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Micropropagation and sanitation of *Citrus sinensis* L. Osbeck from CTV-infected fruits

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Abstract

Plant sanitation is an important tool to ensure disease-free plants, particularly for genetic breeding and germplasm conservation programs. Citrus Tristeza Virus (CTV) is a destructive disease that affects the quality of the seed and causes the plant death. Therefore, it is important to develop a protocol for propagating CTV-free Pineapple sweet orange (*Citrus sinensis* L. Osbeck) plants from infected fruits. When the seed coat was treated with 2% sodium hypochlorite and thermotherapy process at 52°C, not CTV was detected. Similarly, seedlings germinated *in vitro* were negative for CTV after receiving treatment. In addition, 6.9 adventitious shoots were produced using epicotyl with cotyledons as explants in the culture medium supplemented with 1.0 mg L^{-1} of 6-BAP + 0.5 mg L^{-1} of IBA. Furthermore, the highest rate of i*n vitro* rooting percentage was 86% with 4.4 roots per explant using 1.0 mg L^{-1} of IAA + 0.5 mg L^{-1} of IBA. The plantlets *in vitro* rooted and acclimatized showed grafting success with high survival percentages (above 92%). The results of the study allowed us to develop a seed coat sanitation protocol to eliminate the CTV in micropropagated of Pineapple sweet orange plants.

Keywords: Adventitious Shoots; Graft; Propagation; Sanitation

1. Introduction

Pineapple sweet orange (*Citrus sinensis* L. Osbeck) is a fruit tree that has gained wide popularity in the market due to its delicate fragrance, sweet taste, which has promoted the development of new varieties. However, Pineapple sweet orange is not free of pathogenic microorganism's disease that cause significant economic losses in citrus cultivation worldwide. Citrus Tristeza Virus (CTV) is one of the most destructive plant pathogens in citrus plantations worldwide [1, 2]. Losses associated with CTV are estimated at more than 100 million trees around the world with a field prevalence of 60 to 100% in severe infections [3-6]. CTV symptoms in *Citrus sinensis* include stem pitting, which manifests as depressions in the wood of the trunk and branches, leading to the leaf fall, and a decrease in fruit size, quantity, and quality [7]. In the field of phytosanitary, the Pineapple sweet orange is used as a source of plant material for research the CTV. This is because it is a fruit tree that carries the virus, capable of causing symptoms at an early stage in the plant's development.

To eliminate CTV spread by grafting, plant sanitation is an important tool that provides the possibility to obtain disease-free plants and helps in the development of genetic improvement programs or germplasm conservation that require

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optimal phytosanitary qualities. Thus, sanitized plants and seeds are the source of diversity used in conventional breeding or genetic engineering methods to provide solutions to diseases transmitted by grafting.

Plant tissue culture techniques in combination with thermotherapy have shown good results in the fight against certain types of phytopathogenic microorganisms. In this context, citrus sanitation performed by tissue culture using vegetative areas free of phytopathogens as explants, the viral genome combat in the plant through immune mechanism activated by thermotherapy could reduce or eradicate the pathogen presence in the plant or parts of it. Consequently, the combination of these techniques could be efficacious to producing CTV-free Pineapple sweet orange plants.

Therefore, the aim of this study was:

To establish an efficient *in vitro* sanitation and regeneration protocol for CTV-free Pineapple sweet orange plants for use in genetic breeding, biological indexing, and crop production programs.

2. Material and methods

2.1. Plant material

Trees of Pineapple sweet orange grown in a greenhouse were used as plant material. The trees were previously inoculated 12 years ago with isolates of the CTV. Prior to sample collection, the trees were confirmed to be infected with CTV by molecular diagnostics. Likewise, it was confirmed that these samples showed the characteristic CTV-symptoms, such as: spoon-shaped leaves, thickening of the main veins and overall yellowing of the tree. Subsequently, the fruits were washed with a commercial detergent, rinsed with sterile distilled water, and dried with sterile paper towels. Then, pericarp and endocarp of the fruits were removed and CTV diagnosis was performed using RT-PCR. The seeds obtained from the fruits were submerged in a solution of 40 g L^{-1} of agricultural lime for 10 min with constant stirring to eliminate their mucilage. Finally, the mucilage-free seeds were rinsed with sterile distilled water and stored for the subsequent sanitation process.

2.2. Sanitation treatments in seed coat (testa)

The mucilage-free seeds were exposed to three treatments in order to eliminate the CTV presence. The first treatment (T1) consisted in immersing the seed in 70% ethanol for one minute, followed by three rinses with sterile distilled water (Control treatment). The second treatment (T2) consisted in submerging the seeds in 20% sodium hypochlorite for 20 min and then rinsing them three times in sterile distilled water. Subsequently, thermotherapy was performed in water bath at 52°C for 10 min. Afterwards, to avoid saprophytes contamination during post-thermotherapy treatment, the seeds were submerged in 70% ethanol for one minute and rinsed three times with sterile distilled water. The third treatment (T3) was similar to T2, but the sodium hypochlorite immersion time was doubled to 40 min (Table 1). To evaluate the effect of the treatments, the seed coats of the three treatments were extracted, freeze-dried, macerated, and tested to determine the CTV-presence by RT-PCR at day 0 and after 10 days (Table 1).

2.3. *In vitro* seed culture

Seeds free of mucilage and coat were cultured were cultured in 4.43 g L^{-1} of MS salts [8] and 30 g L^{-1} sucrose. Culture medium pH was adjusted to 5.8 with NaOH or HCl (1N) and 2.8 g L^{-1} of phytagel®Sigma was added for the correct solidification. The seeds were placed in total darkness until germination under the following conditions: T = 25°C ± 1, PPFD = 32 µmol photon m^{-2} s^{-1} and 16 h photoperiod supplied by white fluorescent lamps. To determine the presence of CTV on *in vitro* germinated seedlings, a total of three *in vitro* plantlets with five centimeters of height were collected from each treatment (Table 1).

2.4. *In vitro* production of adventitious shoot

For *in vitro* production of adventitious shoots, we used only the CTV-negative plantlets obtained in the T2 treatment. Epicotyl (Epi) and epicotyl segment with attached cotyledon fragment (Epi-Cot) were used as explants. To induce the adventitious shoots, the explants were cultured in MS medium [8] (4.43 g L^{-1}), sucrose (15 g L^{-1}) and supplemented with three different concentrations of 6-Benzylaminopurine (6-BAP) without or with the addition of Indole-3-Butyric Acid (IBA) (Table 2). Culture medium pH was adjusted to 5.8 with NaOH or HCl (1N) and 2.8 g L^{-1} of phytagel®Sigma was added for the correct solidification. The explants were cultured under the following conditions: T = 25°C ± 1, PPFD = 32 µmol photon m^{-2} s^{-1} and 14 h photoperiod supplied by white fluorescent lamps.

2.5. *In vitro* rooting and acclimatization

From T2 treatment, 5 cm long adventitious shoots were obtained and rooted in MS medium [8] (4.43 g L^{-1}), sucrose (15 g L^{-1}), supplemented with Indole-3-Acetic Acid (IAA) and Indole-3-Butyric Acid (IBA) (Table 3). These *in vitro* rooted plantlets were acclimatized in a sealed plastic container filled with a mixture of pine bark, sand, and peat-moss (1:1:1), and were maintained in a closed incubation area for 20 days at 24 °C, 60-80% humidity, and 8-12 h photoperiod. All the plantlets were grafted on rootstocks of rough lemon (*Citrus jambhiri*). After 40 days, the percentage rooting, number and length roots and survival percentage were quantified (Table 3).

2.6. Molecular detection of CTV

Total RNA was extracted from each sample using Trizol® Reagent protocol (Thermo Fischer Scientific) and treated with RQ1 RNAse-free DNAse (Promega) to remove genomic DNA contamination. The concentration and purity of RNA samples were examined by NanoDrop™ 1000 Spectrophotometer (Thermo Scientific NanoDrop Technologies, LLC, Wilmington, DE, USA) and the quality was evaluated by 1.5% agarose gel electrophoresis during 60 min at 85 V. cDNA was synthesized using 100 ηg of RNA, 2.5 µM of random hexamers, and 50 Units/µL MultiScribe™ Reverse Transcriptase (Invitrogen/Life Technologies, CA, USA) according to the manufacturer's recommended protocol. For the CTV detection, RT-PCR reaction was performed using the cDNA obtained from RT as amplification template. The reaction condition was 10X PCR buffer, $MgCl_2$ 2.5 mM, dNTP´s 0.2 mM, 1 µM of each forward (P20 5´-ACAATATGCGAGCTTACTTTA-3´) and reverse primer (P20 5´-AACCTACACGCAAGATGGA-3´) that amplify a fragment of 557 base pairs (bp) [9], 0.5 U of Taq DNA polymerase (Invitrogen) and 2 µL of cDNA, at a final volume of 25 µL. Amplification runs were initiated at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 1 min, with final extension at 72°C for 10 min. Products were analyzed in agarose gels (1.5%), dyed with ethidium bromide and visualized under UV light. To verify the RNA integrity and exclude false negatives, 18S gene amplification was performed according to the conditions described by Yan et al [10]. To determine the presence of CTV, composite samples were collected from CTV-positive plants of Pineapple sweet orange trees grown in the greenhouse, in order to verify the absence of CTV in sanitized plants.

2.7. Statistical analysis

The analysis to evaluate adventitious shoots production and *in vitro* rooting per treatment were established in duplicate with a total of 25 explants per treatment and three repetitions. Analysis of variance test (ANOVA) was used to determine significant difference among treatments. Analysis of variance and the Tukey multiple comparison test were used to detect significant difference between the means (p<0.05) using R Software.

3. Results

3.1. Sanitation protocol

The results showed that when the plants were not treated (T1: control treatment), they all showed the presence of CTV in their seed coats at the beginning of the experiment. Likewise, the number of seed coats infected with CTV was also lower in treatments T2 and T3. At 10 days, the seed coats (T1) showed 100% presence of CTV (7/7), however, the treatments T2 and T3 showed no signs of CTV infection. Interestingly, when the seeds germinated and grew into sprouting plantlets, CTV was absent on these plantlets (Table 1).

Table 1 CTV presence in Pineapple sweet orange seeds coats exposed to different sanitation treatments.

Treatment	Description	RT-PCR frequency (positive samples/total samples)		
		At 0 day	At 10 days	*in vitro* plantlets
T1	Control	7/7	7/7	0/18
T2	20 min NaClO + thermotherapy	3/7	0/7	0/18
T3	40 min NaClO + thermotherapy	4/7	0/7	0/18

3.2. CTV diagnosis by RT-PCR

The results allowed us to confirm the CTV presence in different tissues of Pineapple sweet orange by RT-PCR, including 100% in the shell fruit, 42.8% in the endocarp, and 67% in the seed coat. However, the study also showed that when seed coats were treated with sodium hypochlorite and thermotherapy (T2 and T3), these seed coats did not show the presence of CTV (Figure 1).

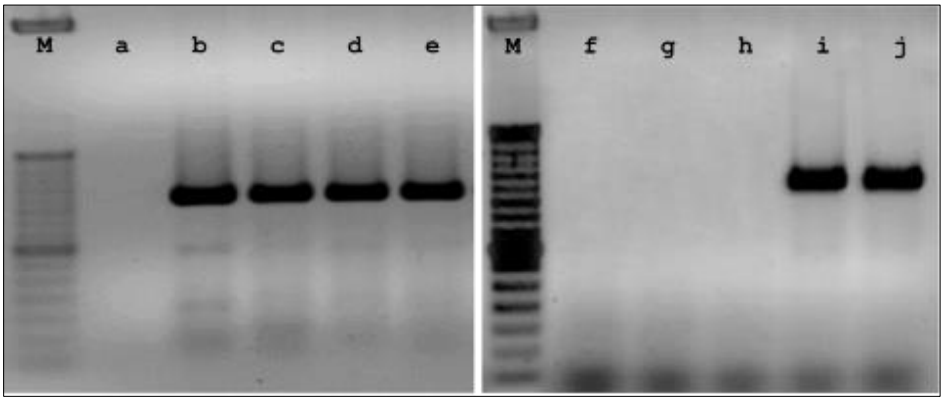

Figure 1 Amplifications obtained from conventional RT-PCR for CTV diagnosis using different tissues of Pineapple sweet orange. Lane M: 1-kb DNA ladder; Lane a: CTV-negative, Lane b: shell fruit, Lane c: Endocarp, Lane d: seed coat, Lane e: CTV-positive, Lane f: CTV-negative, Lane g: seed coat of treatment T2, Lane h: seed coat of treatment T3, Lane i: seed coat of treatment T1 (control; not treated), Lane j: CTV-positive

3.3. Adventitious shoots production

In control treatment (PT1), were obtained 0.96 and 3.24 adventitious shoots using epicotyl with cotyledons (Epi-Cot) and epicotyl explants, respectively. Similar adventitious shoots values were obtained using 0.5 mg L^{-1} IBA (PT2). Although they are not significantly different, the PT3, PT5, PT6, PT8 treatments showed higher adventitious shoots number in Epi-Co and epicotyl explants in comparison to PT1. Interestingly, when 2 mg L^{-1} 6-BAP was added to epicotyl explants, the number of adventitious shoots produced by PT7 treatment (0.64) was lower than that of other treatments. In the PT4 treatment (1.0 mg L^{-1} 6-BAP + 0.5 mg L^{-1} IBA) when Epi-Cot was used as explants, a higher adventitious shoots were produced (6.9) (Table 2).

Table 2 Adventitious shoot production in Pineapple sweet orange under *in vitro* conditions.

Treatment	Description		Number of adventitious shoots per explant	
	6-BAP (mg L^{-1})	IBA (mg L^{-1})	Epicotyl	Epi-Cot
PT1	0	0	0.96 ±0.08 ab	3.24±0.26 c
PT2	0	0.5	0.92 ±0.10ab	3.57 ±0.26 bc
PT3	1	0	1.55 ±0.28 ab	5.20 ±0.11 ab
PT4	1	0.5	1.55 ±0.11 ab	6.96±0.12 a
PT5	1.5	0	1.17 ±0.07 ab	3.53 ±0.30 bc
PT6	1.5	0.5	1.92 ±0.38 a	5.04 ±0.23 bc
PT7	2.0	0	0.64 ±0.24 b	4.79 ±0.14 bc
PT8	2.0	0.5	1.92 ±0.16 a	4.02 ±0.68 bc

* Means with the same letter are not statistically different based on Tukey test (p<=0.05). Epi-Cot: explant cotyledons with an epicotyl segment cultured vertically.

3.4. *In vitro* rooting and acclimatization

The treatment control (RT1) showed 11% rooting with 1 root of 4.5 cm of length. When 1.0 mg L^{-1} of AIA was added (RT2), increased the rooting (86.7%) with 2.7 roots of 5.4 cm of length. In the RT3 treatment, when the plants were cultured in 1.0 mg L^{-1} of IBA, a lower rooting was observed (35.2%) with 1 root generated of 5.3 cm of length. Likewise, the combination of 1.0 mg L^{-1} IAA + 0.5 mg L^{-1} IBA resulted in the highest rooting percentage (86%) with the greatest roots number (4.4) of 5.5 cm of length in comparison to other treatments evaluated. Furthermore, when the survival was evaluated, the plantlets of all treatments showed high survival percentages (between 92 and 96%) (Table 3).

Table 3 *In vitro* rooting and grafting survival of adventitious shoots of Pineapple sweet orange.

Treatment	Description		Rooting (%)	Root number	Length root (cm)
	AIA (mg L^{-1})	IBA (mg L^{-1})			
RT1	0	0	11.1 ±3.0 c**	1.0 ±0.00 b*	4.5 ±0.94 ns
RT2	1	0	86.7 ±5.8 a**	2.7 ±0.49 ab*	5.4 ±0.53 ns
RT3	0	1	35.2 ±4.9 b**	1.1 ±0.06 b*	5.3 ±0.68 ns
RT4	1	0.5	86.0 ±5.4 a**	4.4 ±1.11 a*	5.5 ±0.59 ns

* Means with the same letter in the same column are not statistically different based on Tukey test (p<=0.01**; p<=0.05*; ns=not significant).

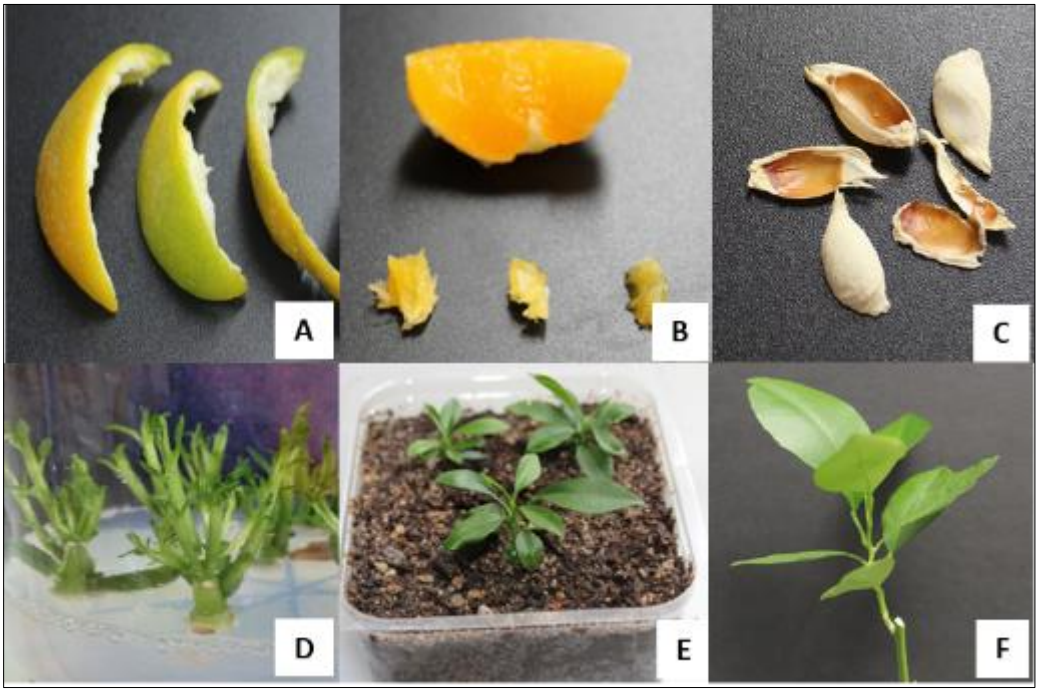

Figure 2 Tissue samples analyzed for CTV diagnosis (A: Shell fruit, B: endocarp, C: seed coat), and Pineapple sweet orange propagation from adventitious shoots (D: Adventitious shoots cultured *in vitro* generated from Epi-Cot explant; E: rooted and acclimatized adventitious shoots; F: plantlets grafted on rootstocks of rough lemon)

4. Discussion

Plant tissue culture techniques using thermotherapy has been shown to be an effective strategy for eliminating phytopathogenic viruses in citrus species [11-14]. In this context, thermotherapy acts by inducing the production of heat shock proteins, which can degrade viral particles and inhibit viral replication, creating a stable cellular environment [15]. Likewise, disinfectants agents such as sodium hypochlorite have been widely used to eliminate plant pathogens during *in vitro* establishment of different species [16-18]. Sodium hypochlorite has the capacity to degrade

the phytopathogenic viruses' capsid that is located on the outside of infected plant tissue [19], thereby changing the pH of the culture medium, inactivating and degrading the viral envelope [20].

Our results showed that 20 min of chlorine treatment and thermotherapy process (T2 treatment) on the seed coat of Pineapple sweet orange could promoted the CTV absence at 10 days. This event suggests that CTV may only exist in the shallow layer of the seed coat that is in direct contact with sodium hypochlorite, so long-term immersion is not required to eliminate the virus. Therefore, this treatment can be used to sanitation CTV-free seeds and sow them in substrate or soil, which is essential for the survival and germination of future Pineapple sweet orange plants. Interestingly, the removal of the seeds coat during *in vitro* culture appears to be essential to ensure health of *in vitro* plantlets, the above, because these *in vitro* plantlets were CTV-free even though they come from seeds treated with or without sodium hypochlorite treatment and thermotherapy (Table 1). This finding underlines the importance of sanitation and proper culture procedures in the *in vitro* propagation of plant material to reduce the risk of transmission of viral diseases. Therefore, removing the seed coat is necessary step for the healthy conventional propagation of Pineapple sweet orange.

The adventitious shoots formation by organogenesis is a consequence of the through dedifferentiation of differentiated cells and reorganization of cell division to create particular organ primordia and meristems [21-22]. In this way, cytokines and auxins are the most pervasive phytohormones that either directly or indirectly accelerate the shoot regeneration process [23]. Previous report demonstrated that the use of 6-BAP cytokine is one of the best alternatives for direct induction of organogenesis *in vitro* of *Citrus latifolia* Tan. [23]; whose effect can be increased with the combination of other growth regulators auxins in *Citrus* plants [24-25]. However, the efficiency of *in vitro* culture protocols varies according to the genotypes, explants, and incubation conditions [26]. Previous studies on *Citrus volkameriana* and *Citrus aurantium* reported that *in vitro* organogenesis occurred using epicotyl and internodal segments as explants (42 and 59%) and that BAP supplementation was essential for development one shoot per explant [26]. The above was corroborated in our study, due to the observed minimum number of adventitious shoots per explant in Pineapple sweet orange when they were cultured in absence of phytohormones. On the contrary, the epicotyl segment with attached cotyledon fragment (Epi-Cot) explant showed a maximum response of 6.9 adventitious shoots per explant with high percentages (89 to 100%) when were cultured in medium supplemented with a combination of 1.0 mg L^{-1} of BAP + 0.5 mg L^{-1} of IBA.

In Mexico, there are several aphid species that can transmit CTV (*Aphis gossypii*, *Aphis spiraecola*, *Toxoptera aurantii* and *Toxoptera citricida*) and cause severe outbreaks of citrus decline because severe variants, therefore, the dispersion of the CTV-disease has progressed in the main citrus-growing regions [27-29]. Furthermore, it is well known that when producers dispersed and planted uncertified citrus seeds, CTV incidence is higher in plantations, ranging from 87.5 to 100% [30]. Therefore, accurate CTV diagnosis of seed coat and *in vitro* plantlets is very important to corroborate the success of the methodology used in our study to confirm the sanitation status of Pineapple sweet orange. Thus, the results allowed us to confirm the CTV absence in seed coats when were treated with sodium hypochlorite and thermotherapy (T2 and T3 treatment).

The results obtained from *in vitro* rooting of Pineapple sweet orange shoots showed the importance of auxin addition in the culture medium to increase the root formation process. The combination of IAA and IBA resulted in the highest rooting percentage (86%) with a greater number of roots (4.4) compared to the control treatment (without phytohormones). Previous study on *Citrus limon* also has showed that the rooting rate of shoots was highest in media containing different concentrations of Indole-3-Butyric Acid (IBA) and Indole-3-Acetic Acid (IAA) [31]. Likewise, IAA+IBA interactions can increase the shoots rooted rates (up to 80%) in *Citrus macrophylla*, *Citrus aurantium*, and *Citrus reshni* [25].

Respect to acclimatization, adventitious shoots rooted and acclimatized *in vitro* showed a higher survival rate at 20 days. Also, when these acclimatized plantlets were grafted on rootstocks of rough lemon (*Citrus jambhiri*), grafting rates were higher (86%) and survival rates were also higher (92-96%). This grafting technique of explants cultured *in vitro* and acclimatized on vigorous rootstocks has allowed us obtained Pineapple sweet orange in a shorter time, which is advantageous because the citrus plants developed from seed, they have to go through a juvenile period of can last three years or more before reaching production. Our results offer valuable information to establish rapid sanitation methods to eliminate CTV on *Citrus sinensis* L. Osbeck, and contribute to improving yields and efficiency in the production of sanitized plants for use in genetic breeding programs and in the citrus industry.

5. Conclusion

A simple and rapid sanitation protocol was established to eliminate CTV from *Citrus sinensis* L. Osbeck fruits with the potential for massive propagation. Our study showed that the application of sodium hypochlorite for 20 min and a thermotherapy period at 52°C for 10 min was effective for obtaining CTV-free plantlets from infected seeds coat. Micropropagation of CTV-free plantlets can be optimized by adding 1 mg L^{-1} 6-BAP to the culture medium to produce adventitious shoots, as well as a combination of 1 mg L^{-1} AIA + 0.5 mg L^{-1} IBA to promote rooting in *Citrus sinen*sis L. Osbeck.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that no conflict of interest exists.

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