Emirates Journal of Food and Agriculture. 2015. 27(11): 857-863
doi: 10.9755/ejfa.2015-07-535
http://www.ejfa.me/

REGULAR ARTICLE

Efficient plant regeneration of Malaysian aromatic rice (*Oryza sativa* L.) through somatic embryogenesis

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ABSTRACT

Aromatic rice variety namely MRQ 74, MRQ 80 and MRQ 50 are the most popular rice in Malaysia. Establishing an efficient regeneration protocols via somatic embryogenesis is required for varietal improvements. An efficient somatic embryogenesis system has been established for three aromatic rice varieties using mature seeds. MS-B5 medium containing 1 mg/L 2, 4-D (2, 4-dichlorophenoxyacetic acid) and 10 mg/L NAA (α-naphthaleneacetic acid) optimized that produced highest embryogenic callus (89%) without any browning effect. The highest whitish somatic embryos frequency (75%) was initiated by incubating embryogenic calli on same medium containing 10 mg/L ABA and 9 g/L Gelrite for 8 weeks. However, the numbers of regenerated plantlets on medium containing NAA and 2,4-D which was previously pre-treated with 10 mg/L ABA, 9 g/L Gelrite and incubation at 8 weeks was the best method for shoots induction of three rice varieties.

Keywords: Aromatic rice; Somatic embryogenesis; Plant growth regulators; Carbon sources

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crop and a primary food source for half of the world's population. Aromatic rice is also named as fine rice, scented rice or fragrance rice. It is very popular in Asia as well as Southeast Asia and has recently gained wider acceptance in the Middle East and Western communities (Sarhadi et al., 2008; Myint et al., 2009; Sakthivel et al., 2009; Hashemi et al., 2015). Aroma is considered one of the most important traits for rice grain quality and MRQ 74, MRQ 80 MRQ 50 are the most popular aromatic rice in Malaysia (Hashemi et al., 2015). Numerous varieties of rice are aromatic, ranging from the famous Basmati to the lesser known Randhunipagal (Mo et al., 2015). Strong fragrance expression plays a significant role in rice trading. Aromatic or scented rice plays a vital role in global rice trading for increasing consumers demand and attractive price (Sakthivel et al., 2009; Hashemi et al., 2013).

Rice improvement through biotechnological approaches depend on an efficient protocols through, *in vitro* callus induction as well as a suitable regeneration protocols (Vega et al., 2009; Khatun et al., 2012; Siddique et al., 2014). *In vitro* rice regeneration can be accomplished through somatic embryogenesis and organogenesis. Somatic embryogenesis of rice is one of the most promising approaches for rapid propagation due to production of large numbers of plantlets and for the application of genetic transformation technology especially against biotic stresses (Zuraida et al., 2011). The success of somatic embryogenesis is highly influenced by a suitable genotype, growth medium, plant growth regulators (PGRs), carbon sources and gelling agent affect the somatic embryogenesis as well as plant regeneration of rice (Deo et al., 2010).

Auxin such as 2,4-D or NAA plays a vital role for cell diversity in callus culture (Su et al., 2009; Cheng et al., 2010; Ding et al., 2010; Rademacher et al., 2012). Auxin stimulates the somatic embryogenesis and plant regeneration on rice callus culture (Vega et al., 2009). Auxin considered as a key factor in somatic embryogenesis which influences osmotic prerequisite and carbohydrate metabolism on shoot regeneration (Lee and Huang, 2014). Huang et al.

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Received: 21 July 2015; **Revised:** 06 September 2015; **Accepted:** 07 September 2015; **Published Online:** 13 September 2015

(2012) highlighted that endogenous auxin stimulate shoot regeneration in rice calli. Abscisic acid (ABA) supplemented medium produces high quality somatic embryos by decreased osmotic water potential compared to the maintenance medium (Klimaszewska et al., 2000; Pérez et al., 2015). Osmotic water potential depends on types of media, carbon sources and gelleting agent concentrations (Hadeler et al., 1995; Klimaszewska et al., 1997; Laine et al., 2000; Triqui et al., 2008).

Hence, selection of appropriate *in vitro* growth medium supplemented with carbon sources and plant growth regulators are essential prerequisite for embryogenic callus culture which is influences on the genetic improvement of aromatic rice. In the present work, we have established an efficient callus induction and regeneration protocol for Malaysian aromatic rice of MRQ 80, 50 and 74 via somatic embryogenesis system.

MATERIALS AND METHODS

Plant materials

Mature seeds of three Malaysian aromatic rice varieties of MRQ 50, MRQ 74 and MRQ 80 were used for the initiation of an embryogenic callus and plant regeneration. Mature dehusked seeds were sterilized sequentially with 100% ethanol for 2 min. and 50% (v/v) clorox (sodium hypochlorite 5.25%) supplemented with 1–2 drops of Tween-20 for 30 min. with shaking, and were then rinsed three times thoroughly with sterile distilled water.

Embryogenic callus induction medium

For embryogenic callus induction three basal media, MS (Murashige and Skoog, 1962), MS-B5 (MS with B5 vitamin) and N6 (Chu, 1975) were used that supplemented with 3% sucrose, 0.3% gelrite, 1.5 mg/L BAP and different concentrations and combination of 2, 4-D (1 and 5 mg/L) and NAA (5 and 10 mg/L). The pH of all media was adjusted to 5.80 with 1 N NaOH or HCl prior to autoclaving for 15 min at 121^{0}C. Inoculated seeds were then incubated at 25±2^{0}C in the dark condition and under cool-white fluorescent light of 30 μmolm^{-2}s^{-1} for 16 hours. Each treatment consisted of 10 seeds per plate with 20 plates and repeated 3 times. Data were recorded after 4 weeks of culture initiation and callus induction rate (%) was calculated as below:

$$\text{Callus initiation (\%)} = \frac{\text{Total number of cultured seed with callus-Total number of intiated callus}}{\text{Total number of cultured seed}} \times 100$$

The fresh calli were transferred onto the same fresh medium and the percentage of browning embryogenic callus was calculated.

Somatic embryogenesis

The best callus induction medium (MS- B5) supplemented with 1.5 mg/L BAP, 1 mg/L 2,4-D and 10 mg/L NAA were used for somatic embryogenesis in this experiment. Selected high quality embryogenic callus were cultured that supplemented with ABA (5 and 10 mg/L) for pre-regeneration purposes. Different concentrations of gelrite (3, 6, 9 and 12 g/L) were tested as a solidifying agent in pre-regeneration medium. Sucrose (10 and 30 g/L) and maltose (10 and 30 g/L) were used as a carbon sources and cultures were incubated in dark condition for 4 - 8 weeks to complete the pre-regeneration process. Data was recorded as the percentage of forming whitish embryos.

Plant regeneration from somatic embryos

Three grams (g) of the initiated 8 weeks old whitish embryos were cultured on MS-B5 medium supplemented with 1.5 mg/L BAP, 1 mg/L 2, 4-D, 10 mg/L NAA and 10 mg/L ABA) and tested on agar concentration (6 and 9 g/L) with carbon sources as sucrose (10 g/L) and maltose (30 g/L). The numbers of regenerated green plantlets were recorded within 8 weeks.

Histological analysis

Whitish embryos were fixed in a FAA solution for 12-24 hours, dehydrated through a series of ethanol solution and embedded in paraffin wax. The specimen were sectioned at 3-4 μm and stained with 0.5% (w/v) fast green and 0.25% (w/v) safranin. The stained samples were observed under a light microscope equipped with a camera connecting to the computer system.

Statistical analysis

All data were calculated by the mean of the three individual experiments. Each experiments were designed in CRD (Complete randomized design) which followed by four replicates. All mean data were analyzed by one way ANOVA via SPSS software version 20. Comparisons of the mean data and standard error (S.E) were determined by Turkey's multiple range tests at $P<0.05$ level of significance.

RESULTS AND DISCUSSION

Callus induction and percentage of browning

The newly induced callus is a mass of unorganized parenchymatus cells derived from mature seeds after four weeks of culture. The significant effects on callus initiation of three Malaysian aromatic rice varieties (MRQ 74, 80 and 50) were evaluated using different callus induction media supplemented with 2,4-D and NAA (Table 1). In this study, 2,4-D (1 and 5 mg/L) were tested either single or in combination with NAA (5 and 10 mg/L) for callus initiation. Inclusion of 2,4-D supplemented with different callus induction media (N6, MS and MS-B5) resulted callus initiation

varying from 41 to 84%. The maximum callus initiation (84%) were recorded from 5 mg/L 2,4-D supplemented with N6 medium of MRQ 74 whereas minimum initiation (41%) were found in 1 mg/L 2,4-D supplemented with MS medium for MRQ 74 and MRQ 80 (Table 1). The combined effect of 2,4-D and NAA influenced significantly for callus initiation of three rice varieties on different callus induction medium (Table 1). Callus induction ranged from 23 to 89% was found in this study. The highest callus induction (89%) was recorded in MS-B5 supplemented with 1 mg/L 2,4-D and 10 mg/L NAA in MRQ 80 and the lowest (23%) was determined as N6 and MS-B5 supplemented with 5 mg/L 2,4-D and 10 mg/L NAA in MRQ 50 and MRQ 74 respectively (Table 1).

Overall, the highest browning (75 %) occurred in MRQ 74 cultured in MS medium supplemented with 5 mg/L 2,4-D. MRQ 80 and MRQ 50 didn't detect any browning incidence form all media supplemented 2,4-D and NAA treatments but MRQ 74 displayed some degree of browning in all cultured media with 2,4-D and NAA. On the basis of result, MS-B5 medium supplemented with 1.5 mg/L BAP, 1 mg/L 2, 4-D and 10 mg/L NAA was suitable for callus induction of three rice varieties.

Callus initiation depends on basal media supplemented with different plant growth regulators. Kaushal et al. (2015) reported that 2,4-D initiated more callus compared to picloram in rice plant and concluded that 2,4-D is the most largely used growth regulator irrespective of the target materials for major cereal crops. Kaushal et al. (2014 a,b) highlighted that callus induction medium supplemented with 2,4-D initiated friable type of callus with low regeneration capability was produced in IR58025eB × Dular. Afrasiab and Jafar (2011) observed that MS medium supplemented with 2 mg/L 2,4-D initiated higher frequency of callus induction in Super Basmati rice. Rossin and Rey (2011) highlighted that 8 mg/l 2,4-D and 12 mg/l picloram enhanced somatic embryogenesis in selected cassava cultivars. Ilahi et al. (2005) pointed out that several mixtures of auxins and cytokinins supplemented modified medium increased the embryogenic callus and effectively multiplied on MS accompanied with 1.0 mg/L of Kin and 0.5 mg/L of NAA. According to Jain et al. (1995), initiation of callus is influenced by several factors such as explants type and plant growth regulators particularly auxins and cytokinins. A similar phenomena was reported by Thengane et al. (2006) with addition of NAA (5.3-10.7 μM) which gave rise to germinating embryos with complete well-developed shoots. In garlic, low level of 2,4-D increased the percentage of explants producing callus and subsequently tend to give rise to fine compact callus formation (Barandiaran, 1999).

Table 1: Callus initiation (%) and percentage of browning of three aromatic rice cultured on three different media supplemented with different combinations of 2,4-D and NAA

Variety	2,4-D (mg/L)	NAA (mg/L)	Callus initiation (%)			Percentage of browning (mean±S.E)		
			N6	MS	MS-B5	N6	MS	MS-B5
MRQ74	1	-	53±7	41±6	63±5	42±2	60±12	61±16
	5	-	84±5	78±4	81±8	51±5	75±7	70±8
	1	5	63±8	54±6	79±11	35±4	55±5	55±3
	1	10	41±7	52±11	84±8	30±6	50±5	45±6
	5	5	47±6	49±5	41±4	40±6	55±6	40±2
	5	10	11±2	31±6	23±5	47±8	47±11	53±3
MRQ80	1	-	64±9	41±3	56±4	24±2	15±3	7±1
	5	-	78±8	62±4	77±11	31±3	5±1	10±2
	1	5	61±6	64±7	84±4	-	-	-
	1	10	85±9	82±15	89±5	-	-	-
	5	5	41±5	72±11	52±3	-	-	-
	5	10	54±7	34±5	55±4	5±0.5	5±1	5±1
MRQ50	1	-	64±6	55±2	41±3	24±5	15±2	-
	5	-	71±6	65±4	64±7	31±6	5±0.5	-
	1	5	71±6	74±15	72±4	-	-	-
	1	10	83±9	84±7	74±7	-	-	-
	5	5	37±3	46±5	43±11	-	-	-
	5	10	23±2	37±5	45±4	10±2	10±3	5±1

The data represent the mean values±standard error. Standard errors (SE) were determined by Turkey's multiple range tests at $P<0.05$ level of significance

Effect of carbon sources, gelrite and ABA on somatic embryogenesis

Proliferated embryogenic callus were cultured to MS-B5 medium supplemented with carbon sources (sucrose and maltose), different concentrations of gelrite and ABA to investigate their potentiality for somatic embryogenesis in MRQ 74, MRQ 80 and MRQ 50. After two months of culture, whitish somatic embryos were calculated as a percentage which derived from embryogenic callus. Compared to carbon sources, maltose helped to initiate more somatic embryos compared to sucrose and 10 g/L maltose initiated highest number of somatic embryos in all rice varieties (Table 2).

MS-B5 medium supplemented with 3 g/L Gelrite could not generate somatic embryos. However, Gelrite between 6 to 12 g/L produced somatic embryos with 9 g/L initiated the maximum number of somatic embryos in all three varieties (Table 2). ABA supplemented media significantly affected to initiate somatic embryos and MS-B5 supplemented 10 mg/L ABA, 10 g/L maltose and 9 g/L Gelrite produced highest number (75%) of somatic embryos in MRQ 80 (Fig. 1a, b). Whereas, lowest number of somatic embryos (5%) were obtained from 5 mg/L ABA supplemented media in all three varieties (Table 2). It was observed that sucrose was not found to be a suitable carbon source for MRQ 74 and showed lower the initiation of somatic embryos in MRQ 80 and MRQ 50. The combination of

Table 2: Effect of agar, ABA and carbon sources on percentage of somatic embryos initiation after 1-2 months of treatments

Varieties	ABA (mg/L)	Carbon sources (g/L)		Somatic embryos initiation (%)			
				Gelrite 3g/L	Gelrite 6 g/L	Gelrite 9 g/L	Gelrite 12 g/L
MRQ74	5	Sucrose	10	0	0	0	0
			30	0	0	0	0
		Maltose	10	0	0	15±3	5±1
			30	0	0	10±3	10±1
	10	Sucrose	10	0	0	0	0
			30	0	0	10±2	0
		Maltose	10	0	0	10±5	10±2
			30	0	0	0	0
MRQ80	5	Sucrose	10	0	0	0	0
			30	0	5±1	30±3	10±2
		Maltose	10	0	10±1	65±5	20±1
			30	0	0	40±5	0
	10	Sucrose	10	0	15±4	35±3	10±1
			30	0	25±5	45±3	20±4
		Maltose	10	0	45±7	75±5	30±34
			30	0	20±3	30±3	0
MRQ50	5	Sucrose	10	0	0	5±1	20±5
			30	0	5±2	45±6	25±1
		Maltose	10	0	20±5	55±6	30±6
			30	0	10±3	20±5	0
	10	Sucrose	10	0	0	15±3	0
			30	0	25±5	35±5	10±3
		Maltose	10	0	35±6	70±8	20±1
			30	0	20±3	45±3	0

The data represent the mean values±standard error. Standard errors (SE) were determined by Turkey's multiple range tests at $P<0.05$ level of significance

6-9 g/L Gelrite, 10 mg/L ABA supplemented MS-B5 medium initiated good somatic embryos in MRQ 80 and MRQ 50. Therefore this treatment was used in the following experiments for regeneration of rice plantlets. Generally, it is well known that carbohydrates not only function as a potential carbon source for metabolism, but also play an important role in the regulation of osmotic potential (Naqvi et al., 2006). Inclusion of maltose in regeneration medium enhanced the regeneration frequency of four japonica upland rice varieties from 27.6 to 43.3% (Geng et al., 2008). Huang et al. (2012) reported that IAA and ABA was very important for organ differentiation and MS medium supplemented with 10 μM IAA and 10 μM ABA initiated maximum embryogenic callus (80%) in rice plantlets. Auxin such as NAA enhances putrescine biosynthesis result in an increase of the putrescine/spermidine ratio which controlled genetically callus browning trait (Friedman et al., 1985).

Histological analysis of somatic embryogenesis

The origin and the developmental methods of somatic embryogenesis in rice were determined by histological analysis. After pre-incubation, the histological sections of embryogenic callus demonstrated intensive mitotic divisions from vicinities of the vascular bundles which provided rise to globular pro-embryogenic masses (gpm) (Fig. 1c, d). The histological analysis also revealed the enlargement of the globular embryo (ge) directly from the callus tissue by its distinct shape (Fig. 1c, d). The intense metabolic and mitotic activity at these sites caused them to expand and emerge rapidly from the callus. The cells of somatic embryos were exhibited dense cytoplasm and reduced number of vacuoles. The intense metabolic and mitotic activity at these sites caused them to expand and emerge rapidly from the callus. The cells of somatic embryos were exhibited dense cytoplasm and reduced number of vacuoles. Feher et al. (2003) reported that meristematic cells have to be dedifferentiated and their cell division cycle has to be activated during the transition from somatic to embryogenic states. Similar observations were also reported in other monocot species of *Curcuma longa* (Raju et al., 2015), *Cocus nucifera* (Buffard-Morel et al., 1992), *Elaeis guineensis* (Schwandiman et al., 1988), *Phoenix dactylifera* (Sane´et al., 2006), dicot species of *Manihot esculenta* (Baba et al., 2008) and *Petiveria alliacea* (Cantelmo et al., 2013).

Regeneration of rice plantlets

For regeneration, whitish somatic embryos cultured on pre-regeneration medium (MS-B5 supplemented with 1.5 mg/L BAP, 1 mg/L 2, 4-D, 10 mg/L NAA and 10 mg/L ABA) and tested on suitable carbon sources with Gelrite concentration. The frequency of green somatic embryos was greatly influenced by carbon sources with agar concentration and the best results achieved at 10 g/L maltose and 9g/L of Gelrite supplemented media for all varieties (Table 3). The maximum number of plantlets (14%) was recorded from the variety of MRQ 50, which was statistically similar to variety of MRQ 80 and the lowest number of plantlets (2%) was observed in the variety of MRQ 74 (Fig. 1e, f, g, h; Table 3).

In this study, although green somatic embryos occurred with 10 and 30 g/L of maltose, higher masses occurred more frequently with maltose compared to sucrose. Maltose between 10 and 30 g/L was more conducive to development of somatic embryos compared to sucrose. Similar results were recorded for regenerated rice plantlets using maltose at 10 and 30 g/L which produce higher number of regenerated plantlets (Fig. 1h; Table 3). Chemical desiccation using maltose instead of sucrose in MS medium proved to be best for the improvement of both development of green somatic embryos and their regeneration into rice plantlets. It was also noted that regeneration without somatic embryo induction medium with maltose caused callus to become darker and browning appearances were observed (Fig. 1f). Production of embryogenic callus with high regeneration

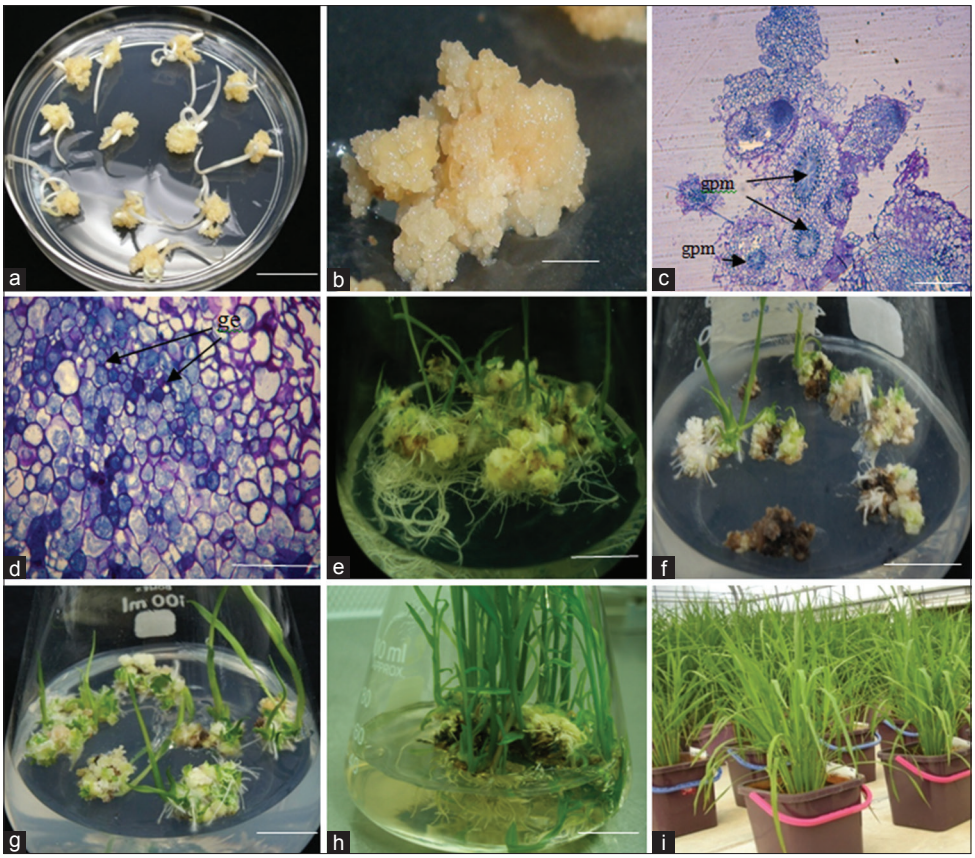

Fig 1. Regeneration of aromatic rice plantlets via somatic embryogenesis system. Callus initiation and proliferation of embryogenic callus (a-b), histological observation of embryogenic callus in early stage of globular pro-embryo, 50X (c) and 200X (d), early phase regeneration of somatic embryogenesis rice MRQ 80 (e), MRQ 74 (f) and MRQ 50 (g), regeneration of rice plantlets in regeneration medium (h) and potted rice plants grown in glasshouse (i). GPM: Gpro-embryogenic mass, GE: Globular embryo and Bars in a, b = 1cm; c, d = 100 µm; e, f, g, h = 2cm.

Table 3: Effect of carbon sources and agar on the number of regenerated plantlets of three Malaysian aromatic rice varieties

Varieties	Carbon sources (g/L)		Gelrite 6 g/L	Gelrite 9 g/L
MRQ74	Sucrose	10	0	0
		30	0	0
	Maltose	10	0	2±0.5
		30	0	0
MRQ80	Sucrose	10	0	0
		30	0	0
	Maltose	10	4±0.5	13±3
		30	3±0.5	8±1
MRQ50	Sucrose	10	0	0
		30	2±0.1	5±1
	Maltose	10	6±0.3	14±3
		30	3±0.4	7±1

The data represent the mean values±standard error. Standard errors (SE) were determined by Turkey's multiple range tests at $P<0.05$ level of significance

capacity is a prerequisite for highly efficient genetic transformation in rice.

Acclimatization of *in vitro* grown palnts

The shoots produced, healthy were transferred for acclimatization. The roots appeared within 3 weeks during the acclimatization process and no morphological changes were observed after transfer to large pots and grown in the greenhouse (Fig. 1i). After 117 - 125 days, all the rice plantlets from the three varieties attained maturity with all plants reaching 74–100 cm in height and produced numerous grains.

CONCLUSION

The present study confirmed the production of embryogenic callus, somatic embryos as well as regeneration of plantlets in MRQ 74, MRQ 80 and MRQ 50 aromatic rice varieties were established. The somatic embryogenesis was confirmed through histology studies. As a result, MS-B5 medium supplemented with 1.5 mg/L BAP, 1 mg/L 2, 4-D, 10 mg/L NAA, 10 mg/L ABA, 10 g/L maltose and 9 g/L Gelrite is a suitable combination for somatic embryogenic callus induction as well as plant regeneration for all studied aromatic rice varieties.

ACKNOWLEDGMENTS

The authors would like to thank to the Malaysian Ministry of Science and Technology (MOSTI) and MARDI for supporting grant for this research work.

Author contributions

Z. A. R.: Were involved in overall planning, conceived the idea, and designed the experiments. A. R.: Made a major contribution to conducting experiments. H. H.: Made a major contribution to conducting experiments. R. K.: Made a major contribution to conducting experiments. Z. A. S.: Made a major contribution to conducting experiments. A. N. O.: Made a major contribution to conducting experiments. Z. Z.: Made a major contribution to conducting experiments. J. U.: Carry out statistical analysis and contributed in writting. S. S.: Were involved in overall planning and improvement of article.

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