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In vitro multiplication and acclimatization of black galingale (*Curcuma Aeruginosa* Roxb.)

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ABSTRACT

Black galingale (*Curcuma aeruginosa* Roxb.) is an important medicinal plant that is widely distributed in South and South-east Asia; however, its utilities in Indonesia are still limited due to superior varieties and seedling availability. Micro-propagation could provide rapid and true to type seedlings to meet the black galingale demand. The objectives of this study were to develop the best *in vitro* medium composition for shoot multiplication and to compare the field growth of plants originated from the rhizome and *in vitro* plantlet. The *in vitro* experiment was arranged in a randomized complete block design with two factors and five replications. The first factor was BAP concentration consisted of 0, 2, 4, 6, and 8 mg l^{-1}. The second factor was IAA concentration consisted of 0, 0.5, and 1.0 mg l^{-1}. Our results showed that number of shoots, number of leaves, and plantlet height were significantly affected by the interaction of IAA and BAP. The highest number of shoots was obtained in the medium containing 6 mg l^{-1} BAP with no IAA. The *in vitro* grown plantlets were acclimatized and grown in the field. The growth of these *in vitro* originated plants was compared with those propagated through the rhizome.

INTRODUCTION

Zingiberaceae family is a group of plants widely used as raw materials for traditional medicine (Harit *et al*., 2012), spices and herbs (Gowda *et al*., 2012), natural colorant for food and fabric (Behura *et al*., 2002; Velayudhan *et al*., 2012), food industry (Jan *et al*., 2012), and as insecticide (Damalas, 2011; Tavares *et al*., 2013). Curcuma genus is naturally spread in the tropics and subtropics, from India to Thailand, Indochina, Malaysia, Indonesia, to Northern Australia (Maknoi *et al*., 2005; Ding *et al*., 2011). More than 80 species from Curcuma genus are originated from Indomalayan region (Cousin *et al*., 2007).

Curcuma aeruginosa Roxb. (Zingiberaceae) or known as black galingale is widely spread in South-eastern Asia (Srivilai *et al*., 2011) and it is one of many medicinal plants that are available in Indonesia. This plant has been known and cultivated as a medicinal plant in Malaysia, Cambodia, and Myanmar. Compared to other plants from genus Curcuma, black galingale has the distinct property that is its bluish rhizome (Nurcholis *et al*., 2016; Setiadi *et al*., 2017). Black galingale rhizome is used as raw material for traditional medicine because of its biochemical compounds, such as saponin, triterpenoid (Nurcholis *et al*., 2016), flavonoid, polyphenol, guaianin (Takano *et al*., 1995), and glycan (Ranjini and Vijayan, 2005). According to Nugrahaningtyas *et al*. (2005), essential oil of black galingale contains 1.8-sineol. Phytochemical research on black galingale rhizome found three sesquiterpene groups, which were identified as zedoarol, curcumenol, and isocurcumenol (Sukari *et al*., 2007), curcumin (Srivastava *et al*., 2006), aeruginon, and curcuminon are identified as distinct compounds of black galingale (Atun *et al*., 2012). Black galingale rhizome is used as traditional tonic and anti-inflammation (Reanmongkol *et al*., 2006) to relief cough, asthma, and skin diseases (Nasrullah *et al*., 2010), anti-microbial (Angel *et al*., 2012), anti-fungi (Srivastava *et al*., 2006), anti-oxidant (Choudhury *et al*., 2013; Nurcholis *et al*., 2015), and anti-androgen (Srivilai *et al*., 2011).

Multiplication of black galingale is usually achieved through asexual propagation using rhizome (Theanphong *et al*., 2010). Rhizome utilization as plant propagation material also

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has constriction, which takes a long time for mass propagation. Plant tissue culture is a technique to isolate a part from the plant, cell, tissue, or organ, and grow it in aseptic condition, controlled environment, and nutrition until new plants are formed (Hussain *et al*., 2012). Tissue culture technology had been applied in a broad scale, especially in plant propagation. Tissue culture has advantages such as more efficient, and propagules can be produced in high number and quality (Sama *et al*., 2015), and true-to-type (Hussain *et al*., 2012; Mohanty *et al*., 2011). Growth response and *in vitro* multiplication rate are different among plant species, even among genotypes within species. Several *in vitro* multiplication protocols have been reported on several Zingiberaceae family members. However, *in vitro* multiplication protocol development on black galingale is still limited. Therefore, best *in vitro* medium for *in vitro* multiplication for plant production is needed, as an alternative to produce high-quality propagule with distinct varieties.

MATERIALS AND METHODS

This research was conducted in Tissue Culture Laboratory, Department of Agronomy and Horticulture, Bogor Agricultural University. Rhizome was originated from Kendal, Central Java. Shoot from rhizome with the size of 1–2 cm was washed with flowing water for 30 minutes. Sterilization was conducted by submerging shoots in 20% (m/v) *streptomycin sulphate* and 80% (m/v) *mancozeb* for 12 hours, continued with rinsing the rhizome using sterilized distillate water. Sterilization then continued by submerging the explants with *sodium hypochlorite* (NaOCl) 0.5% and 1.0% for 5 and 10 minutes, respectively, inside the laminar air flow cabinet. The last sterilization step was the addition of 0.6 ml *povidone iodine* 10% for 5 minutes. Explant was planted in precondition medium (medium without nutrition and growth regulator, MS0) and then kept in culture chamber for 2 weeks with 24 hours light and 23°C of chamber temperature. Subculture to MS0 medium was conducted 2 weeks after culture (WAC).

Research was arranged according to Completely Randomized Block Design (CRBD) with two factors. First factor was the concentration of benzylaminopurine (BAP) with five levels, which were 0, 2, 4, 6, and 8 mg l^{-1} and the second factor was IAA concentration with three levels, which were 0, 0.5, and 1.0 mg l^{-1} with five replications, which made 75 experimental units. Each experimental unit contained three explants of black galingale shoots, so there were 225 plantlet bottles. Observations included contamination percentage, first shoot emergence, number of shoots per plantlet, explant height, and number of leaves per plantlet.

Acclimatization was conducted using compost and charcoal husk (1:1 v/v) that had been sterilized using an autoclave for 45 minutes at 121°C as a medium. Addition of 20% (m/v) *streptomycin sulphate* and 80% (m/v) *mancozeb* with a dosage of 15 ml per plants was conducted in early preparation of plant medium to prevent bacterial and fungal development. Before acclimatization, black galingale plantlets in bottles were kept in a chamber with room temperature for 24 hours. The variables observed at acclimatization were plant survival percentage, plant height, and number of leaves.

RESULTS AND DISCUSSION

Explant that has been planted in experimental medium showed shoot initiation at 2 WAC. Based on the explants observation, shoot growth occurred after the root formations. The first shoot emergence happened on medium with 6 mg l^{-1} BAP (2 WAC), while the latest shoot emergence happened on explants that were cultured in the medium with IAA 0.5 and 1.0 mg l^{-1} (Fig. 1). Variance analysis showed that addition of BAP and IAA had a significant effect on first shoot emergence. Research using *6-Benzyladine* (BA) on *Zingiber officinale* explants also showed a similar result, in which the shoot emergence was faster at 2 WAC with an additional BA (Rout *et al*., 2001). While research conducted by Behera *et al*. (2010) showed different results, the addition of BAP on *Curcuma longa* L. explants inducted slower shoot emergence at 12–16 WAC.

Shoot multiplication is a very important step for *in vitro* plant propagation. At this step, plantlet that produced more shoot and could acclimatize well to the field increasing number of plant material that can be planted in the field. Variance analysis showed that BAP and IAA concentration had a significant effect on number of shoots per explant. Addition of BAP at concentrations of 2, 4, 6, and 8 mg l^{-1} without auxin was able to induct more shoots compared to explants that were grown without growth regulator (Fig. 2). Research by Theanphong *et al*. (2010) on black galingale explant showed that medium without growth regulator is not able for shoot induction, contrary with this research where all explant planted on medium without growth regulator also able for shoot induction with a different number of shoots. Cytokinin in plant tissue culture is involved in cell division, shoot proliferation, and root growth inhibitions. This research was similar with Yaacob *et al*. (2014) that showed additional BAP could improve number of shoots in curcumin. The optimal medium for obtaining the highest number of shoot (3.38 shoots per explant) in black galingale is with BAP of 6 mg l^{-1}. This result was higher compared to Rahayu and Adil (2012) on galingale which was two shoots per explant and Chong *et al*. (2012) on white galingale which was 1.7 shoots per explant. The result showed that BAP addition without auxin is effective enough to improve number of shoot in black galingale plantlet.

Analysis of variance showed that interaction between the concentration of BAP and IAA had a significant effect on number of leaves and plantlet height (Table 1). Highest average number of shoot was 6.25 leaves per plantlet in medium with 6 mg l^{-1} BAP + 0 mg l^{-1} IAA. Lowest number of leaves obtained in plantlet with the medium of 0.5 mg l^{-1} IAA without BAP, 2.13 leaves per plantlet (Table 1).

Highest plantlet was 2.5 cm, obtained in medium with 6 mg l^{-1} BAP + 0 mg l^{-1} IAA, while the shortest was 0.63 cm obtained in medium with 0 mg l^{-1} BAP + 1 mg l^{-1} IAA. Research conducted by Shukla *et al*. (2006) showed that plantlet height was between 3 cm on medium MS0 + 0 mg l^{-1} BAP and 0.63 cm in the medium of 0 mg l^{-1} BAP.

Acclimatization is the last step of *in vitro* plant propagation before plantlet can be planted to field (Kumar and Rao, 2012). Acclimatization is conducted by removing plantlet from culture bottle to acclimatization medium with low light exposure and high humidity (Yahya *et al*., 2015). The success of acclimatization is depending on gradual acclimatization number and good seedling handling (Shahihnozzaman *et al*., 2013). Seedling size and roots differentiation must be considered for plantlet acclimatization (Hazarika, 2003). Black galingale plantlet

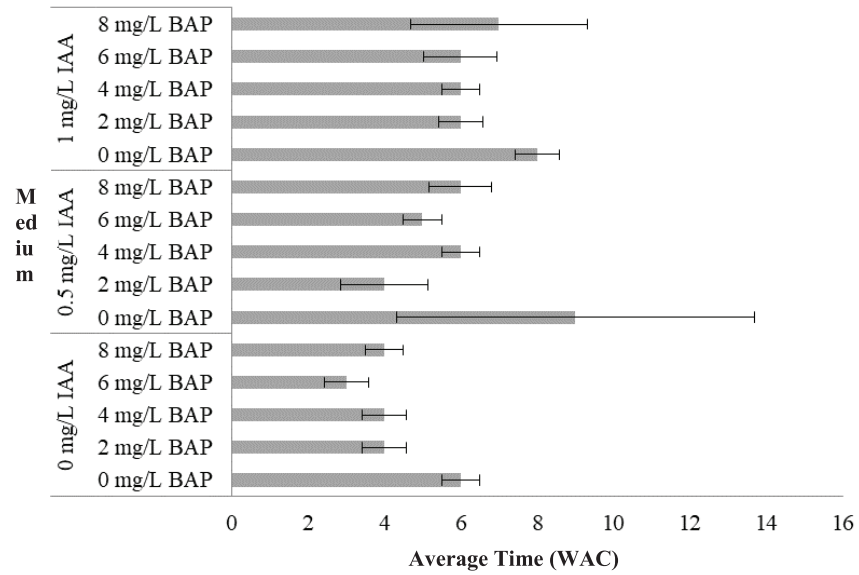

Figure 1. Average time of first shoot emergence of black galingale. Data are expressed as means (n = 5).

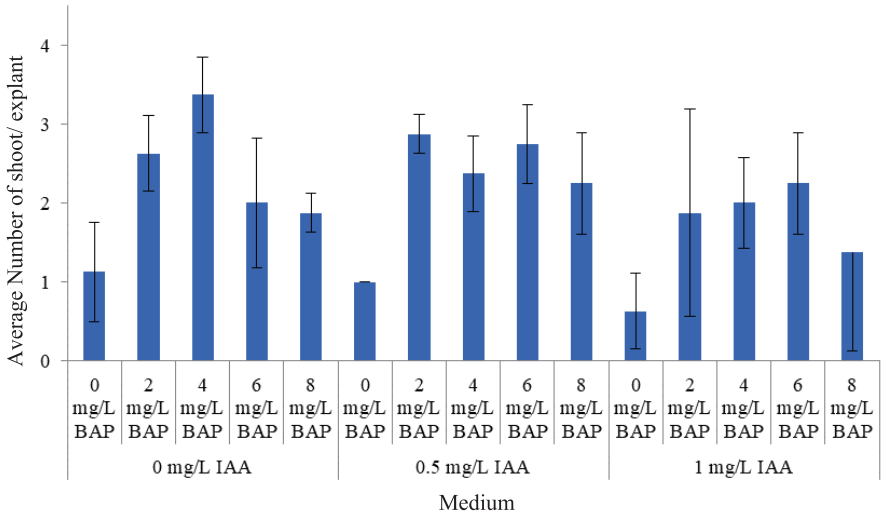

Figure 2. Average number of shoot of black galingale Kendal accession on several BAP and IAA concentration at 12 WAC. Data are expressed as means (n = 5).

Table 1. The effect of IAA and BAP concentration on average number of leaves and plantlet height of black galingale Kendal accession.

BAP concentration	IAA concentration (mg l^{-1})					
(mg l^{-1})	Average Number of leaves			Average Plantlet height (cm)		
	0	0.5	1	0	0.5	1
0	2.88cA	2.13cA	3.17bA	1.80bA	0.64cB	0.63bB
2	3.88abA	4.75bA	3.50bA	2.15abA	2.39aA	1.34abA
4	5.88aA	3.50bA	5.36aA	1.79bA	2.03bA	2.05aA
6	4.75abA	6.25aA	2.75bB	2.51aA	1.73bB	1.72abB
8	5.00abA	4.63bA	2.83bB	1.93abA	1.97abA	0.70bB

Notes: Number followed by the same small letter at the same column of variable and number followed by the same capital letter at the same line means not significantly different based on DMRT at level of α = 5%.

Figure 3. Black galingale rhizome from *in vitro* multiplication after acclimatization for 6 MAP. Cross section of mother rhizome (a), fleshy root (b), black galingale rhizome from *in vitro* multiplication (c), scale = 2 cm.

Figure 4. Black galingale performance that originated from *in vitro* multiplication. *Notes*: Red circles showed purple streak in leaf.

Table 2. Average number of leaves and plant height from acclimatization of black galingale Kendal accession plantlet at 2 WAC.

IAA concen-tration	BAP concentration (mg l^{-1})									
(mg l^{-1})	Average number of leaves					Average plant height (cm)				
	0	2	4	6	8	0	2	4	6	8
0	4.7 ± 0.58	6.7 ± 1.16	5.0 ± 0.82	6.3 ± 2.31	6.3 ± 0.98	9.8 ± 1.46	13.8 ± 6.00	12.3 ± 4.27	20.3 ± 4.92	12.2 ± 3.76
0.5	6.3 ± 0.58	7.0 ± 0.82	3.3 ± 0.96	5.7 ± 0.58	4.7 ± 0.58	17.8 ± 3.75	17.7 ± 2.76	16.8 ± 6.03	14.0 ± 4.00	19.0 ± 4.36
1	4.0 ± 0.00	4.7 ± 1.53	3.3 ± 0.58	5.7 ± 1.15	5.0 ± 1.00	9.2 ± 1.56	9.4 ± 1.00	11.3 ± 1.21	3.0 ± 0.20	3.8 ± 0.35

Note: ** = significantly correlated at α = 1%, * = significantly correlated at α = 5%.

Table 3. Effect of fertilizer rate on the growth and productivity of black galingale originated from *in vitro* culture.

Fertilizer rate	Plant height (cm)	Stem diameter (cm)	Leaf number	Leaf length (cm)	Leaf width (cm)	Rhizome fresh weight (g)	Number of mother rhizome	Number of primary rhizome	Number of secondary rhizome
P1	25.7 ± 0.20	0.76 ± 0.00	4.9 ± 0.20	11.9 ± 8.28	4.0 ± 3.56	16.2 ± 17.71	1.0 ± 0.0	4.2 ± 2.9	6.3 ± 0.6
P2	17.9 ± 8.10	0.4 ± 0.01	5.1 ± 0.20	9.2 ± 5.10	3.2 ± 0.95	13.0 ± 11.23	1.6 ± 0.70	4.8 ± 3.1	4.9 ± 1.3
P3	20.6 ± 1.60	0.5 ± 0.14	5.0 ± 0.80	10.1 ± 2.28	3.7 ± 0.31	6.2 ± 3.37	1.0 ± 0.0	2.5 ± 0.2	3.3 ± 0.8
Average	21.42	0.56	5.00	10.4	3.6	11.8	1.2	3.8	4.8

Note: P1 = 20 ton ha^{-1} of manure, P2 = 10 ton ha^{-1} of manure, 200 kg ha^{-1} Urea, 100 kg ha^{-1} SP18, 100 kg ha^{-1} KCl, P3 = 20 ton ha^{-1} of manure, 200 kg ha^{-1} Urea, 100 kg ha^{-1} SP18 and 100 kg ha^{-1} KCl.

that had poor root development also had poor growth that leads to plantlet death because of the bad rotting system which makes the plantlet cannot absorb the water nor nutrients from the medium. Only 40 black galingale plantlets had normal growth, while 25% of total plantlet had no growth until the end of observation. We assumed that the harsher environment in the experimental site (i.e., rocky and drier soils compared to the acclimatization conditions) caused the low living percentage of the transplanted seedlings.

Analysis of variance results showed that addition of BAP in the culture medium had no significant effect on number of leaves and plant height of black galingale after acclimatization. Highest number of leaves was shown in medium with 4 mg l^{-1} BAP + 0.5 and 1 mg l^{-1} IAA. Plant height after *in vitro* acclimatization did not get affected by BAP and IAA concentration and shows various results on this character. Plant height observed was between 3.0 cm on medium 6 mg l^{-1} BAP + 1 mg l^{-1} IAA and 20.3 on medium 6 mg l^{-1} BAP + 0 mg l^{-1} IAA (Table 2).

Black galingale performance in acclimatization showed wide variation in plant height and number of leaves. At the same medium composition, treatment showed a different response on plant height and number of leaves. Purple shades could be observed on leaves in the medium with additional of 6 mg l^{-1} BAP + 0 mg l^{-1} IAA at 4 MAP (month after planting).

Observation on black galingale rhizome from acclimatization was conducted after all leaves were fallen (6 MAP). Observation result showed that fleshy root was formed in rhizome with bigger size than the mother rhizome. Primary rhizome and secondary rhizome could not be found in every rhizome from acclimatized black galingale. Cross section of rhizome showed grayish color in mother rhizome and could not be found in the cross section of the fleshy root (Fig. 3a and b). Rhizome from acclimatized black galingale had smaller size and mass compared to rhizome from the field, a similar result to Salvi *et al*. (2002) in curcumin, where propagules from acclimatization had smaller rhizome size. Black galingale rhizome from acclimatization is shown in Figure 3c.

Harvested black galingale rhizome from *in vitro* propagation was replanted in medium with compost and charcoal husk (1:1 v/v). The emergence of shoot from *in vitro* originated rhizome occurred at 1 MAP, and had similar performance with the previous acclimatized plant (Fig. 4). Therefore, acclimatized plantlet can be used for propagation propagules.

Adelberg *et al*. (2013) reported that nutrient affected the growth of turmeric during acclimatization; therefore, the black galingale mature rhizome and seedlings from *in vitro* culture were planted with different fertilizer rates in a separated experiment. Fertilizer rate significantly affects the growth and productivity of plant originated from *in vitro* culture. Manure application of 20 ton ha^{-1} resulted in higher plant, larger stem diameter, longer and wider leaf lobe, and higher rhizome fresh weight compared to those fertilized by manure and inorganic fertilizers (Table 3). *T*-test was performed to compare the growth and production of different planting material (rhizome *versus* seedlings from *in vitro* culture) and the data are presented in Table 4. Plants grown from *in vitro* culture showed lower living percentage (54%) compared to those grown from the mature rhizome (98%). The rhizome fresh weight of plants originated from *in vitro* culture was also lower compared to those originated from the mature rhizome.

Table 4. *T*-test results of several variables on black galingale plants from matured rhizome and acclimatized plantlet.

Variables	Rhizome	Plantlet	*t*-test
Percentage of growing plant (%)	98	54	**
Number of leaves	7.60	3.40	**
Plant height (cm)	53.8	17.7	**
Stem diameter (cm)	1.20	0.50	**
Leaf length (cm)	32.2	10.3	**
Leaf width (cm)	10.9	3.40	**
Fresh rhizome weight (gram)	309.23	11.81	**
Number of mother rhizome	1.80	1.20	*
Number of primary rhizome	6.50	3.80	**
Number of secondary rhizome	9.10	4.80	**

The photosynthetic organ of seedlings originated from *in vitro* culture might still not function properly. Naz *et al*. (2009) reported that the *in vitro* environment (i.e., supplemented nutrients and sucrose) lead to a limited photosynthetic cycle in the *in vitro* condition and caused morphological abnormalities on stomata and cuticle. The low photosynthetic capacity of the seedlings might cause low survival in the field. Seedlings might also have undeveloped rooting system during the *in vitro* culture as reported by Kozai *et al*. (2005) which also might lead to low survival in the field. In contrast, the rhizome is a well-developed planting material with energy storage which supports plant growth (Girija and Shree, 2014) and leads to a higher survival rate in the field.

The number of stomata on plant leaf is tightly related to leaf transpiration rate, where the higher transpiration rate means higher number of opening stomata, consequently allowing more CO_2 absorption to be used for plant photosynthesis process (Lawson, 2009). The number of stomata on the lower part of black galingale leaf that originated from the matured rhizome and *in vitro* rhizome was not significantly different for fertilizer treatment. Average number of stomata on black galingale originated from matured rhizome was 108.4 per mm^2, while the number of stomata originated from *in vitro* rhizome was 92.52 per mm^2 (Table 5). Research conducted by Jadhao *et al*. (2015) on genus Curcuma showed that number of stomata was around 94–120 per mm^2. The number of stomata on plant originated from matured rhizome and *in vitro* rhizome was not significantly different based on *t*-test (Table 4). The use of acclimatized plantlet as propagation propagules give the same potency as matured rhizome does.

Qualitative characters of acclimatized black galingale had no difference with its mother plants. On every observed plant, whether stem or rhizome vegetative characters had the same character with the mother plants, such as the presence of purple streak on the leaves and grayish white color of the rhizome (Table 6). The presence of purple streak in black galingale leaves can be observed on the fourth leaves (Fig. 4). However, this result also in accordance with Setiadi *et al*. (2017) research that conducted characterization on the morphology of several black galingale accession in Indonesia.

Table 5. Number of *Curcuma aeruginosa* Roxb. stomata in plant originated from matured rhizome and acclimatized plantlet with different fertilizer rates (6 MAP).

Fertilizer Rate	Number of stomata (per mm²)	
	Rhizome	Plantlet
P1	109.6 ± 23.4	73.89 ± 0.0
P2	110.4 ± 83.3	89.87 ± 1.7
P3	105.3 ± 11.5	113.80 ± 8.4
Average	108.4	92.52

Notes: P1 = 20 ton ha^{-1} manure, P2 = 10 ton ha^{-1} manure, 200 kg ha^{-1} Urea, 100 kg ha^{-1} SP18, 100 kg ha^{-1} KCl, P3 = 20 ton ha^{-1} manure, 200 kg ha^{-1} Urea, 100 kg ha^{-1} SP18 and 100 kg ha^{-1} KCl.

Table 6. Qualitative character of acclimatized black galingale plant and rhizome from *in vitro* multiplication.

Fertilizer Rate	Leaf color	Presence of purple streak	Rhizome skin color	Rhizome flesh color	Presence of fleshy root
P1	Green	Present	Bright brown	Grayish white	Present
P2	Green	Present	Bright brown	Grayish white	Present
P3	Green	Present	Bright brown	Grayish white	Present

CONCLUSION

Result from *in vitro* multiplication induction of Kendal accession showed that there was an interaction between plant growth regulator BAP and IAA to the number of shoot, number of leaves, and plantlet height variables. Culture medium with 0 mg l^{-1} IAA combined with 6 mg l^{-1} BAP resulted in the highest number of shoot (3.38 shoot per explant) and tallest plantlet (2.51 cm), while the highest number of leaves (6.25 leaves per plant) was produced from medium with 6 mg l^{-1} BAP + 0.5 mg l^{-1} IAA. Black galingale plantlet can be acclimatized and produce rhizome that can be used as propagules for black galingale propagation.

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CONFLICT OF INTERESTS

Authors declare that there is no conflict of interest.

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