International Journal of Agricultural Technology 2026 Vol. 22(3):1319-1330
DOI: https://doi.org/10.63369/ijat.2026.22.3.1319-1330
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Study on the regeneration of sausage tree (*Kigelia africana*) by tissue culture

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Poeaim, S. and Poeaim, A. (2026). Study on regeneration of sausage tree (*Kigelia africana*) by tissue culture. International Journal of Agricultural Technology 22(3):1319-1330.

Abstract This study investigated the regeneration of sausage tree (*Kigelia africana*) using tissue culture techniques, focusing on callus induction, shoot regeneration from seeds, and root induction from shoot explants. Seeds were cultured on solid MS medium supplemented with various concentrations of BA (0.5, 1, 2, 3, and 5 mg L^{-1}) for 10 weeks. Callus formation was observed at all BA concentrations, with the largest mean callus volume (5214.17 mm^3) and highest induction rate (80%) achieved at 5 mg L^{-1}. Shoot regeneration occurred across all BA treatments, with the highest number of shoots (45 shoots; 86.54%) obtained on medium containing 1 mg L^{-1} BA. Root induction was studied using shoot segments derived from seed-induced shoots cultured on MS medium supplemented with IAA, NAA, or IBA for 4 weeks. IAA at 0.5 mg L^{-1} induced roots in 33.33% of explants with an average root length of 6.68 mm. NAA at 2 mg L^{-1} promoted the highest root induction (70.83%), though roots were shorter, while 1 mg L^{-1} NAA produced fewer roots but with greater length (7.00 mm). IBA at 3 mg L^{-1} induced rooting in 33.33% of explants with an average root length of 3.78 mm and also stimulated callus formation, with the largest callus volume observed at 2 mg L^{-1}. These results demonstrate that BA is effective for callus and shoot induction, whereas IAA, NAA, and IBA promote root formation, with distinct responses at different concentrations. The optimized protocols provide a foundation for *in vitro* propagation, mass multiplication, and conservation of *K. africana*, with potential applications in biotechnology and secondary metabolite production.

Keywords: Plant regeneration, Root induction, Shoot induction, Sausage tree

Introduction

Kigelia africana (African sausage tree or sausage tree) is a distinctive plant known for its elongated, sausage-like fruits, which can reach up to 1.2 meters in length and weigh several kilograms. The tree has a smooth, gray trunk that develops fissures with age, and its flowers are reddish-orange and rely on birds and bats for pollination (Imran *et al*., 2021). The characteristics of the trunk, leaves, fruits, flowers, and fruit pulp are shown in Figure 1. This species is widely distributed in forests across South Africa and has diverse applications in

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medicine, food, materials, and ornamental use. Phytochemical studies indicate that *K. africana* contains multiple bioactive compounds, including iridoids, flavonoids, naphthoquinones, coumarins, and terpenes. Specific compounds, such as verbascoside, verminoside, pinnatal, and kigelinole, contribute to its antioxidant, antibacterial, antitumor, and anti-inflammatory activities, highlighting its potential for medicinal and cosmetic applications (Olatunji and Olubunmi, 2009; Bello *et al*., 2016; Assanti *et al*., 2022).

In Thailand, *cultivation of K. africana* is limited and mainly occurs in ornamental gardens, such as Nong Nooch Garden in Chonburi Province. Direct propagation from seeds is challenging due to a dormancy period of up to 1 year and the fruit pulp's susceptibility to insect damage, which reduces seed viability. Germination can be enhanced by brief hot-water treatment of seeds or through plant tissue culture techniques (Pandey and Pandey, 2015). Only one study has investigated the effect of fertilizers on the growth of *K. africana* seedlings, namely that of Jayeoba *et al*. (2017), which reported that 2.5 g of poultry manure produced the greatest average height, whereas 7.5 g of cow dung resulted in the highest leaf number and stem diameter. Given its wide-ranging uses and propagation difficulties, the present study aimed to develop a tissue culture protocol for *K. africana* to improve germination rates and to increase plant production within a shorter period.

Figure 1. Characteristics of *Kigelia africana*: (A) whole tree, (B) leaves, (C) fruit, (D) flower and (E) fruit pulp

Materials and methods

Culture medium

All cultures were established on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 30 g L^{-1} sucrose, 2.6 g L^{-1} gellan gum, and the required plant growth regulators (PGRs). The medium pH was adjusted to 5.7 ± 0.1 before autoclaving at 121 °C (15 psi, 15 min). Cultures were incubated in a growth room at 25 ± 2 °C with a 16 h light/8 h dark cycle.

Seed sterilization

The ripe fruits were collected from Bangkok. Before cutting the fruit to separate the seeds from the fruit pulp, the outer surface was washed thoroughly with water, then immersed in 95% ethanol for 1 minute. The seeds were surface-sterilized in sterile distilled water containing 0.2 % mercuric chloride, 2–3 drops of Tween-20, streptomycin, 0.1 % Plant Preservative Mixture (PPM), and Zefozan for 30 minutes. After this treatment, the seeds were transferred to a bottle containing sterile distilled water supplemented with streptomycin, 0.1 % PPM, and 1:1000 (v/v) Zefozan, shaken for 10 minutes, and the procedure was repeated twice. Finally, the seeds were rinsed in sterile distilled water for 5 minutes and air-dried on sterilized paper.

Seed callus induction

Sterilized seeds were placed onto solid MS medium containing 30 g·L^{-1} sucrose and 2.6 g·L^{-1} Phytagel. The plant growth regulator (PGR), 6-benzyladenine (BA) or 2,4-dichlorophenoxyacetic acid (2,4-D), was added at concentrations of 0.5, 1, 2, 3, and 5 mg·L^{-1}. Five replicate bottles were prepared for each concentration, containing four seeds (20 seeds per treatment). Cultures were maintained under room culture conditions, with a 16 h light/8 h dark photoperiod at 25 ± 2 °C. After 12 weeks of culture, callus formation was evaluated: callus size was measured using a vernier caliper, and the percentage of seeds producing callus was calculated.

Shoot induction

Sterilized seeds were placed on solid MS medium containing 30 g L^{-1} sucrose and 2.6 g L^{-1} phytagel supplemented with 6-benzylaminopurine (BA) at 0.5, 1, 2, 3, or 5 mg L^{-1}, pH 5.6–5.8. For each BA concentration, 13 cultures were prepared with four seeds per jar (total = 52 seeds per concentration).

Observations began at week 4 and continued every two weeks (weeks 4, 6, 8, and 10) for a total culture period of 10 weeks. Plant growth was assessed by measuring: shoot height (from the stem base to the shoot tip), leaf length (from leaf base to tip), and root length (from the stem base to the root tip) using a vernier caliper. The number of shoots, leaves, and roots was counted for structures ≥ 1 mm long. The percentage of seedling regeneration was then calculated.

Root induction

Shoots obtained from the plantlets in shoot induction were trimmed into segments 2–3 cm in length and placed on solid MS medium containing 30 g L^{-1} sucrose and 2.6 g L^{-1} Phytagel supplemented with different concentrations of auxins: indole-3-acetic acid (IAA) and naphthaleneacetic acid (NAA) at 0.25, 0.5, 1, 2, and 3 mg L^{-1}, and indole-3-butyric acid (IBA) at 0.5, 1, 2, 3, and 5 mg L^{-1}, pH 5.6–5.8. Cultures were maintained under a 16 h light/8 h dark photoperiod at 25 ± 2 °C for 4 weeks. At the end of the culture period, root length was measured from the cut end of the shoot to the root tip using a vernier caliper, and roots ≥1 mm in length were counted. The percentage of root induction was calculated.

Selected plantlets with well-developed roots from the root-induction stage were carefully removed from the medium, rinsed to eliminate residual agar, and transplanted into sterile potting substrate. The pots were covered with transparent plastic bags to maintain high humidity for 2 weeks and kept under a 16 h light/8 h dark photoperiod at 25 ± 2 °C with moderate watering. After removing the covers, the plantlets were maintained in a nursery for growth.

Results

Effect of the plant growth regulator on callus induction

Seed culture of *K. africana* produced callus on MS solid medium containing BA at all concentrations tested, with a light-green appearance (Figure 2A-E). The highest callus induction (80 %) and the largest mean callus volume (5214.17 mm^3) were obtained with 5 mg L^{-1} BA. At 0.5, 1, 2, and 3 mg L^{-1} BA, mean callus volumes were 2371.99, 4984.11, 4482.24, and 4332.95 mm^3, respectively (Table 1). On MS medium supplemented with 2,4-D, callus developed at all concentrations but appeared brown (Figure 2F-J). The highest induction rate (65 %) and a mean callus volume of 3797.73 mm^3 occurred with 0.5 mg L^{-1} 2,4-D. At 1 and 5 mg L^{-1} 2,4-D, callus induction was identical (50 %), but the mean callus volume at 1 mg L^{-1} (4392.86 mm^3) exceeded that at 5 mg L^{-1} (3106.84 mm^3) (Table 1).

Table 1. The number of seeds, percentage of callus induction, and callus size of *K. africana* cultured for 12 weeks on MS medium containing different concentrations of BA and 2,4-D

PGR type[1]	Concentrations (mg·L⁻¹)	No. of seeds	Callus induction (%)	Callus size (mm³)
BA	0.5	20	7 (35)	2371.99
	1.0	20	15 (75)	4984.11
	2.0	20	14 (70)	4482.24
	3.0	20	12 (60)	4332.95
	5.0	20	16 (80)	5214.17
2,4-D	0.5	20	13 (65)	3797.73
	1.0	20	10 (50)	4392.86
	2.0	20	9 (45)	2204.84
	3.0	20	5 (25)	3515.75
	5.0	20	10 (50)	3106.84

[1] PGR type = type of plant growth regulator

Table 2. Shoot and root growth parameters of *K. africana* seedlings on MS medium with varying BA levels after 10 weeks

BA Conc. (mg L⁻¹)	No. of seedlings	Shoot induction (%)	No. of shoots	Shoot length (mm)	No. of leaves	Leaf length (mm)	No. of roots	Root length (mm)
0.5	29	55.77	84	56.48	76	20.51	21	18.38
1	45	86.54	78	51.22	92	18.68	23	21.71
2	38	73.80	97	49.58	62	20.54	20	29.97
3	42	80.77	116	56.31	78	20.72	29	22.41
5	26	50.00	79	50.08	56	18.23	24	30.68

Note: Each treatment was initiated with 52 seeds

Shoot induction from seeds

The seed culture of *K. africana* on MS medium supplemented with BA at 0.5, 1, 2, 3, and 5 mg L⁻¹ for 10 weeks produced shoots at all concentrations, with 26–45 seeds germinating or forming shoots out of 52 seeds (Table 2). The highest shoot number was obtained on medium containing 1 mg L⁻¹ BA, yielding 45 shoots (86.54%), followed by 3 mg L⁻¹ BA, which produced 42 shoots (80.77%). In week 4, seedlings exhibited two large cotyledons and small shoots and roots. By week 6, leaves had elongated along the stem, numerous small shoots had emerged, and roots formed near the seed base. In week 8, multiple shoots and roots were observed along the stem, showing clear effects of BA on shoot number, root number, shoot length, and leaf production (data for weeks 4, 6, and 8 are not shown). Ten plants exhibited well-developed leaves along the stem, numerous small shoots and roots, and callus formation at the base at 10 weeks (Figure 3A-E). The medium supplemented with 3 mg L⁻¹ BA yielded the highest numbers of

shoots and roots (116 and 29, respectively). The greatest mean shoot length (56.48 mm) was recorded at 0.5 mg L^{-1} BA, while the highest number of leaves (92) was obtained at 1 mg L^{-1} BA (Table 2).

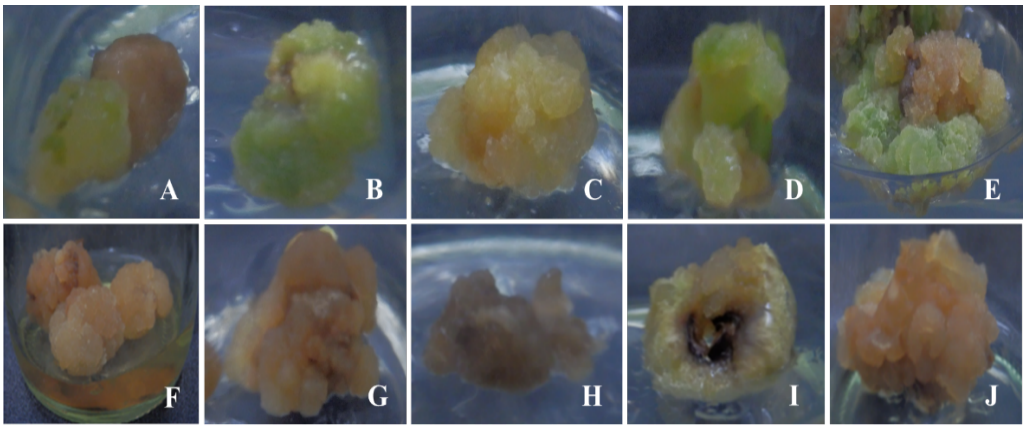

Figure 2. Callus formation of *K. africana* seeds cultured on MS solid medium supplemented with different concentrations of plant growth regulators for 12 weeks: (A–E) BA at 0.5, 1, 2, 3, and 5 mg L^{-1}, (F–J) 2,4-D at 0.5, 1, 2, 3, and 5 mg L^{-1}

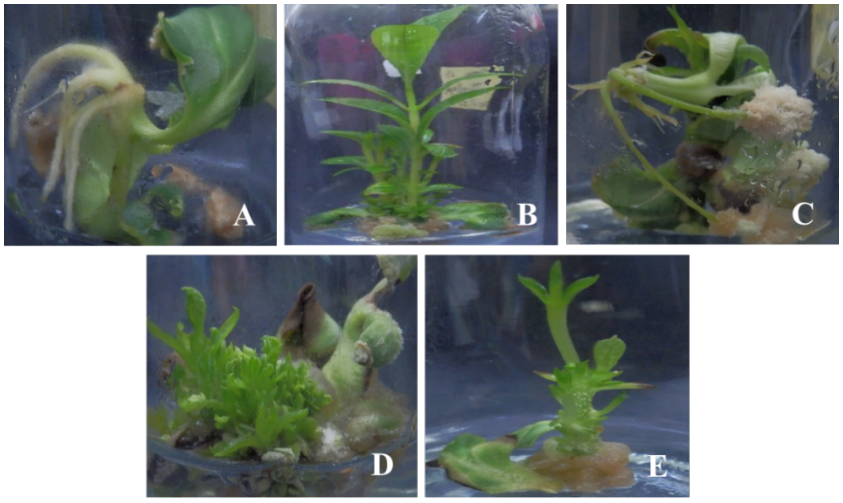

Figure 3. Shoot development of *K. africana* on MS solid medium supplemented with BA at concentrations of (A) 0.5, (B) 1, (C) 2, (D) 3, and (E) 5 mg L^{-1} for 10 weeks

Root induction from shoots

The growth of *K. africana* revealed that when shoot segments derived from seed-induced shoots were cultured on MS solid medium for 4 weeks (Table 3), root formation occurred only in the treatment supplemented with 0.5 mg L^{-1} IAA. At this concentration, 33.33 % of the explants produced roots with a root length of 6.68 mm (Figure 4A–E). In the NAA treatments, the medium containing 2 mg L^{-1} NAA induced the highest root formation (70.83 %), with a mean root length of 3.90 mm. NAA at 0.25 and 1 mg L^{-1} each promoted 16.66 % root induction; however, the 1 mg L^{-1} concentration yielded the greatest mean root length (7.00 mm) and the largest mean callus volume (1867.86 mm^3), whereas 0.25 mg L^{-1} produced a root length of only 0.97 mm (Figure 4A–E). For the IBA treatments, root formation was observed only at 3 mg L^{-1}, where 33.33 % of the explants developed roots with a root length of 3.78 mm. Callus formation also occurred on explants cultured with IBA, producing light-green calli. The largest callus volume (3767.05 mm^3) was recorded at 2 mg L^{-1} IBA (Figure 4K–P). Root induction was successfully achieved on MS medium containing 2 mg L^{-1} NAA for 1 month (Figure 5A). Following acclimatization for 2 weeks under plastic covers (Figure 5B) and 1 month of nursery growth, the selected plantlets developed healthy shoots and roots and showed normal growth in pots (Figure 5C).

Table 3. Root induction, root length, and callus volume of *K. africana* cultured on MS medium supplemented with IAA, NAA, and IBA for 4 weeks

PGR type[1]	Conc. ($mg \cdot L^{-1}$)	No. of shoots	Root induction (%)	Root length (mm)	Mean callus size (mm^3)
IAA	0.25	3	0 (0)	0	665.58
	0.5	3	1 (33.33)	6.68	1500.78
	1.0	3	0 (0)	0	356.50
	2.0	3	0 (0)	0	376.34
	3.0	3	0 (0)	0	485.24
NAA	0.25	7	1 (16.66)	0.97	1049.68
	0.5	7	0 (0)	0	887.93
	1.0	7	1 (16.66)	7.00	1867.86
	2.0	7	5 (70.83)	3.90	872.12
	3.0	7	0 (0)	0	585.58
IBA	0.5	3	0 (0)	0	3690.66
	1.0	3	0 (0)	0	2577.88
	2.0	3	0 (0)	0	3767.05
	3.0	3	1 (33.33)	3.78	3417.04
	5.0	3	0 (0)	0	1719.47

[1] PGR type = type of plant growth regulator

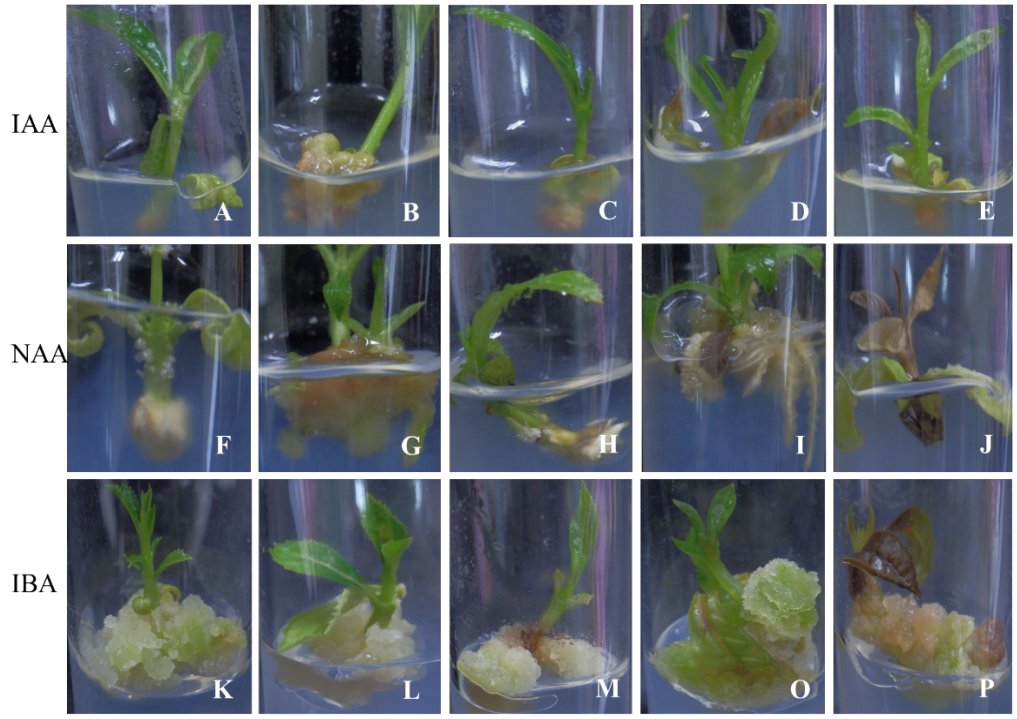

Figure 4. Root induction of *K. africana* cultured on MS solid medium supplemented with different concentrations of plant growth regulators for 4 weeks: (A–E) IAA at 0.25, 0.5, 1, 2, and 3 mg L^{-1}, (F–J) NAA at 0.25, 0.5, 1, 2, and 3 mg L^{-1}, (K–P) IBA at 0.5, 1, 2, 3, and 5 mg L^{-1}

Figure 5. Root induction and acclimatization of *K. Africana*: (A) shoots rooted on MS medium with 2 mg L^{-1} NAA for 1 month, (B) plantlets transferred to pots and covered with plastic bags for 2 weeks, (C) selected plantlets with well-developed shoots and roots showed normal growth after 1 month in pots

Discussion

The effect of plant growth regulators on callus induction was evaluated using two: BA, a cytokinin, and 2,4-D, an auxin. Seed culture of *K. africana* showed the highest callus induction (80 %) and mean callus volume (5214.17 mm^3) on MS medium supplemented with 5 mg L^{-1} BA. On MS medium supplemented with 2,4-D, callus formation occurred at all tested concentrations and exhibited a brown coloration. The highest induction rate (65 %) was observed at 0.5 mg L^{-1} 2,4-D. Browning of callus under 2,4-D supplementation has been reported in many species (Liu *et al.*, 2024). At higher auxin levels, phenolic oxidation and necrosis occur, resulting in unhealthy, brown callus with low regeneration potential.

Comparison of callus induction percentages between BA and 2,4-D treatments showed that 5 mg L^{-1} BA produced the highest callus induction. At BA concentrations of 1, 2, 3, and 5 mg L^{-1}, callus induction was consistently higher than with 2,4-D concentrations. This agrees with Saxena *et al*. (2000), who found that a high cytokinin/low auxin ratio (10 mg L^{-1} Kn with 0.1 mg L^{-1} 2,4-D) effectively promoted callus formation in *Pelargonium graveolens*. However, at 0.5 mg L^{-1}, 2,4-D induced a higher percentage of callus than BA. Similarly, comparison of mean callus volumes revealed that 5 mg L^{-1} BA produced the largest callus size. BA at 1, 2, 3, and 5 mg L^{-1} also yielded larger callus volumes than 2,4-D at the same concentrations, whereas at 0.5 mg L^{-1}, 2,4-D produced a larger mean callus volume than BA.

The shoot segments of *K. africana* formed roots only on MS medium supplemented with 0.5 mg L^{-1} IAA, where 33.33 % of the explants developed roots with an average length of 6.68 mm. Similarly, root formation in the IBA treatments was observed only at 3 mg L^{-1}, where 33.33 % of the explants produced roots with an average length of 3.78 mm. Comparable results were reported by Dennis Thomas and Shankar (2009), who found 100 % rooting in *Sarcostemma brevistigma* shoot segments (1.5–2.0 cm) cultured on MS medium containing 3 µM IBA. Harahap *et al*. (2014) also demonstrated that mangosteen (*Garcinia mangostana* L.) cultured on MS medium containing 3 mg L^{-1} IBA achieved up to 85% rooting with a mean root length of 1.49 cm. Likewise, Nissar *et al*. (2013) reported that 3 mg L^{-1} IBA induced 71 % rooting in *Orthosiphon stamineus*, with a mean root length of 7.20 ± 2.14 cm.

In the case of NAA, the treatments effectively promoted root induction. The concentration of 2 mg L^{-1} produced the highest root formation (70.83 %), although the roots were relatively short, whereas 1 mg L^{-1} resulted in fewer roots but with greater average length (7.00 mm). These findings are consistent with those of Dennis Thomas and Shankar (2009), who observed that 2 µM NAA

induced 71% rooting, with an average of 2.1 roots per shoot segment. Furthermore, Lin *et al*. (2010) reported that low concentrations of NAA (0.05 mg L^{-1}) were most effective for callus induction in *Catalpa bungei*, while higher concentrations produced reduced callus growth that turned brown. In addition, Naomita and Ravishankar (2004) showed that a combination of 2.69 µM NAA with 5.71 µM IAA induced the highest root formation (91.6 %) in *Oroxylum indicum* Vent.

K. africana seeds and shoot segments exhibited distinct in vitro responses to PGRs: BA efficiently promoted callus and shoot formation, whereas IAA, NAA, and IBA favored root induction, with NAA enhancing root number and IBA also stimulating callus development. These findings highlight that careful optimization of PGR type and concentration can improve in vitro propagation of *K. africana*, facilitating mass propagation, conservation, and potential biotechnological applications. The study's strengths lie in its comprehensive assessment of PGR effects on shoot, root, and callus development, while limitations include the relatively short culture period and lack of long-term acclimatization and biochemical characterization, indicating the need for future research on acclimatization, rooting optimization, and secondary metabolite analysis.

Acknowledgements

This work was supported by King Mongkut's Institute of Technology Ladkrabang (KMITL), Grant No. 2562-01-05-15.

Conflicts of interest

The authors declare no conflict of interest.

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(Received: 30 September 2025, Revised: 22 March 2026, Accepted: 7 May 2026)