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BIOLOGY

Somatic embryogenesis in leaf explants of genipap genotypes

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ABSTRACT: The genipap (*Genipa americana* L.) is a non-endemic species native to Brazil belonging to the family Rubiaceae. It is a species that unites socioeconomic and environmental pillars. The study objective was to establish the induction of indirect somatic embryogenesis in foliar explants of genipap genotypes. Leaf explants of UMB, SAL, JSA, SC, and CER accessions cultivated in half the salt concentration of Murashige & Skoog (MS), 30 g/L sucrose, and 3g/L Phytagel™ with the following combinations of NAA × BA regulators were used: M1: 4.0/4.0 mg/L NAA and BA, M2: 4.0/6.0 mg/L NAA and BA, and M3: 6.0/4.0 mg/L NAA and BA. At 60 days of culture, they were transferred to secondary embryogenic callus multiplication medium supplemented with 2.21 mg/L of 2,4-D. At 30 and 60 days, the increment (%) and fresh mass (g) of primary callus and at 120 days the presence of embryogenic callus were evaluated. The primary medium with 4.0 mg/L of NAA and 6.0 mg/L of BA induced embryogenic primary callus in leaf explants of the genipap accessions SAL, SC, and JSA. The secondary medium was promising for the multiplication of embryogenic callus. Cytochemical analysis confirmed the presence of embryogenic cells in SAL, SC, and JSA accessions.
Key words: *Genipa americana* L., Rubiaceae, plant tissue culture, *in vitro* propagation.

Indução da embriogênese somática em explantes foliares de genótipos de jenipapeiro

RESUMO: O jenipapeiro (*Genipa americana* L.) é uma espécie nativa não endêmica do Brasil pertencente à família Rubiaceae. É considerada uma espécie que une os pilares socioeconômicos e ambientais. O objetivo deste trabalho foi estabelecer a indução da embriogênese somática indireta em genótipos de jenipapeiro. Foram utilizados explantes foliares dos acessos UMB, SAL, JSA, SC e CER cultivados em meio primário composto por ½ MS, 30 g/L de sacarose e 3g/L de Phytagel™ com as combinações dos reguladores de ANA x BAP M1: 4,0/4,0 mg/L de ANA e BAP, M2: 4,0/6,0 mg/L de ANA e BAP e M3: 6,0/4,0 mg/L de ANA e BAP. Aos 60 dias de cultura foram transferidas para meio secundário de multiplicação de calos embriogênicos suplementado com 2,21 mg/L de 2,4-D. Aos 30 e 60 dias foram avaliados o incremento (%) e a massa fresca (g) dos calos primários e aos 120 dias a presença de calos embriogênicos. O meio primário com 4,0 mg/L de ANA e 6,0 mg/L de BAP induziu calos primários embriogênicos em explantes foliares dos acessos SAL, SC e JSA de jenipapeiro. O meio secundário foi promissor para multiplicação de calos embriogênicos. A análise citoquímica confirmou a presença de células embriogênicas nos acessos SAL, SC e JSA.
Palavras-chave: *Genipa americana* L., Rubiaceae, cultura de tecidos de plantas, propagação *in vitro*.

INTRODUCTION

Genipap (*Genipa americana* L.) is a non-endemic native species of Brazil belonging to the Rubiaceae family, occurring in the North, Northeast, Midwest, Southeast, and South regions of Brazil (FLORA DO BRASIL, 2020). The Plants of the Future Program of the CNPq/World Bank/Global Environment Facility/Ministry of the Environment (MMA)/Probio recognized *G. americana* as among the ten species with very high priority, with potential for immediate use among fruit trees native to Brazil (CORADIN et al., 2018).

It is considered a species that unites the socio-economic and environmental pillars. Its food, pharmacological, textile, and construction uses drive large industrial sectors. Its primary highlights are its cultural importance for the Indians and ecological importance due to its edaphoclimatic characteristics in projects to recover degraded areas and urban afforestation (BELLÉ et al., 2018). The wood is used in construction, joinery, furniture making, tool handles, and carpentry in general. The bark is used in tanneries to treat leather as it is rich in tannins. Its fruits, when still green, provide a bluish dye due to the presence of genipin. This coloring property was already well known by the Indians to dye fabrics, ornaments, and ceramics, and paint the body in religious ceremonies (SILVA et al., 2018). Genipin, an iridoid belonging to the monoterpene class, and has the potential to

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replace cross-linking agents in obtaining enzymes applied to the production and processing of products in the pharmaceutical and fuel industries, although it is mainly used in the food industry (BELLÉ et al., 2018). Based on this, the growing market for food enzymes stands out, valued at US$ 12.27 billion in 2022, reinforcing the greater interest of both industry and consumers in the use of enzymes in processes to benefit the health and nutritional quality of food (GRAN VIEW RESEARCH, 2022). Additionally, genipin has anticholestatic, anti-inflammatory, antidepressant, anticancer, and anti-obesity properties, among other curative and pharmacological actions (FENG et al., 2011).

Indiscriminate extractive exploitation jeopardizes the genetic diversity of the species, compromising natural regeneration and making it impossible to expand and improve its uses (OLIVEIRA et al., 2017). According to CHILUKAMARRI et al. (2021), new propagation techniques with greater efficiency and sustainability are needed in the horticultural industry to satisfy the global demand for food from an ever-growing population.

Plant tissue culture has become a great tool for agriculture, with multiple functions, such as plant propagation and conservation, genetic transformation, and germplasm exchange. Promising results were obtained for the species in terms of in vitro propagation (SÁ et al., 2016; OLIVEIRA et al., 2017) and cryopreservation (NASCIMENTO et al., 2020).

Somatic embryogenesis is a morphogenic route for obtaining complete plants, without the fusion of gametes. It can occur directly, with a somatic embryo formed from the explant, or indirectly, including the callogenesis stage (MÉNDEZ-HERNÁNDEZ et al., 2019), allowing clones with genetic fidelity and phytosanitary quality to be obtained quickly. It can be used as a tool to increase the quality and productivity of elite genotypes (SUN et al., 2022). It involves three stages, as follows: induction of embryogenic callus, somatic embryogenesis, and development of somatic embryos (ELHITI et al., 2013). Each stage comprises the interaction of multiple factors, for example, external signals, the changes in the endogenous concentrations of different plant growth regulators (PGRs), and expression of numerous genes. Different studies support the theory that the early stages of somatic embryogenesis are characterized by the induction of numerous stress-related genes (MÉNDEZ-HERNÁNDEZ et al., 2019).

Most in vitro asexual regeneration protocols for genipap occur via organogenesis, with the species having good organogenic potential (SILVA et al., 2018). However, the establishment of somatic embryogenesis protocols is still unknown. This research studied the induction of somatic embryogenesis in genipap from leaf explants, and analyzed different types of genotypes and concentrations of growth regulators.

MATERIALS AND METHODS

Origin of the explants

Seedlings previously established in vitro from five accessions of the BAG Genipap of Embrapa Tabuleiros Costeiros, Sergipe, Brazil were used as a source of explants: accession CER from Núcleo Bandeirante, DF (15°51'55.72"S; 47°57'34.59"W); accession UMB from Umbaúba, SE (11°22'34"S; 37°39'11"W); accession JSA from Coronel João Sá, BA (10°17'3"S; 37°55'37"W) accession SC from São Félix, BA (12°40'0"S; 40°43'60"W) and accession SAL from Salvaterra, PA (0°45'50.32''S; 48°30'40.44''W).

To obtain in vitro seedlings, seeds from ripe fruit were aseptically treated in a laminar flow chamber by immersing them in 70% alcohol for 5 min, 20 min in a 1% sodium hypochlorite solution, and washed three times in autoclaved sterile water. The seeds were then inoculated into MS medium (Sigma-Aldrich, M5519) supplemented with 30 g/L sucrose and gelled with 3 g/L Phytagel™ (Sigma, P8169) (PEREIRA et al., 2007).

Embryogenic callus induction and multiplication tests

Rectangular leaf explants were obtained from the central leaf zone with an area of approximately 0.5 cm^2 from five genipap accessions (CER, UMB, SC, JSA, and SAL). The explants were inoculated into embryogenic callus induction medium with three combinations of the growth regulators NAA (naphthalene acetic acid) and BA (6-benzylaminopurine). The following combinations were evaluated: M1: 4.0 mg/L NAA and 4.0 mg/L BA; M2: 4.0 mg/L NAA and 6.0 mg/L BA; M3: 6.0 mg/L NAA and 4.0 mg/L BA. The leaf explants were placed with the adaxial side in contact with the culture medium and inoculated into sterile polystyrene Petri dishes (50 × 10 mm) containing 20 mL of culture medium with half the salt concentration of Murashige & Skoog (1962) - MS (½ MS) supplemented with 30 g/L sucrose, 100 mg/L hydrolyzed casein, 400 mg/L malt extract (BARTOS et al., 2018), and gelled with 3 g/L Phytagel™. The pH of the culture medium was adjusted to 5.8 and then sterilized in an autoclave

at 120 °C ± 1 °C for 20 min. The cultures were placed in the dark for 60 days in a growth room at a temperature of 25 °C ± 2 °C and an average relative humidity of approximately 70%. After 60 days of culture, they were transferred to secondary medium for the multiplication of embryogenic calluses (secondary medium: primary medium + 2.21 mg/L of 2,4-dichlorophenoxyacetic acid [2,4-D]) and after 90 days to a photoperiod of 12 hours of light with a light intensity of 60 µmol/m^2/s. At 30 and 60 days, the fresh mass of the embryogenic calluses (g) was assessed and the increase (%) in callus mass over the period from 30 to 60 days was determined. A precision scale (0.001 g) was used to obtain the fresh mass callus. The presence of embryogenic callus at 120 days was detected using a LEICA Z4 magnifying glass.

The experiment was set up in a completely randomized design with a 5 × 3 factorial scheme (5 accessions ×3 combinations NAA x BA) and six replicates, each experimental unit consisting of five Petri dishes with four explants each.

Cytochemical analysis

For the cytochemical analysis, callus cultures from the previous trial were randomly selected after 90 days of cultivation. Samples of 100 mg of callus were stained with 3–5 drops of Evans blue-AE (0.1%) for 3 min, and after removing the excess dye, 3–5 drops of acetic carmine-CA (2%) were added for 3 min (GUERRA, 1999). Somatic embryos were visualized using a LEICA EZ4 magnifying glass at 4× and 10× magnification.

Statistical analysis

The data obtained was submitted to analysis of variance using the F test at 5% significance. The means were compared using the Tukey's test at 5% significance in the SISVAR statistical program (FERREIRA, 2019).

RESULTS AND DISCUSSION

At 15 days of culture, callus formation was observed on the leaf explants in primary medium. For the fresh mass of callus, a significant effect existed of the single factor genipap accessions and the interaction between accessions and NAA and BA combinations at 30 and 60 days of in vitro culture (Table 1). The UMB accession exhibited afresh mass of embryogenic callus at 30 and 60 days (0.44 and 1.18 g, respectively) on M2 medium (4.0 mg/LNAA and 6.0 mg/L BA). The SC accession reached the highest callus mass (0.84 g) only when exposed to M3 medium (6.0 mg/L NAA and 4.0 mg/L BA) and CER and SAL accessions did not exceed 0.60 g in all the combinations of regulators studied. The JSA accession showed good fresh callus mass production at 60 days in M1, M2, and M3 (0.93, 0.70, and 0.74 g, respectively).

Although, the SAL accession achieved the lowest average fresh mass (0.45 g) in the presence of 4.0 mg/L NAA and 4.0 mg/L BA at 60 days, the mass value was triple of that obtained by OLIVEIRA et al. (2018), who obtained only 0.15 g at 60 days in the combination of 2.0 mg/L 2.4-D and 1.77 mg/L BA. The CER accession showed a different response to that observed by OLIVEIRA et al. (2018) in leaf explants in the presence of 2.0 mg/L 2.4-D and 1.77 mg/L BA, accumulating an average fresh mass of 0.096 g, showing the influence of the growth regulator on the in vitro behavior of the accessions.

For the increase in the fresh mass of embryogenic calluses from 30 to 60 days, a significant interaction effect was observed between the factors accessions and NAA and BA combinations (Table 1).The UMB accession on M2 medium achieved the greatest increase in fresh embryogenic callus mass from 30 to 60 days (74.14%). The other accessions; however, did not achieve a 50% increase in fresh mass in the combinations of regulators tested, with the exception of JSA accession in M1 (60.22%). According to reports by TUSKAN et al. (2018), individual species vary in their ability to form callus. Therefore studies of individual genotypes are recommended for a more complete analysis of the ability of the species to form callus, and subsequently, somatic embryos.

After 120 days of cultivation, a significant effect was observed of the interaction between accessions and combinations of NAA and BA on the formation of embryogenic callus (Table 1). The SAL accession, which had the lowest variation in increment (18–25%) of primary callus, had 100% induction of embryogenic callus in two combinations of NAA and BA (M1 and M2). This result shows that despite the late development of the SAL accession, with low production synchronicity in the primary medium, it showed greater multiplication intensity in the secondary medium.

The UMB accession, which obtained good results in the primary medium, at this stage showed high oxidation in the cultures started in media M1 and M2. To a lesser extent, accession SC in medium M3 had less oxidation in some cultures. Oxidation is considered a limiting factor in plant tissue culture. When it occurs, the cultures darken

Table 1 - Fresh mass of callus (g), increase (%) at 30 and 60 days and induction of embryogenic callus at 120 days of in vitro cultivation of accessions of *Genipa americana* L. in different combinations of NAA and BA.

Time (Days)						
	30			60		
Accessions	Fresh mass of callus (g)					
	M1	M2	M3	M1	M2	M3
UMB	0.37 Aa	0,44 Aa	0.35 ABa	0.80 ABb	1.18 Aa	0.66 ABb
JSA	0.33 Aba	0,24 Ba	0.30 ABa	0.93 Aa	0.70 Ba	0.74 ABa
SC	0.22 BCb	0,24 Bb	0.38 Aa	0.56BCa	0.62BCa	0.84 Aa
CER	0.25 BCa	0,21 Ba	0.24 BCa	0.60BCa	0.53 BCa	0.57 ABa
SAL	0.19 Ca	0,13 Ba	0.13 Ca	0.45Ca	0.31 Ca	0.42 Ba
VC (%): 18.87					VC (%):21.26	
Callus increase (%)						
Accessions		M1		M2	M3	
UMB		42.83 ABb		74.14 Aa	31.69 Ab	
JSA		60.22 Aa		45.90 Ba	43.62 Aa	
SC		33.56 Ba		38.81 ABa	46.07 Aa	
CER		35.42 ABa		31.99 ABa	32.82 Aa	
SAL		25.75 Ba		18.00 Ba	25.46 Aa	
VC (%): 27.09						
Induction of embryogenic callus (%)						
Accessions		M1		M2	M3	
UMB		0.00 Ba		0.00 Ba	50.00 Aa	
JSA		25.00 Bb		100.00 Aa	25.00 Ab	
SC		25.00 Bb		100.00 Aa	50.00 Aab	
CER		0.00 Ba		25.00 Ba	0.00 Ba	
SAL		100,00 Aa		100.00 Aa	50.00 Aa	
VC (%): 84.27						

Averages followed by the same uppercase and lowercase letters in the column and row, respectively, do not differ according to the Tukey's test at 5% probability. M1: 4.0 mg/L NAA and 4.0 mg/L BA; M2: 4.0 mg/L NAA and 6.0 mg/L BA; and M3: 6.0 mg/L NAA and 4.0 mg/L BA. Accessions: UMB - Umbaúba- SE, JSA- Coronel João Sá- BA, CER -Núcleo Bandeirante- DF, SC -São Félix- BA and SAL-Salvaterra- PA.

due to the release of phenolic compounds, impairing callus growth (CID & TEIXEIRA, 2014). The oxidation should be considered in the multiplication rate of embryogenic calluses. After a certain time of in vitro cultivation, the calluses showed a dark cream color with low embryo conversion rates. Generally, oxidation is influenced by the type of explant, genotype, culture medium components, and culture conditions, and can cause losses in the experiment due to the death of the explant.

The combination of regulators in the M2 culture medium was promising, inducing 100% of embryogenic callus in the JSA, SC, and SAL accessions and the M3 medium for the UMB accession. The CER accession had the lowest response to the combinations of regulators tested, showing a low embryogenic potential, since all the accessions were kept under the same cultivation conditions. These results indicated the need to adjust the NAA and BA balance, with a higher concentration of NAA, for the induction of embryogenic callus in CER and a greater response from UMB accession.

The morphological analysis of the cultures (Figure 1) showed that the embryogenic calluses have similar characteristics to those of *Coffea arabica* L. BARTOS et al. (2018) described them as intense yellow in color, with reduced growth.

Considering the CER accession in the C2 culture medium, the induction of primary callus characteristic of type 2 was observed, with a whitish

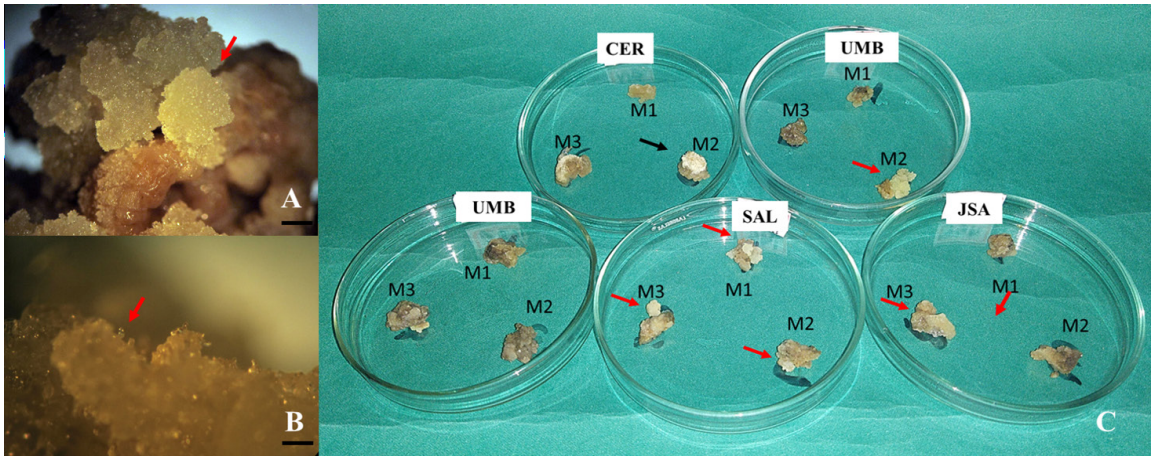

Figure 1 - (A) Embryogenic calli from leaf explants of *Genipa americana* L. (B) Globular embryo (C) Embryogenic calli from accessions cultivated in MS médium with different combinations of NAA and BA.

M1: 4.0 mg/L NAA and 4.0 mg/L BA; M2: 4.0 mg/L NAA and 6.0 mg/L BA; and M3: 6.0 mg/L NAA and 4.0 mg/L BA. Red arrows: embryogenic callus and globular embryo (B); black arrow: type 2 callus. Accessions: UMB: Umbaúba, SE; JSA: Coronel João Sá, BA; CER: Núcleo Bandeirante, DF; SC: São Félix, BA; and SAL: Salvaterra, PA. Bar: 50 µm.

color. According to BARTOS et al. (2018), type 2 callus compete with and inhibit the formation of embryogenic calluses.

The cytochemical test of the accessions with the AE 0.1% and CA 2% dyes and positive reaction of the cells to the CA dye confirmed the presence of pro-embryogenic cells in the SAL and SC accessions for the three combinations of regulators tested and in the JSA accession in the M3 combination (Figure 2). The stained and unstained cells in AE correspond to non-embryogenic cells, found in large quantities in the UMB and CER accessions in the combinations of regulators tested. It is possible to observe the presence of blue and red colored calluses, indicating the presence of both embryogenic and non-embryogenic cells in the same material.

Embryogenic cultures present the following two cell morphologies: rounded with dense cytoplasm that can stain red with CA or elongated with the presence of a vacuole stained blue by AE (GUPTA & DURZAN 1987; STEINER et al., 2016). According to LOPES et al. (2016) callus and pro-embryonic masses react vigorously to CA and weakly to EA and for STEINER et al. (2016) the positive CA reaction is linked to the competence of the cell for development.

The dual dye cytochemical test is widely used in various studies of embryogenic potential. SANTOS et al. (2015) verified in response to the cytochemical test that the callogenesis of *Jatropha curcas* L. (jatropha, Euphorbiaceae) is optimized in MS medium supplemented with 4.52 mg/L 2.4-D and 2.22 mg/L BA, inducing an embryogenic response in the species.

In vitro somatic embryogenesis is affected by many factors such as explant, genotype, growth regulators, nutrient medium, physical culture environment (DESAI et al., 2022). Considering these aspects, a somatic embryogenesis protocol was established covering these factors that make it possible to choose the best genotype associated with this culture medium are extremely important.

CONCLUSION

Genipa americana L. genotypes have embryogenic potential in the presence of NAA and BA. The primary medium with 4.0 mg/L NAA and 6.0 mg/L BA induces primary embryogenic callus in leaf explants of the genipap accessions SAL, SC, and JSA. The secondary medium supplemented with 2.21 mg/L 2.4-D is promising for the multiplication of

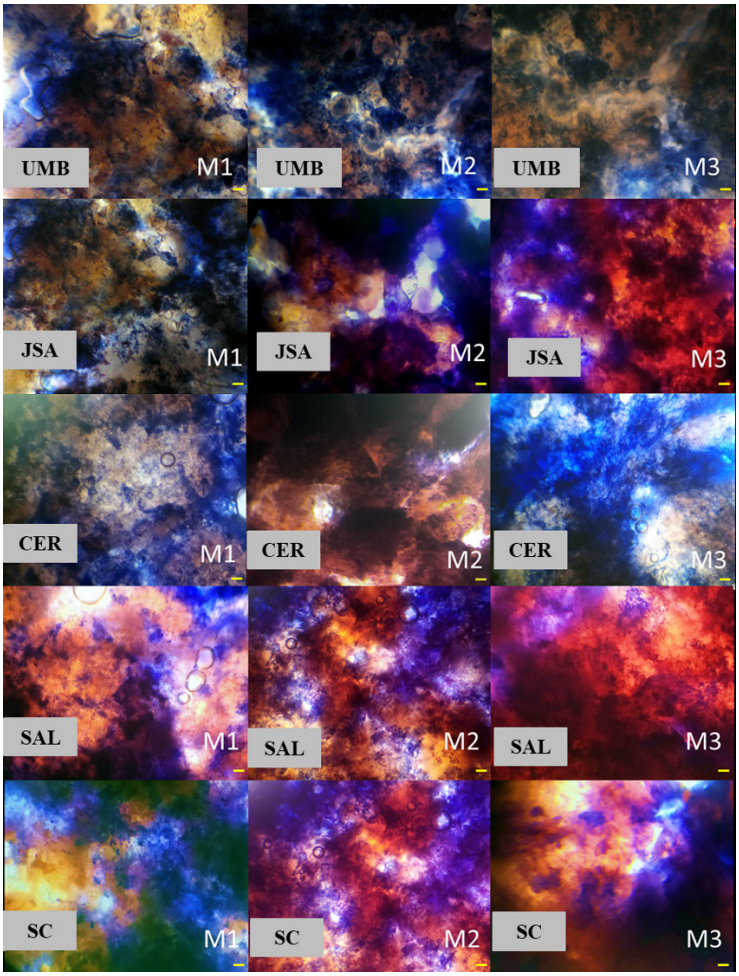

Figure 2 - Acetic carmine and Evans blue analysis of embryogenic calluses from leaf explants of five accessions of *Genipa americana* L. grown in MS with different combinations of NAA and BA (mg/L).

M1: 4.0 mg/L NAA and 4.0 mg/L BA; M2: 4.0 mg/L NAA and 6.0 mg/L BA; and M3: 6.0 mg/L NAA and 4.0 mg/L BA. Bar: 100 µm. Accessions: UMB: Umbaúba, SE; JSA: Coronel João Sá, BA; CER: Núcleo Bandeirante, DF; SC: São Félix, BA; and SAL: Salvaterra, PA.
El João Sá, BA, CER -Núcleo Bandeirante, DF, SC -São Félix- BA e SAL-Salvaterra, PA.

embryogenic callus. Cytochemical analysis confirms the presence of embryogenic cells in the SAL, SC, and JSA accessions.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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