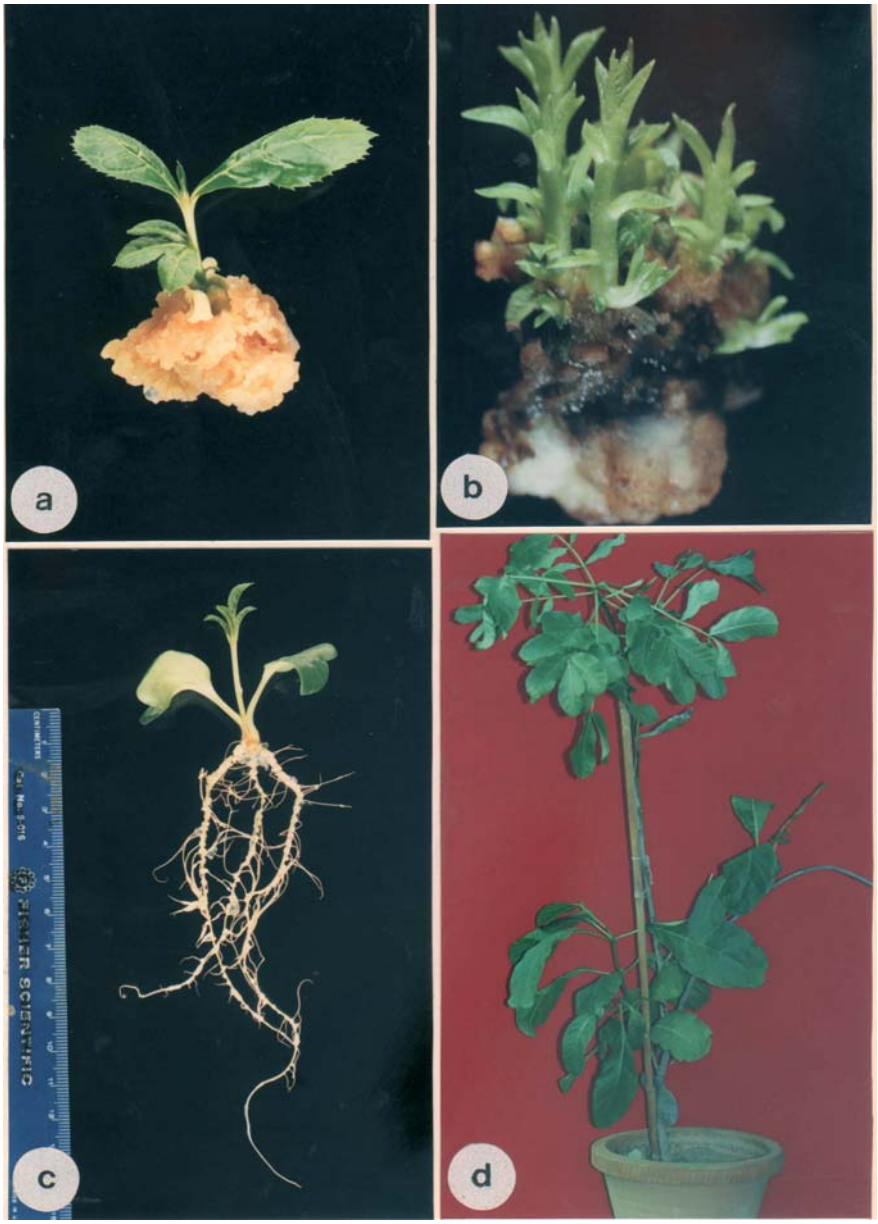

Figure 1. Different stages in the shoot organogenesis and complete plant regeneration in *Kigelia pinnata*. a, A nodal segment culture taken out from MS medium supplemented with 2, 4-D (3 μM) 45 d after culture. Luxuriantly growing morphogenic friable calli formed at the basal cut end of the nodal segment (×1.2); b, High frequency in vitro callus regeneration on MS medium supplemented with TDZ (3 μM) and NAA (0.5 μM). Photographs were taken 45 d after culture. Well developed, healthy shoots emerged from the calli (×3.2); c, Rooting of regenerated shoots ½ MS medium with 4 μM IBA for 45 d. Note the long healthy roots with root hairs (×1.8); d, A one-year-old acclimatized plant growing in the green house. The plant developed two new branches (×0.1).

Rooting

Root differentiation was observed after about 1 week of culture. The optimal medium for rooting contained MS + IBA (4 μM), on which a maximum of 100% shoots rooted with an average 4.4 roots per shoot and an average root length of 11.3 cm (Table 3; Figure 1c). Complete plants were obtained 45 d after the regenerated plantlets were transferred to this medium. IBA is the most commonly used auxin for root induction. The superior role of IBA in root induction has been reported in several plants (Fracaro and Echeverrigaray, 2001; Abrie and Van Staden, 2001; Thomas, 2003).

Transplantation and Acclimatization of Regenerated Shoots

Primary transplantation was carried out in order to increase the survival percentage. From earlier trials we found that survival of transplants improved by 20-30% if transplantation was carried out in two steps. In the secondary transplantation, plantlets were transplanted to four different potting mixtures (Table 4). Of these, the potting mixture containing autoclaved garden soil, soil rite, and VAM showed maximum response (100%), in terms of survival percentage after 60 d of transplantation. The medium containing autoclaved garden soil, and VAM was also found suitable for transplantation, with 95% survival (Table 4). After 30 d the emergence of new leaves was observed. The better survival rate of VAM inoculated plants may be due to the beneficial physiological features imparted to the plant by the symbiotically associated VAM fungi. The beneficial physiological features of these plants include, development of a superior root system, increase in water conducting capacity, increase in phosphorous uptake, enhanced nitrogen levels, increased photosynthetic efficiency, aversion of possible attack by soil born pathogens, enhanced uptake of micro and immobile nutrients, and alleviated environmental stress (Puthur et al., 1998; Sharmila et al., 2000). Plants transferred to the greenhouse have been established well in the soil (Figure 1d).

Histology

The histological analysis of the regenerating calli clearly showed that the shoot buds had emerged from the periph-

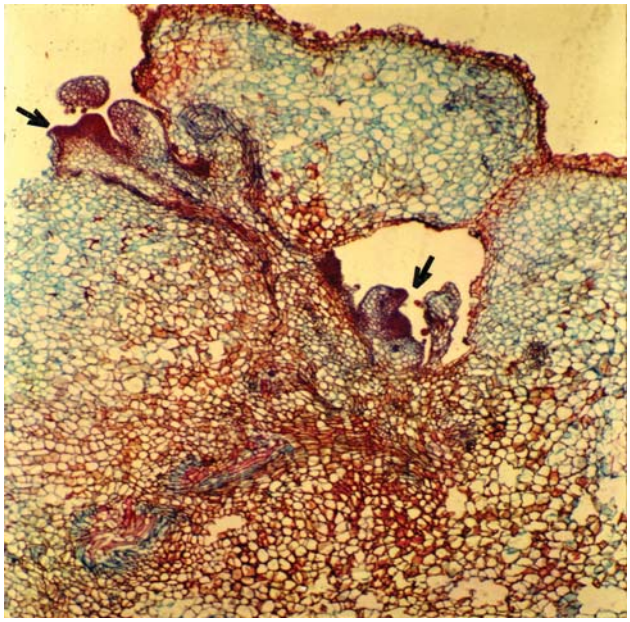

Figure 2. Histological analysis of the regenerating callus. A section of a 15-d-old callus showing initiation of shoot (arrow marked) from the peripheral meristematic tissue (×40).

Table 3. Effect of half strength MS medium supplemented with different concentrations of IBA on rooting of the shoots in *Kigelia pinnata* after 45 d in culture. The values represent the mean (±S.E.) of three independent experiments. At least 24 cultures were raised for each experiment. Means within a column followed by the same letter are not significantly different by Duncans's multiple range test (P>0.05).

IBA treatment (μM)	% Shoots rooted	No. of roots per shoot	Mean root length (cm)	Time required for root initiation (d)
0.0	0	0	0	0
1	47a	2.2±0.3a	6.8±0.3a	9±1.9c
2	62b	2.3±0.4a	8.9±0.8b	8±2.1c
3	79c	2.9±0.2a	9.8±0.2b	7±2.2b
4	100d	4.4±0.2c	11.3±0.5c	7±1.8b
5	88c	3.2±0.6b	9.7±1.2b	5±1.4a

Table 4. The survival percentage of micropropagated plantlets of *Kigelia pinnata* after secondary transplantation to various potting mixtures. Data collected 30 d and 60 d after secondary transplantation.

Potting mixtures	% Survival after 30 d	% Survival after 60 d
Autoclaved garden soil	66a	61a
Autoclaved garden soil + VAM	98b	95b
Autocalved garden soil + soilrite (1:2)	68a	59a
Autoclaved garden soil + soilrite (1:2) + VAM	100c	100c

eral nodular structures, which consisted of closely arranged and highly cytoplasmic cells. In some shoots the vascular supply was found to be continuous with the vasculature of the callus (Figure 2).

Conclusion

In conclusion this paper describes a procedure for micropropagation of *K. pinnata* via callus regeneration and a successful adaptation of plants to field conditions. To the best of our knowledge this is the first report on the micropropagation of *K. pinnata*. The present study showed that inducing high frequency organogenesis in nodal segments derived calli by using thidiazuron and NAA is possible. This efficient plant regeneration system can play an important role in generating transgenic plants of this multipurpose tree.

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Thidiazuron 誘導紫葳科 *Kigelia pinnata* L. 癒傷組織之高頻率的莖器官形成

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Kigelia pinnata L. 乃紫葳科之一多用途樹種，我們對此發展出一套有效的再生系統。我們在試管內培養節 (node) 之部份，找出誘導癒傷組織形成之最適植物生長調控素。脆的、具器官形成能力之癒傷組織乃得自節之底切處。當所用之MS培養基輔以 3 μM 2,4-D 的時候具形態潛力的癒傷組織之產率可達100%。此癒傷組織在所用之癒傷誘導基質之前四次繼代培養維持高的再生能力。當MS培養基輔以 3μM thidiazuron（N-phenyl N' 1,2,3-thidiazol-5-yl urea）（TDZ）及 0.5 μM NAA 時，可 100% 再生最大數目之莖 (28/culture)，從癒傷組織之多莖形成經組織學方法確認。再生後之莖在 ½ MS 培養基輔以 4 μM indole-3-butyric acid 時可達最大發根狀態。我們檢驗 vesicular arbuscular mycorrhizae 之附著是否有益減緩移植所導致的衝擊，結果發現非常有幫助：移植 60 天後仍有 100% 之存活率。本文所述之高效的再生系統提供生產此多用途樹種之轉殖植物的基礎。

關鍵詞：*Kigelia pinnata* L.；藥用植物；植物再生；Thidiazuron；組織培養；Vesicular arbuscular mycorrhizae。