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In-vitro plant regeneration of mature adlai (*Coix lacryma-jobi* L.) embryos using coconut water as a natural alternative growth promoter

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ABSTRACT: *In-vitro* culture has paved the way for the development of plant cultivars with enhanced morphophysiological characteristics. Adlai (*Coix lacryma-jobi* L.), a grain-producing crop with promising economic potential, has benefited from pioneering studies using synthetic hormones to induce regeneration. Although proven to be effective, the use of these hormones makes tissue culture an expensive technique. This study aimed to provide a cost-effective alternative by evaluating the efficiency and determining the optimal concentrations of coconut water as a natural alternative for 6-BAP (6-benzylaminopurine), while still producing plantlets of comparable quality. After 18 days of incubation, the morphological and physiological characteristics of the plantlets were recorded. Among the treatments, 7.5% matured coconut water consistently supported balanced growth, including optimal root elongation, shoot height, high biomass accumulation, and favorable stomatal dimensions, indicating enhanced water-use efficiency and gas exchange. Meanwhile, 15% matured coconut promoted leaf proliferation and the highest chlorophyll content, induced smaller stomatal apertures and reduced structural biomass. Overall, this study supports the potential of coconut water as a natural and cheaper alternative to synthetic hormones, thereby reducing the overall costs associated with tissue culture technology.

KEYWORDS: *6-BAP, adlai, coconut water, plant regeneration, tissue culture*

INTRODUCTION

Adlai (*Coix lacryma-jobi* L.) is a grain-producing member of the Poaceae family, characterized by its erect, branched, tall shoot with long, broad, flat leaves, and teardrop-shaped seeds. This species originated in Southeast Asia and is distributed in many countries, including the Philippines [1]. Its grains are rich in carbohydrates, protein, fat, dietary fibers, and minerals. Research has also shown that adlai possesses pharmacological properties, including antioxidant, anti-inflammatory, anti-cancer, antitumor, hypoglycemic, and analgesic effects [2].

The Philippine government, through the Department of Agriculture, has been actively promoting adlai as either a full substitute for or as a blend with rice [3]. With an energy content of 365 kcal, it exceeds the 110-kcal provided by white rice [4]. Moreover, it holds great potential as a commercial crop, indicating that cultivation has substantially increased farmers' income. Despite this, Laxmisha et al. [5] noted that there has been limited progress in improving the crop. Biswas and Das [6] emphasized the need for the development of high-yielding, short-duration, dwarf, drought- and lodging-resistant varieties with synchronous maturation and high harvest index to enhance adlai's success.

Over the years, tissue culture technology has been instrumental in developing novel plant cultivars that are resistant to pests and diseases and more tolerant to varying environmental conditions. For adlai, studies conducted by Li et al. [7] and Sun and Chu [8] focused on using synthetic hormones for plant regeneration. While these hormones demonstrated great results, their use makes tissue culture an expensive technique. In contrast, Bernardo et al. [9] explored the use of organic additives such as agnihotra ash and cow dung ash for similar purposes.

Coconut water is the liquid endosperm found in young coconuts and is one of the most widely used organic growth additives in tissue culture. It contains organic acids, vitamins, carbohydrates, amino acids, minerals, and other substances

[10]. Its phytohormone components, which play crucial roles in regulating plant growth, have already been documented. In particular, cytokinin exerts various roles in cell division, formation and activity of shoot meristems, induction of photosynthesis gene expression, and root growth [11]. The use of matured coconut water as a natural alternative presents a cost-effective solution to the high costs associated plant hormones.

This study sought to evaluate the efficiency of coconut water as an alternative to the synthetic hormone 6-BAP (6-benzylaminopurine) in promoting plant regeneration of adlai by characterizing the morphological and physiological traits of the plantlets. Additionally, the optimum concentration of matured coconut water required in the culture media for in vitro regeneration of adlai was determined.

MATERIALS AND METHODS

Plant materials and sterilization

Mature, dry seeds of adlai *(C. lacryma-jobi)* obtained from Davao del Sur, Philippines were manually dehusked. Seed surface sterilization was performed using a modified protocol based on the methods by Bernardo et al. [9]. Dehusked seeds were washed with sterile distilled water followed by 70% ethanol for 1 minute. Then, the seeds were sterilized twice for 3 minutes using 1% NaOCl (sodium hypochlorite) and rinsed thrice with sterile distilled water.

Preparation of culture media

Half-strength MS (Murashige & Skoog) supplemented with 11 mg/L 6-BAP was used as positive (+) control (Table 1). To test the effect of coconut water on plant regeneration, varying concentrations of coconut water (7.5%, 15%, 30%) were added to the MS basal media. Before autoclaving, the pH was adjusted to 5.6-5.7, and approximately 20 ml of the prepared media was poured into individual glass containers. When autoclaved at 121 °C, some of the heat sensitive compound of coconut may degrade and some of its heat-sensitive compounds are degraded particularly vitamins (ascorbic acid) and certain growth regulators. However, coconut water retains sufficient nutritional and hormonal activity to support plant tissue development in many species of plants [10-12].

Inoculation and incubation of culture media

The procedure for seed dissection followed the description by Nandariyah et al. [13]. Mature embryos were aseptically dissected from the caryopsis, slightly damaging the radical portion, and then cultured with the scutellum in contact with the medium. For each treatment, a total of 10 explants were planted. The cultures were maintained in a plant growth chamber with a photoperiod of 16 hours light and 8 hours dark, using cool white, fluorescent lamps at an intensity of approximately 60 µmol m^{-2} s^{-1}, relative humidity of 40% at 25°C.

Morpho-physiological characterization

The morpho-physiological characteristics of the plantlets' shoots, roots, and leaves were assessed after 18 days of incubation.

Emergence of shoot, roots, and leaves

The percent emergence of induced shoot, root, and leaves were computed following the formula:

$$\text{Emergence (\%)} = \frac{\text{total no. of emerged shoot/root/leaves}}{\text{total no. of explants planted}} \text{ x } 100$$

Shoot length and width, root length

Using a Vernier caliper, the length of the shoot and roots were measured from the base to the tip. Additionally, the width of the shoots was recorded by taking the diameter of the base.

Leaf area (mm^2)

For each plantlet, the individual number of leaves formed was recorded. Then, the length of each leaf from the base to tip, and the width at its widest point were measured. The leaf area was obtained by multiplying the length and width. For plantlets with multiple leaves, the area of each leaf was first measured individually, then the total leaf area was obtained by adding these individual areas.

Daily growth

The values for the average daily growth of the plantlets were obtained following the formula:

$$\text{Daily growth} = \frac{\text{shoot length} + \text{root length}}{\text{total no of days incubated}}$$

Fresh and oven-dry weight

The total fresh weight of the plantlets from each treatment was measured using an analytical balance. To determine the oven-dry weight, the samples were then placed in a drying oven at a controlled temperature. After 48 hours, the samples were removed, and the dry weight was recorded. The percent weight loss was calculated using the following formula:

$$\text{Weight loss (\%)} = \frac{\text{fresh weight} - \text{dry weight}}{\text{fresh weight}} \text{ x } 100$$

Total chlorophyll content

The fresh samples were weighed, cut into smaller segments, and soaked in 80% acetone for 40 hours. The total amount of solvent used was recorded before transferring the samples to the cuvettes. The optical density (absorbance) was then measured using a spectrophotometer with wavelengths of 645 nm and 663 nm. Three readings were collected for each treatment and the following measurements were used to determine the total chlorophyll content of each:

$$\text{Chlorophyll a} = \frac{(12.7)(A663) - (2.63)(A645)\text{x V}}{1000 \text{ x W}}$$

$$\text{Chlorophyll b} = \frac{(22.9)(A645) - (4.68)(A663)\text{x V}}{1000 \text{ x W}}$$

$$\text{Total chlorophyll} = \text{Chlorophyll a} + \text{Chlorophyll b}$$

Where: A = absorbance, V = total volume of the solvent (80% acetone), W = fresh weight of the sample

Stomatal density

To analyze the stomata, the nail polish imprint technique was performed. A thin layer of clear nail polish was applied to the abaxial surface of the leaf. Once dried, a piece of clear adhesive tape was pressed onto the nail polish. The tape was then carefully peeled off and mounted onto a glass slide. The slide was viewed under a microscope at 400x magnification, and images were captured and processed using ImageJ (1.54 g) software. The following formula was used to obtain the density:

$$\text{Stomatal density} = \frac{\text{number of stomata}}{\text{area of the field of view}}$$

Data analysis

The study followed a completely randomized design, with ten replicates for each treatment. The data were analyzed by using the analysis of variance (ANOVA). The means were compared by the Tukey Test at a 5% significance level. All statistical analysis was done using Statistical Package for the Social Sciences (SPSS) software.

RESULTS AND DISCUSSION

The efficiency of matured coconut water as an alternative to 6-BAP for promoting plant regeneration in adlai was tested using MS basal media supplemented with increasing concentrations of matured coconut water. After 18 days of incubation, the morphological characteristics of the shoots, roots, and leaves were assessed along with the growth rate, fresh weight, and oven-dry weight. Additionally, physiological parameters such as chlorophyll content and stomatal density were recorded.

Shoot characteristics

The length and width of the shoots are important determinants of the plantlets health. Generally, longer shoots indicate good growth [13]. Both coconut water and 6-BAP serve as sources of the phytohormone cytokinin, which promotes protein synthesis and stimulates organogenesis of shoot cultures [14]. On the other hand, plants deficient in cytokinin typically exhibit stunted shoots and smaller apical meristem [15]. Presented in Table 1 are the shoot characters such as percentage emergence, shoot length and the shoot width of adlai. The longest and widest shoots of adlai were recorded in MS media with 15%(102.52mm) and MS with BAP (1.14mm), respectively. Results also revealed that there is no statistical difference among all treatments, which means the comparable effect of the matured coconut water with 1 mg/L 6-BAP.

Table 1. Shoot characteristics of in vitro cultured adlai

Treatments	Emergence (%)	Shoot length (mm)	Shoot width (mm)
MS + 7.5% MCW	90	91.51 ± 69.31[a]	1.07 ± 0.44[a]
MS + 15% MCW	90	102.51 ± 70.93[a]	0.78 ± 0.40[a]
MS + 30% MCW	90	95.19 ± 75.38[a]	0.86 ± 0.41[a]
MS + 1 mg/L 6-BAP	100	91.73 ± 43.76[a]	1.14 ± 0.35[a]

Means with the same letter are not significantly different.

Matured coconut water also contains other phytohormones such as auxin, gibberellins, and other undefined chemical components that may exert synergistic effects with cytokinin [11]. This synergistic interaction could explain the observed morphological characteristics of the plantlets. To identify the healthiest plantlets, a larger width and shorter length similar to the observations in the positive control are desirable. This suggests that adding 7.5% coconut water to MS basal media shows comparable results. This further supports that phytohormones are most effective at lower concentrations and might lead to toxicity when used in excess.

Root characteristics

As shown in Table 2, among the treatments, MS medium supplemented with 7.5% MCW yielded the most favorable root development, achieving 100% emergence and the longest mean root length (70.89 ± 41.10 mm). This suggests that moderate MCW supplementation provides an optimal balance of natural cytokinin, auxins, and organic nutrients that promote both root initiation and elongation [16, 17]. Meanwhile, increasing MCW concentration to 15% and 30% resulted in a marked decline in emergence (60%, 80%) and significantly shorter roots (29.48 ± 34.33 mm, 37.34 ± 23.96 mm), indicating potential osmotic stress or hormonal imbalance at higher levels. Excessive hormone levels, such as high concentrations of BAP, can further suppress root initiation by antagonizing auxin-mediated pathways, leading to poor root primordia formation or complete inhibition of rhizogenesis.

Table 2. Root characteristics of in vitro cultured adlai

Treatments	Emergence (%)	Root length (mm)
MS + 7.5% MCW	100	70.89 ± 41.10[a]
MS + 15% MCW	60	29.48 ± 34.33[b]
MS + 30% MCW	80	37.34 ± 23.96[b]
MS + 1 mg/L 6-BAP	100	51.13 ± 22.11[a]

Means with the same letter are not significantly different

Roots, as the primary organs for nutrient absorption, are crucial for plant growth. Hence, a longer root generally indicates a healthy plantlet. The induction of rooting and the increased root length observed from MS medium with 7.5% coconut water is also attributed to its phytohormone content. While cytokinin is a major component, coconut water also contains indole-3-acetic acid (IAA), the primary type of auxin in plants [18,19]. Auxin plays a central role in the formation of main, lateral, and adventitious roots [20, 21] . Initially, auxins are located in the shoot tip and later transported to the root tip, where they promote cell division and elongation, and root growth [18]. In a separate study, Muhammad et al. [21] noted that rooting is correlated with changes in auxin concentration. Contrary, the high cytokinin content in 6-BAP has a negative regulatory effect on root growth [11] and inhibits root initiation [13]. This, along with the minimal amount or absence of auxin in the media, may explain the shorter roots observed in the plantlets from positive control.

Leaf characteristics of *in vitro* cultured adlai

Table 3 presents the leaf characters of the in-vitro grown adlai in MS medium supplemented with coconut water. Overall, MS medium +15% MCW is optimal for promoting both leaf emergence and expansion in Adlai tissue culture, with the highest percentage leaf emergence, number of leaves and the largest leaf area of 70%, 8, and 219.40mm^2 ± 163.79, respectively in terms of leaf area, it is statistically comparable with MS medium + 7.5% coconut water with 176.08 mm^2 . Meanwhile those grown in MS medium with BAP has the least of 48.39mm^2.

The leaves are the major structures for photosynthesis. Its formation is also governed by cytokinin. Yong et al. [16] emphasized that these hormones are essential for driving the cell division cycle at normal rates and for obtaining the required number of divisions to produce a normal leaf size. In a study by Matloob et al. [20], a significant increase in the number of leaves was observed in the media containing 12% coconut water. However, they also noted that higher concentrations of coconut water led to a decline in overall growth and morphological features, as well as the development of abnormal plantlets. Similar observations were made in the current study, where the largest leaf area and higher number of individual leaves were recorded at lower concentrations (7.5 and 15%), while reduced leaf formation and smaller areas were noted in the media with 30% coconut water.

Table 3. Leaf characteristics of *in vitro* cultured adlai

Treatments	Emergence (%)	No. of Leaves	Area (mm^2)
MS + 7.5% MCW	40	5	176.08 ± 271.38[a]
MS + 15% MCW	70	8	219.40 ± 163.79[a]
MS + 30% MCW	30	4	83.88 ± 131.99[b]
MS + 1 mg/L 6-BAP	20	3	48.39 ± 102.80[c]

Means with the same letter are not significantly different.

Daily growth of *in vitro* cultured adlai

The average daily growth refers to the increase in the total plant height, obtained by adding both shoot and root lengths and dividing by the number of days under incubation. The growth of plantlets is influenced by the interaction of several factors. One of which is the condition of the explant, including its genotype, source, physiological stage, size, and age of tissues used [13]. Another critical factor is the culture media which consists of macro- and micronutrients, vitamins, amino acids, carbon sources, organic supplements, solidifying agents, and growth regulators [18].

Among the treatments, MS + 7.5% MCW produced the highest total plant height (162.40 ± 87.92 mm) and daily growth rate (9.02 ± 4.88 mm/day), compared to higher MCW concentrations (15% and 30%) and the synthetic cytokinin treatment (MS + 1 mg/L 6-BAP). This implies that lower

concentrations of coconut water may be more effective in promoting shoot elongation and overall vigor but excessive concentrations may lead to nutrient imbalances or inhibitory effects [11].

Interestingly, the synthetic cytokinin 6-BAP, commonly used to induce shoot proliferation, resulted in moderate growth (142.86 ± 44.34 mm height; 7.94 ± 2.46 mm/day), suggesting that natural additives like coconut water can match or even surpass synthetic hormones in promoting vegetative growth under certain conditions. This aligns with findings by Bernardo et al. [9], who reported that coconut water enhanced *Adlay* seedling development and responsiveness to hormonal treatments in *in vitro* culture.

Fresh and oven-dry weight of *in vitro* cultured adlai

The fresh weight of the plant includes both the weight of the plant material and the water content in its tissues [23]. In contrast, dry weight represents the actual biomass, serving as a more accurate indicator of the plant's performance in response to photosynthesis, nutrient uptake, and environmental conditions. Apart from the controls, the highest and lowest percent loss was recorded in the basal media with 7.5% and 30% coconut water, respectively (Table 5). Since the weight measurements were taken collectively for each treatment, statistical analysis was not performed. Among the treatments, MS + 7.5% MCW yielded the highest fresh weight (0.9325 g) and oven-dry weight (0.065 g), indicating superior biomass production and tissue hydration. This supports previous findings that moderate MCW supplementation enhances nutrient uptake and cellular expansion due to its rich composition of natural cytokinins, amino acids, and sugars [16, 24]. On the other hand, MS + 15% MCW and MS + 30% MCW resulted in lower fresh weights (0.5886 g and 0.5475 g, respectively) and reduced oven-dry weights, suggesting that higher MCW concentrations may inhibit biomass accumulation due to hormonal oversaturation that impairs metabolic efficiency [25]. While MS + 1 mg/L 6-BAP produced a moderate fresh weight (0.6983 g) but the lowest oven-dry weight (0.032 g), resulting in the highest percentage weight loss (95.42%). This implies that while BAP may stimulate initial tissue expansion, it may also lead to excessive water retention and reduced structural biomass, consistent with reports of cytokinin-induced hyperhydricity and poor tissue density [26].

Table 4. Total plant height and mean daily growth of *in vitro* cultured adlai

Treatments	Total plant height	Daily growth
MS + 7.5% MCW	162.40 ± 87.92[a]	9.02 ± 4.88[a]
MS + 15% MCW	131.99 ± 97.42[a]	7.33 ± 5.41[a]
MS + 30% MCW	132.53 ± 91.04[a]	7.36 ± 5.06[a]
MS + 1 mg/L 6-BAP	142.86 ± 44.34[a]	7.94 ± 2.46[a]

Means with the same letter are not significantly different

Table 5. Fresh and oven-dry weight of *in vitro* cultured adlai

Treatments	Fresh weight (g)	Oven-dry weight (g)	% Loss
MS + 7.5% MCW	0.9325	0.065	93.03
MS + 15% MCW	0.5886	0.0485	91.76
MS + 30% MCW	0.5475	0.0494	90.98
MS + 1 mg/L 6-BAP	0.6983	0.032	95.42

Means with the same letter are not significantly different

Chlorophyll content of *in vitro* cultured adlai

Chlorophyll is the green photosynthetic pigment located on the thylakoid membranes of chloroplasts. Total chlorophyll content is an important index of photosynthetic efficiency, reflecting the plant's productivity. A study by Dobránszki & Mendler-Drienyovszki [27] reported that the total chlorophyll content depends on the concentrations of cytokinin applied.

Table 6 presents the total chlorophyll content of *in vitro* cultured Adlai (*Coix lacryma-jobi* L.) under varying concentrations of matured coconut water (MCW) and 6- -BAP. All MCW-supplemented treatments produced statistically similar chlorophyll levels (29.07 ± 0.88, 30.03 ± 2.27, and 29.22 ± 1.71 mg/g fresh weight, respectively). This suggests that MCW, regardless of concentration, consistently supports chlorophyll biosynthesis,

likely due to its natural supply of cytokinins, vitamin B-complex, and organic acids that enhance plastid development and pigment stability [16, 24]. In this study, it was observed that chlorophyll content is not directly proportional to the concentration of phytohormones. This aligns with Yu [28] who noted no definite correlation between cytokinin concentration and chlorophyll content. Additionally, this observation may be attributed to the interaction of other media components, such as mineral salts, amino acids, and sugars which all contribute to the plant's chlorophyll content [29].

Table 6. Total chlorophyll content of in vitro cultured adlai

Treatments	Chlorophyll content
MS + 7.5% MCW	29.07 ± 0.88[a]
MS + 15% MCW	30.03 ± 2.27[a]
MS + 30% MCW	29.22 ± 1.71[a]
MS + 1 mg/L 6-BAP	25.17 ± 0.97[b]

Means with the same letter are not significantly different.

Stomatal density and dimensions of *in vitro* cultured adlai

Stomata are small pores on the surfaces of leaves that facilitate gas exchange and transpiration. Stomatal density is a measure of the number of stomata per unit area. According to Kumar et al. [30], low density does not necessarily lead to reduced photosynthesis. In contrast, a study by Tanaka et al.[32] presented that increased density enhanced leaf photosynthetic capacity. The stomatal density can vary [33] and these modifications occur throughout the entire cycle of plant growth to adjust to the surrounding environment [34,35]. Further, these changes are brought by plants' signaling mechanisms, involving phytohormones. Similar to other parameters measured, this is also influenced by varying levels of cytokinin, as reported by Farber et al. [36].

Based from the results of the present study (Table 7), MS + 15% MCW resulted in the highest stomatal density (102.08 ± 16.40 mm^{-2}), suggesting enhanced epidermal differentiation and possibly increased transpiration potential. However, this treatment also showed the smallest aperture (31.26 ± 6.68 µm) and reduced stomatal dimensions, which may reflect a compensatory mechanism to regulate water loss under elevated MCW concentrations [16,24,26]. MS + 7.5% MCW produced the widest stomata (101.24 ± 14.94 µm) and the longest stomata (151.00 ± 20.81 µm), with moderate density (68.75 ± 10.21 mm^{-2}), indicating a balance between gas exchange efficiency and water conservation. This treatment also had a relatively large aperture (36.50 ± 5.91 µm), supporting optimal photosynthetic activity and transpiration control.

The MS + 30% MCW and MS + 1% 6-BAP treatments showed intermediate stomatal densities (79.11 and 82.33 mm^{-2}, respectively) and relatively large apertures (~38–39 µm) but differed in dimensional traits. Notably, MS + 1 mg/L BAP yielded the longest stomata (157.09 ± 17.34 µm), which may reflect cytokinin-induced cell expansion, though excessive BAP is also known to disrupt stomatal patterning and increase susceptibility to hyperhydricity [13,14,18].

Table 7. Stomatal characteristics of in vitro cultured adlai

Treatments	Density	Aperture (µm)	Width (µm)	Length (µm)
MS + 7.5% MCW	68.75 ± 10.21[b]	35.60 ± 5.91[ab]	101.24 ± 14.94[a]	151.00 ± 20.81[a]
MS + 15% MCW	102.08 ± 16.40[a]	33.16 ± 6.68[bc]	85.14 ± 12.11[b]	135.90 ± 13.41[bc]
MS + 30% MCW	79.17 ± 44.00[a]	32.29 ± 6.03[c]	90.97 ± 10.73[b]	143.24 ± 17.14[b]
MS + 1 mg/L 6-BAP	58.33 ± 11.79[b]	33.09 ± 8.32[bc]	85.31 ± 9.56[b]	157.09 ± 17.34[a]

Means with the same letter are not significantly different.

The present study found no correlation between stomatal density and plant productivity, as measured by oven-dry weight. This aligns with Kumar et al.[23] and Tanaka et al. [35] , where neither high nor low stomatal density affected plant biomass. Finally, this suggests that plant productivity is influenced by a combination of factors, and not dependent on stomatal density alone [28, 36].

The results of the present study demonstrate that the physiological and morphological responses of *in vitro* cultured Adlai are highly dependent on the type and concentration of media additives. Among the treatments, 7.5% MCW consistently supported balanced growth across multiple traits, including optimal root elongation, shoot height, high biomass accumulation, and favorable stomatal dimensions, indicating enhanced water-use efficiency and gas exchange. Meanwhile, 15% MCW promoted leaf proliferation and the highest chlorophyll content, though it also induced smaller stomatal apertures and reduced

structural biomass, suggesting a shift toward surface expansion rather than tissue density. In contrast, addition of 30% MCW showed reduced leaf area, shorter roots, and moderate chlorophyll levels. Overall, the data suggest that MCW supplementation, at 7.5–15%, offers a more favorable biochemical environment for *in vitro* Adlai supporting both morphogenic competence and physiological integrity. This would lead to utilization of MCW which is most often treated as waste in the Philippines since only the coconut meat of the matured coconut are commonly used. This supplementation in new to in vitro culture of adlai.

CONCLUSION

In conclusion, this study found that coconut water effectively induces plant regeneration from mature adlai embryos. Specifically, incorporating 7.5% to 15% coconut water into the basal media produced plantlets with morpho-physiological characteristics comparable to, and in some cases superior to those treated with 6-BAP.

DECLARATION

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Authorship Contributions

Conceptualization, Experimentation, Writing: Jean Pontillas; Concept Review: Joseph Diaz, Nonnatus Bautista; Concept, experimentattion, writing : Mary Jhane Valentino.

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Competing interests

The authors declared that there is no conflict of interest.

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