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Optimized *In vitro* Propagation and Somatic Embryogenesis of Camu Camu (*Myrciaria dubia*) Cultivar Vitahuayo for Enhanced Commercial Production

Pedro M. Adrianzén[1,2], Sergio F. Pinedo[3], Barbara S. Valles[2], Jorge L. Marapara[2], Marianela Cobos[1,2], Hicler N. Rodríguez[1,2], Juan C. Castro[1]

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ABSTRACT

Background: *Myrciaria dubia* "camu-camu" a fruit-bearing shrub native to the Amazon, is known for its high vitamin C content. However, variations in vitamin C biosynthesis and accumulation among different cultivars present challenges for commercial production. This study aimed to establish an efficient *in vitro* regeneration protocol for the Vitahuayo cultivar through callus induction and somatic embryogenesis.

Methods: Stem and leaf explants were cultured on Murashige and Skoog (MS) medium supplemented with 24-dichlorophenoxyacetic acid (24-D) and 6-benzylaminopurine (BAP) to induce callus formation. Somatic embryos were induced using Woody Plant Medium (WPM) supplemented with 2,4-D, thidiazuron (TDZ), indole-3-butyric acid (IBA), naphthaleneacetic acid (NAA) and kinetin (KIN).

Result: The best results for callogenesis were obtained with 1 mg/L of 24-D and 0.5 mg/L BAP. Somatic embryos were successfully induced with treatments T2 (2,4-D at 3 mg/L, NAA at 20 mg/L and KIN at 15 mg/L) showing 90% efficiency, while T3 (KIN at 0.3 mg/L and BAP at 0.05 mg/L) achieved 57.5% effectiveness. These findings offer great potential for the large-scale propagation of Vitahuayo, the optimization of its commercial production and the standardization of vitamin C biosynthesis.

Key words: Ascorbic acid, Callus induction, Plant regeneration, Somatic embryogenesis.

INTRODUCTION

Myrciaria dubia (Kunth) McVaugh "camu-camu" is an Amazonian shrub known for its exceptional ability to produce and store remarkable amounts of vitamin C in its fruits, with concentrations exceeding 2000 mg/100 g of pulp, as well as pharmacologically important compounds (Castro *et al.,* 2019). Its high demand in the local, national and international markets (Iman, 2001) has promoted camu-camu production programs to develop sustainable agriculture in the Peruvian Amazon. However, despite these efforts, the global demand for camu-camu has fluctuated in recent years (MINCETUR, 2021). One of the main reasons for these demand fluctuations is the inconsistent vitamin C content in camu-camu fruits, both in natural plantations and in farmer plots. This was evidenced in *M. dubia* germplasm bank accessions at the National Institute of Agricultural Innovation (INIA), which varied from 650 to 2475 mg vitamin C/100 g pulp (Córdova *et al.,* 2014). To overcome this limitation, it is essential to establish strategies to clone genotypes with high vitamin C production. Plant biotechnology, particularly plant tissue culture, can offer a solution (Bijalwan and Shilpa, 2021; Levitus *et al.,* 2010) by significantly improving the multiplication rates of camu-camu, addressing low natural propagation rates and ensuring a sustainable supply (Ewa, 2018). To date, *in vitro* propagation studies of camu-camu have provided insufficient results (da Rocha *et al.,* 2016). This underscores the need to explore alternative strategies, such as indirect somatic embryogenesis through callus induction. In light of this, the following question arises: what concentrations of growth regulators are most effective in inducing callus formation and promoting somatic embryogenesis *in vitro* in *M. dubia* cultivar (cv) INIA 395 - Vitahuayo? Previous studies have evaluated the effects of different hormone concentrations and disinfection treatments on the *in vitro* propagation of

[1]Specialized Unit of Biotechnology Research Laboratory, Natural Resources Research Center of UNAP, Universidad Nacional de la Amazonía Peruana (UNAP), Iquitos 16001, Peru.

[2]Academic Department of Biomedical Sciences and Biotechnology, Faculty of Biological Sciences, Universidad Nacional de la Amazonía Peruana (UNAP), Iquitos 16001, Peru.

[3]San Roque Agricultural Experiment Station, Direction of Genetic Resources and Biotechnology, Instituto Nacional de Innovación Agraria (INIA), Iquitos 16001, Peru.

Corresponding Author: Pedro M. Adrianzén, Specialized Unit of Biotechnology Research Laboratory, Natural Resources Research Center of UNAP, Universidad Nacional de la Amazonía Peruana (UNAP), Iquitos 16001, Peru.
Email: pedro.adrianzen@unapiquitos.edu.pe

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M. dubia (Córdova *et al.,* 2014; da Rocha *et al.,* 2016; Pinedo *et al.,* 2013). Based on these studies, the present study aimed to determine the optimal concentrations of growth regulators to induce callus formation and promote somatic embryogenesis *in vitro* in *M. dubia* cv INIA 395-Vitahuayo.

MATERIALS AND METHODS

The research was conducted between 2021 and 2023 in the Plant Tissue Culture Laboratories of the National University of the Peruvian Amazon (UNAP) and the National Institute of Agricultural Innovation (INIA), Iquitos, Peru.

Botanical samples (bud branch) were obtained through simple random sampling from *M. dubia* "camu camu" plants of the new cultivar called "INIA 395 - Vitahuayo. This cultivar is officially recognized according to Jefatural Resolution No. 0040-2021-INIA (INIA, 2021). The samples were collected from the "El Dorado" Experimental Field of the San Roque Agrarian Experimental Station of INIA, located on the Iquitos-Nauta Road.

The bud branch was immersed in 0.60% quaternary ammonium solution for 24 h. Each branch was then cut into two parts to obtain a bud branch of 20 cm in length, with a horizontal cut (upper part) and a bevel cut (lower part). They were placed in 150 × 25 mm test tubes with 20 mL of tap water and a water-soluble antifungal paste (Cupravit) was applied to the horizontal cut area. The bud branch was moistened daily with sprayed water and the water in each tube was changed every three days. Additionally, 5 mL of 0.01% Vitrofural was added to each tube and the buds of the bud branch every weekend. The bud branch was sprayed with 2% Bayfolan solution one month after collection.

Shoots with 2 to 4 nodal segments were cut and immersed in a 0.2% Benomyl fungicide solution (commercial product Benzomil 500) for transport to the plant tissue culture laboratory. The shoots were then agitated for 1 hour and rinsed with sterile water up to four times. Four treatments were tested for explant disinfection, each with 0.3%, 0.5%, 1.0% and 1.5% NaClO and a control without disinfectant. For each liter of disinfectant, 100 µL of Tween 20 was added. Four disinfection times (5, 10, 15 and 20 min) were applied for each treatment. The shoots were then rinsed with sterile distilled water and 1 cm^2 leaf area and 1 cm stem or internode segments were cut.

After disinfecting the plant explants, they were placed in Petri dishes containing MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.8% agar. The pH of the solution was adjusted to 5.7. Twelve treatments were used for callus induction in the leaf and stem explants, with four explants per dish and three replicates. The phytohormonal treatments were: T1: 2,4-D 0.0 mgL^{-1} + BAP 0.0 mgL^{-1}, T2: 2,4-D 0.0 mgL^{-1} + BAP 0.1 mgL^{-1}, T3: 2,4-D 0.0 mgL^{-1} + BAP 0.5 mgL^{-1}, T4: 2,4-D 1.0 mgL^{-1} + BAP 0.0 mgL^{-1}, T5: 2,4-D 1.0 mgL^{-1} + BAP 0.1 mgL^{-1}, T6: 2,4-D 1.0 mgL^{-1} + BAP 0.5 mgL^{-1}, T7: 2,4-D 2.0 mgL^{-1} + BAP 0.0 mgL^{-1}, T8: 2,4-D 2.0 mgL^{-1} + BAP 0.1 mgL^{-1}, T9: 2,4-D 2.0 mgL^{-1} + BAP 0.5 mgL^{-1}, T10: 2,4-D 3.0 mgL^{-1} + BAP 0.0 mgL^{-1}, T11: 2,4-D 3.0 mgL^{-1} + BAP 0.1 mgL^{-1} and T12: 2,4-D 3.0 mgL^{-1} + BAP 0.5 mgL^{-1}. Leaf explants were placed on the adaxial side in contact with the culture medium and stem explants were placed in a horizontal position. They were kept in the dark for 15 days at 25±2°C and then exposed to a photoperiod of 16 hours light and 8 hours dark. Callus development, contaminants (fungi and bacteria) and oxidation were evaluated weekly (Ronquillo, 2005). Three replicates of 32 explants were evaluated for both the leaves and stems.

To induce somatic embryos, stem calli were transferred to a woody plant medium (Lloyd and McCown, 1980), supplemented with 3% sucrose, 0.4% PVP and 0.6 g/l agar, with pH adjusted to 5.7. For the embryogenic treatment, four calli were cultured per plate. Seven phytohormonal treatments were established: T1: 2,4-D 3.0 mgL^{-1} + NAA 20.0 mgL^{-1}, KIN 2.0 mgL^{-1}, T2: 2,4-D 3.0 mgL^{-1} + NAA 20.0 mgL^{-1}, T3: KIN 0.3 mgL^{-1} + BAP 0.05 mgL^{-1}, T4: 2,4-D 3.0 mgL^{-1} + NAA 2.0 mgL^{-1}, T5: TDZ 1.5 mgL^{-1} + IBA 0.5 mgL^{-1}, T6: NAA 1.0 mgL^{-1} + BAP 1.0 mgL^{-1} + GA*ƒ* 1.0 mgL^{-1}, T7: NAA 0.5 mgL^{-1} + KIN 3.0 mgL^{-1}. They were kept in the dark for 15 days at 25±2°C and then exposed to a photoperiod of 16 hours light and 8 hours dark. The cultures were evaluated weekly to determine which phytohormonal treatments induced embryo formation.

A completely randomized design was used in this study. To assess the normality of the data anderson-Darling tests, variance analysis, Kruskal-Wallis, Tukey's multiple comparison and Mann-Whitney tests were applied, with a significance level of $p<0.05$. Statistical analyses were conducted using Minitab version 21.4 and Microsoft Office Excel 2021.

RESULTS AND DISCUSSION

Contamination and oxidation of explants

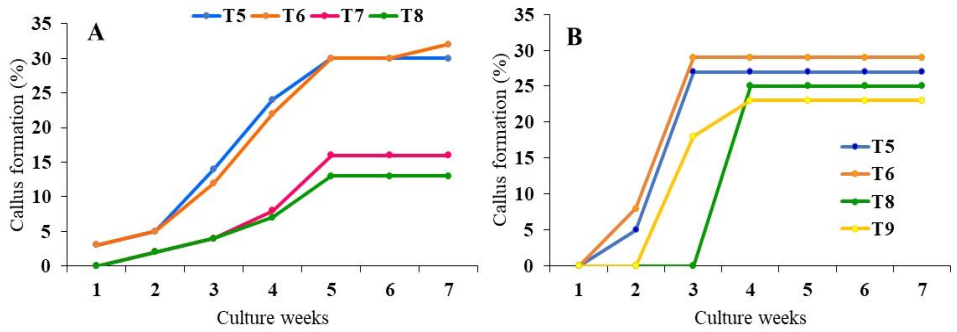
Using 1% NaClO resulted in a significantly higher number of explants without contamination or oxidation, with an effectiveness of 51.7%, outperforming the other concentrations. Although some fungal and bacterial contamination was observed, the oxidation rate was considerably lower than the 1.5% and 2% concentrations, which showed high oxidation rates (41.4% - 48.5%). These results indicated that the optimal concentration of NaClO to minimize contamination and oxidation of explants was 1%. Contamination of explants is a crucial challenge for in vitro callus induction. Despite maintaining controlled conditions, the presence of fungi, bacteria and explant oxidation are issues already reported in in vitro cultures (Córdova *et al.,* 2014; Pinedo *et al.,* 2013), including the presence of endophytic microorganisms or laboratory contaminants (Cassells, 1997). In addition to microorganism contamination, explant oxidation was another issue, especially in stems. This could be due to the lower concentration of ascorbic acid compared to leaves, which

have a better antioxidant response (Córdova *et al.,* 2014). Oxidation is common in woody plants because of the presence of lignin and phenolic compounds (Kaur *et al.,* 2020) and the stress caused by cutting and exposure to sodium hypochlorite solutions (Slater *et al.,* 2008). Reducing the concentration of hypochlorite decreased oxidation, but this remained a challenge in the in vitro cultivation of Vitahuayo.

Callogenesis induction

In stem explants, white-green, friable and compact calluses were observed, mainly at the ends and periphery, covering up to 75% of the surface. In contrast, leaf explants also generated calluses, although in smaller quantities (30%) (Fig 1). In stem explants, the treatments with development in the first week were T5 (1 mgL^{-1} 2,4-D - 0.1 mgL^{-1} BAP) and T6 (1 mgL^{-1} 2,4-D - 0.5 mgL^{-1} BAP), followed in the second week by T7 (2 mgL^{-1} 2,4-D - 0 mgL^{-1} BAP) and T8 (2 mgL^{-1} 2,4-D - 0.1 mgL^{-1} BAP) (Fig 1A). Tukey's Multiple Comparison Test indicated that T5 and T6 had significantly higher means than did the other treatments ($p<0.05$) (Table 1). For leaf explants, the treatments that showed development from the second week were T5 (1 mgL^{-1} 2,4-D + 0.1 mgL^{-1} BAP) and T6 (1 mgL^{-1} 2,4-D + 0.5 mgL^{-1} BAP), followed in the third week by T3 (0.0 mgL^{-1} 2,4-D + 0.5 mgL^{-1} BAP) and T9 (2 mgL^{-1} 2,4-D + 0.5 mgL^{-1} BAP) and in the fourth week by T8 (2 mgL^{-1} 2,4-D - 0.1 mgL^{-1} BAP) (Fig 1B), with T5 and T6 showing the best response.

The Kruskal-Wallis test shows that T6 is above the global median (Z=2.41), *i.e.*, it has a higher average rank compared to other treatments ($p<0.05$), while the Mann-Whitney test indicates that T6 (1 mgL^{-1} 2,4-D - 0.5 mgL^{-1}



(A) Percentage of callus formation in stem explants. (B) Percentage of callus formation in leaf explants. The values correspond to the averages of the treatments with 3 replications. Significant differences for stem explants were determined using Tukey's test ($p<0.05$) and for leaf explants using Kruskal-Wallis and Mann-Whitney tests ($p<0.05$).

Fig 1: Callus induction in cv. Vitahuayo.

Table 1: Effects of different concentrations and combinations of hormones on callus induction from stem and leaf explants of cv. Vitahuayo.

Treatments		Callus in steam			Callus in leaf				
2,4-D (mgL^{-1})	BAP (mgL^{-1})	% Formation (Steam)	Quality (Steam)	Mean (Steam)	% Formation (Leaf)	Quality (Leaf)	Mean (Leaf)	Z-value (Leaf)	Medians M-W (Leaf)
0	0.1	15.6	++	2.85b	25.0	++			
0	0.5	18.7	++	3.29b	25.0	++	10.9	-2.06	
1	0.1	93.7	+++++	19.43a	84.4	+++++	22.2	1.22	27,00
1	0.5	100.0	+++++	19.14a	90.6	+++++	26.4	2.41	29,00
2	0.1	40.6	+++	7.43b	78.1	++++	15.6	-0.70	
2	0.5	31.2	+++	5.57b	71.9	+++	15.0	-0.87	
3	0.1	18.7	++	3.42b	40.6	++			
3	0.5	21.8	++	4.29b	43.75	++			

Data from 3 replicates of 32 explants each. The data comes from 3 replicates of 32 explants each.

Means followed by the same letter are not significantly different according to Tukey's multiple range test, $p<0.05$. Means followed by the Z value according to the Kruskal-Wallis test with $p<0.05$.

Quality rating: + (bad) ~ +++++ (good). Medians according to the Mann-Whitney test with $p<0.05$.

Quality Rating: + (poor) ~ +++++ (good).

BAP) has a greater effect compared to other treatments ($p<0.05$) (Table 1).

After reviewing experiments with various combinations of hormones, including auxins, cytokinins and gibberellins, the synthetic auxins 2,4-D and cytokinin BAP were selected based on previous studies on callus formation (Chagas *et al.,* 2023; Córdova *et al.,* 2014; da Rocha *et al.,* 2016; Pinedo *et al.,* 2013). The analysis of variance showed no significant differences in callus formation between treatments T5-T6 and T7-T8. However, T5 and T6 exhibited early formation (in the first week), whereas T7 and T8 did so in the second week. A significant auxin/cytokinin ratio favors callus formation (Ikeuchi *et al.,* 2013). Similar results in callus induction in *M. dubia* explants were obtained by Pinedo (Pinedo *et al.,* 2013), who used a BAP concentration 5-40 times higher than that used in this study. Similarly, Córdova (*Córdova et al.,* 2014) and da Rocha (da Rocha *et al.,* 2016) used twice the concentration of 2,4-D, whereas the BAP concentration was similar to that used in this study. Additionally, Chagas (Chagas *et al.,* 2023) used twice the concentration of 2,4-D and a BAP concentration that was 5-25 times higher. The authors obtained calluses at 30 days, except for Córdova (Córdova *et al.,* 2014), who obtained calluses in the second week. In the present study, calluses were obtained starting from day seven, particularly in stem explants for T5:1 mgL^{-1} 2,4-D and 0.1 mgL^{-1} BAP and T6:1 mgL^{-1} 2,4-D and 0.5 mgL^{-1} BAP.

The lethargy in the callogenic response observed in certain experiments with plant explants may be due to the autoclaving of phytohormones along with the medium, which affects their efficacy in inducing callus formation. This sterilization can degrade or alter hormonal activity, thereby affecting cellular responses. Research on other species, such as Rauvolfia serpentina (Gupta *et al.,* 2014), highlights the importance of precise concentrations of phytohormones and application methods in tissue culture. It is essential to follow established protocols for the handling and application of phytohormones to ensure successful callogenic responses and efficient tissue culture techniques.

Somatic embryo induction

Somatic embryo induction was observed starting from the sixth week in only two hormone treatments: T2 and T3. T2, consisting of 2,4-D (3 mgL^{-1}) + NAA (20 mgL^{-1}) + KIN (15 mgL^{-1}), was 90% effective in inducing somatic embryos. On the other hand, T3, which included KIN (0.3 mgL^{-1}) + BAP (0.05 mgL^{-1}), had 57.5% effectiveness of the total exposed calluses. It is important to note that the other treatments were ineffective in inducing embryos. Statistical analysis revealed no significant differences in the embryogenic response between treatments T2 and T3 (Kruskal-Wallis $p>0.05$), suggesting that both treatments can be equally effective in inducing somatic embryos. In contrast, the callogenic mass presented as a compact mass with green and brown colors, as shown in Fig 2(A-B-C). The embryos at the heart stage appeared as globular structures with yellow-brown and green colors, as shown in Fig 2(D-E). Finally, mature embryos in the third developmental stage showed clear differentiation into the torpedo stage (Fig 2F).

The low response to callus formation and embryogenesis in woody species may be influenced by the high exudation of phenols and other compounds, which affects the efficiency of somatic embryogenesis. Studies on various woody plants, such as *Larix kaempferi*, *Pinus koraiensis* and *Theobroma cacao*, have highlighted the importance of phenolic compounds in regulating embryogenic potential

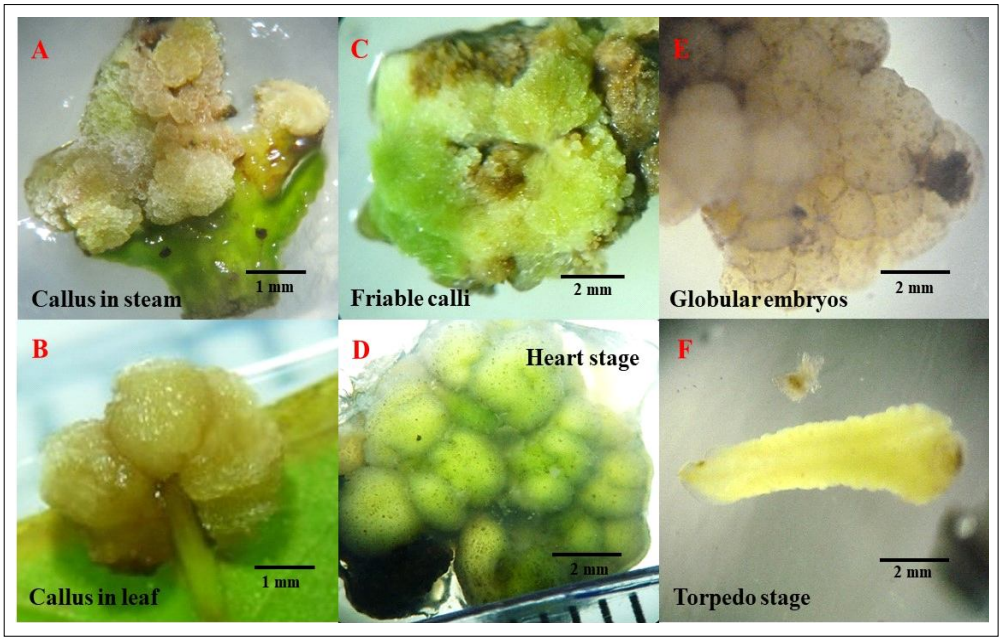


Fig 2: Calli obtained from stem and leaf explants (A-B-C) and the stages of somatic embryo development in cv. Vitahuayo (D-E-F).

(Gao *et al.,* 2021; Wang *et al.,* 2022). These compounds can affect the redox state of the culture medium, thereby influencing cell proliferation rates and the induction of embryogenic calluses. Additionally, the accumulation of phenolic compounds in tissue cultures can hinder further development and affect the production of somatic embryos (Jang *et al.,* 2016).

The combination of 2,4-D and naphthaleneacetic acid (NAA) has been widely studied for its role in the induction of callus formation and promotion of somatic embryogenesis (Phuong *et al.,* 2021). Research on *Coffea arabica* and *Coffea canephora* has shown that 2,4-D, a synthetic auxin, plays a crucial role in promoting proliferation and dedifferentiation of somatic cells, leading to the formation of somatic embryos (Arimarsetiowati *et al.,* 2023; Oliveira *et al.,* 2022). Furthermore, studies on rice cultivars have highlighted the importance of plant growth regulators, such as 2,4-D and kinetin, in enhancing callus growth and somatic embryogenesis efficiency (Sidek *et al.,* 2022). Similarly, experiments with pineapple plants showed that a combination of 2,4-D and BAP (6-benzylaminopurine) resulted in high rates of somatic embryo formation, emphasizing the importance of specific growth regulator combinations in morphogenic responses (Kessel-Domini *et al.,* 2022). However, research on cytokinins such as kinetin (KIN) and 6-benzylaminopurine (BAP) highlights their role in promoting cell division, bud formation and somatic embryogenesis when combined with auxins in specific ratios (Ash *et al.,* 2020; Martins *et al.,* 2022). Studies have shown that KIN and BAP induce shoots with distinct anatomical and biochemical characteristics, where BAP leads to underdeveloped features, while KIN stimulates differentiation over proliferation and a combination of both can mitigate the negative effects of BAP on growth (Avilez-Montalvo *et al.,* 2022).

The synergistic effects of 2,4-D, NAA, KIN and BAP have been well documented in promoting callus induction and somatic embryogenesis in various plant species, with the balance between auxins and cytokinins being crucial for directing somatic embryogenesis. However, the explant type (da Rocha *et al.,* 2016) and species genotype (Dias *et al.,* 2018) also influence these processes. Therefore, callus induction and somatic embryogenesis in cv. Therefore, Vitahuayo should focus on optimizing protocols to achieve adequate dedifferentiation and seedling formation.

CONCLUSION

This study successfully optimized *in vitro* propagation protocols for *M. dubia* cv. Vitahuayo, a high vitamin C cultivar. Key findings include optimal callus induction using 1 mg/L 24-D and 0.5 mg/L BAP, particularly effective for stem explants and efficient somatic embryogenesis with a 90% success rate using WPM supplemented with 3 mg/L 24-D, 20 mg/L NAA and 15 mg/L KIN. These protocols address variability in vitamin C content, potentially enhancing camu camu's commercial viability and genetic conservation. While promising, further research is needed to refine techniques, extend to other cultivars and overcome challenges like contamination and oxidation. This work marks a significant step towards sustainable, large-scale production of Vitahuayo, with potential applications beyond the Peruvian Amazon. As demand for vitamin C-rich crops grows, these biotechnological tools will be crucial in meeting global agricultural and nutritional needs.

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Disclaimers

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Informed consent

Does not apply.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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