



Comparative study of axenisation protocols for aquatic plants

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Received: 21 January 2025 / Accepted: 25 April 2025 / Published online: 21 July 2025
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Abstract

Obtaining in vitro plant cultures free of parasites or pathogens, namely axenic cultures is a major challenge both in the field of scientific research and in agronomy or biotechnology. For aquatic macrophytes, plants free from contamination are required for aquaculture or aquariums, and necessary to prevent microbial growth in medium containing dissolved organic carbon. Then, various protocols have been elaborated to facilitate the production of different qualities of plant cultures from “clean” to sterile. The performance of various calcium hypochlorite axenisation protocols in aquatic plants was evaluated. Through trials with variations in hypochlorite concentration and exposure time, it became possible to draw up a response table for three first species (*Ceratophyllum demersum*, *Lemna minor* and *Myriophyllum spicatum*). Additional results on other species were used to define the response of different species to axenisation, so that each species could be oriented towards a particular technique. A trial with successive axenisation steps was thus successfully tested on the two branchy but resilient species (*C. demersum* and *M. spicatum*). The effectiveness of protocol variations (e.g. addition of sucrose and use of rinsing breaks during hypochlorite exposures) were also evaluated. This resulted in recommendations that we propose, primarily the banning of sugars during the early stages of axenisation, contrary to previous recommendations in literature protocols.

Key message

Sterile in vitro aquatic plant cultures are a major challenge in scientific research, agronomy, and biotechnology. Axenisation trials with varying exposure times and hypochlorite concentrations were successfully tested on three macrophytes.

Keywords Aquatic macrophytes · Axenia · Calcium hypochlorite · Carbohydrates · In vitro culture

Introduction

The acquisition of ready-to-use plants, a critical requirement in scientific research, agronomy, and biotechnology, has presented a significant challenge over the past few decades (Ailstock et al. 1991). The necessity for plants devoid of

parasites such as algae, molluscs, insects, nematodes, and pathogens, including fungi, bacteria, and viruses, has become paramount for plant commercialization and global trade. Plants that are devoid of bacteria and other epiphytic or endophytic microorganisms are classified as axenic cultures. In the context of aquatic macrophytes, the demand for plants free from microbial contamination is high, particularly for aquaculture or aquariums, with substantial investments made by companies in this sector. In scientific research, axenic cultures are essential for preventing microbial proliferation in media containing dissolved sugars or other organic carbon sources (Godmaire and Nalewajko 1986), and for maintaining pure strains of certain model species for omic analyses (Ailstock et al. 1991). The utilization of axenic plants for studying photosynthesis or nutrient uptake is widely acknowledged (Godmaire and Nalewajko 1986). Moreover, genomic and transcriptomic analyses benefit from the purity of genetic material obtained from axenic

Communicated by Patricia Marconi.

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cultures (Barrera-Redondo et al. 2020). Furthermore, plants have been extensively employed for phytoremediation, and sterile conditions are required to conduct tests solely with plants to differentiate the relative abilities of the plant and its microbiota in detoxifying chemical compounds (Khellaf and Zerdaoui 2010; Liu et al. 2019).

To address the challenges associated with obtaining suitable plants, protocols have been developed to facilitate the production of various qualities of plant cultures, ranging from “clean” to sterile, without compromising plant survival and development potential (prerequisite for strain maintenance and propagation for commercial or scientific purposes). For example, the standardised OECD protocols on *Lemna minor* and *Myriophyllum spicatum* (OECD 2014. Test No. 221 and 238), which are widely adopted and used for chemical risk assessment (Nuttens and Gross 2017), require axenisation based on sodium hypochlorite treatment (NaOCl). The technique, which has been used for several decades, can be modulated by varying the concentration of the disinfectant, the duration of exposure, and intermediate washes of the plants. Other molecules such as H_2O_2 , $\text{Ca}(\text{OCl})_2$, ethanol, HgCl_2 have also been used for axenization (Wetzel and McGregor 1968; Godmaire and Nalewajko 1986; Kane and Gilman 1991; Henry et al. 2006; Aasim et al. 2015). Among these, $\text{Ca}(\text{OCl})_2$ is the most commonly used disinfectant but NaOCl has a broader spectrum of activity.

However, the currently available axenisation protocols are largely empirical, and the details of axenia are inadequately documented. The literature generally lacks quantitative data to assess or compare the effectiveness (survival rates and sterility success) of the different proposed protocols. Moreover, such protocols have been documented for only a limited number of aquatic plant species, raising questions about their applicability to other aquatic species.

This study employs an opportunistic sampling approach, utilizing diverse data collected during a PhD thesis. Although not based on a dedicated experimental design, this approach offers valuable insights by exploring a range of conditions. This will be particularly relevant for experimenters who need to choose an axenisation protocol for aquatic plants, given the scarcity of comparable data in the literature. The aim of this study is not to criticize the protocols already established but to explore the use of calcium hypochlorite for various plant species and to test a range of exposure time/concentration combinations. We will also discuss the relevance of adding different compounds to the protocol, including sugars. The potential benefits of starting cultures from seeds, especially in *Ceratophyllum demersum* will be discussed, along with ways to identify microorganisms to improve axenisation protocols and success rates. In our approach, we aimed to quantify the success rate of

axenia and explant survival, which is essential to better anticipate the requirements of the protocols and optimise the chances of success.

Materials and methods

Plant materials and culture protocol

The fourteen strains (belonging to six species) used are presented in Table 1. Each strain was maintained in a 200 L outdoor container since their collections or, for *L. minor*, in 500 mL Erlenmeyer flasks. Four to six weeks before axenisation steps, all plants were stored in a phytotron, at 22 °C with a photoperiod of 16:8, and $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, in 20 to 40 L aquariums or in 500 mL Erlenmeyer flasks. At this step, *L. minor* fronds were then stored in sterile 250 mL Falcon flasks and transplanted every month to renew the culture medium. The other species were transplanted up to every three months.

Two types of culture media were used for growing in phytotron. The first one, specific to *L. minor*, contains KNO_3 –60.66 mg.L^{-1} ; NH_4NO_3 –10.88 mg.L^{-1} ; K_2HPO_4 –7.308 mg.L^{-1} ; KH_2PO_4 – 16.2 mg.L^{-1} ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ –36.95 mg.L^{-1} ; NaHCO_3 – 63 mg.L^{-1} ; FeEDTA – 0.5 mg.L^{-1} ; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ – 150 mg.L^{-1} ; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ –73.52; H_3BO_3 – 1.95 mg.L^{-1} ; $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ –7 $\mu\text{g.L}^{-1}$; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ – 1 $\mu\text{g.L}^{-1}$; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ – 22 $\mu\text{g.L}^{-1}$; H_2SeO_3 –1.6 $\mu\text{g.L}^{-1}$; $\text{Na}_2\text{Mo}_4 \cdot 2\text{H}_2\text{O}$ – 24 $\mu\text{g.L}^{-1}$ (adapted from Roubeau Dumont et al. 2019). The medium was buffered at pH 5.8 and conductivity was 445 $\mu\text{S cm}^{-1}$. For the other species, the medium was adapted as following, removal of NH_4NO_3 , use of different concentrations for K_2HPO_4 , KH_2PO_4 and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (respectively: 6.98, 3.26 and 75 mg.L^{-1}), and pH buffered at 7.0. Both media were autoclaved at 121 °C for 20 min and stored in the dark at 5 °C.

Experimental design

Each axenisation trial was performed in a microbiological safety workstation (Faster, BHA 36). The explants were pre-collected, rinsed with sterile tap water and then sterilised with calcium hypochlorite solution. Exposure times ranged from 30 s to 6 min, and concentrations ranged from 0.1 to 3% (i.e. 1 g/L to 30 g/L). Moderate agitation in the tube improves the contact of the solution with the whole plant, especially for those with a branchy structure. After exposure, the explant was immediately placed in a 50 mL Falcon flask filled with the culture medium dedicated to the species. Depending on the duration and concentration used, the plant was largely discoloured and even the leaves could fall off. Multisequential exposure to hypochlorite was also tested

Table 1 Plant species and strains used for axenisation assays

Species	Strains	GPS coordinates	Collection year	Waterbody type	Surrounding land use	Trophic status
<i>Ceratophyllum demersum</i>	Ramonville (Hte Garonne, FR)	43.541056, 1.490556	2019	Canal du midi	Urban	Eutrophic
<i>Egeria densa</i>	Saint-Nicolas (Lot-et-Garonne, FR)	44.140337, 0.760565	2021	Garonne, River	Urban and agricultural	Eutrophic
<i>Lemna minor</i>	Authume (Jura, FR) ¹	47.12256, 5.50560	2015	Pond	Urban and agricultural	Eutrophic
	Toulouse (Hte Garonne, FR) ¹	43.559863, 1.474494	2015	Pond	Urban	Eutrophic
	Saint-Aignan (Hte Garonne, FR)	44.025050, 1.075448	2015	Garonne, River	Urban and agricultural	Eutrophic
	Avillers (Meuse, FR) ¹	49.02943, 5.71536	2015	Drainage ditch	Agricultural	Eutrophic
<i>Myriophyllum spicatum</i>	Villemur (Tarn, FR) ¹	43.88671, 1.487307	2015	River	Agricultural	Mesotrophic
	Besançon (Doubs, FR) ¹	47.23153, 6.02252	2015	Pond	Urban	Mesotrophic
	Arles (Bouches-du-Rhône, FR) ¹	43.48083, 4.64703	2015	Camargues, Drainage ditch	Agricultural	Eutrophic
	Roques (Hte Garonne, FR)	43.504105, 1.368130	2017	Garonne, River	Urban and agricultural	Mesotrophic
	Plön (Schöhsee lake, Germany) ¹	54.16624, 10.44114	1991	Lake	Urban and agricultural	Mesotrophic
	Le-Cannet-des-Maures (Var, FR)	43.387389, 6.360889	2021	Lake	Urban and agricultural	Mesotrophic
	Seilh (Hte Garonne, FR)	43.697639, 1.369222	2020	Garonne, River	Urban and agricultural	Mesotrophic
<i>Ranunculus fluitans</i>	Seilh (Hte Garonne, FR)	43.697639, 1.369222	2020	Garonne, River	Urban and agricultural	Mesotrophic
<i>Vallisneria spiralis</i>	Saint-Nicolas (Lot-et-Garonne, FR)	44.140337, 0.760565	2015	Garonne, River	Urban and agricultural	Eutrophic

¹ Strains already used for previous experimentations (Roubeau Dumont et al. 2019, 2020). Localisations are specified in brackets including the department and the country

to minimise the negative effect: for example sequences of 3 one-minute periods interspersed with rinsing with sterile tap water were tested, especially on *M. spicatum*. The flasks were placed in the phytotron and monitored daily. During the first few days post-sterilisation, light exposure was reduced to prevent extra stress.

Another protocol was specifically tested with *C. demersum*, which grows with difficulty after axenisation. To evaluate, the possible requirement of growth factors from natural environment, extracts from a pond colonised by *C. demersum* (43.56515 N, 1.47148 E) were added to the culture medium containing the explants after exposure to hypochlorite. Two formulations were tested at a concentration of 5 mL/L: an extract filtered twice with folded standard filter paper (Fisherbrand) and 0.20 µm cellulose acetate syringe filter (FP30/0.2 CA-S Whatman), or an unfiltered extract autoclaved for 20 min at 121 °C.

The axenia of explants was verified in two steps: after 1 week of culture, observation of possible contamination (chlorophyllous microorganism, bacterial biofilm or hyphae of fungi are very visible). If the explant appeared clean, a microbial culture analysis was performed. 400 µL of the culture medium was cultivated on Petri dishes, containing Trypticase Soy Agar (TSA) (Zimbro et al.) or Sabouraud agar (Motta Ferreira 2016), incubated at 28 °C in the dark

and analysed after 24 h, 48 h and 7 days. If the test was negative, a sample of the culture medium was run on CytoFLEX (Beckman Coulter) flow cytometer with SYTO 9™ green-fluorescent nuclear and chromosome counterstain (Thermo Fisher Scientific).

Statistics

To model the effects of the different axenization conditions on explant survival and sterility, we applied generalized linear models (GLM) with a binomial family and logit link function. This approach was used to assess the relationship between the response variables (survival and sterility) and the predictor variables, including macrophyte species, hypochlorite concentration, hypochlorite exposure time, exposure mode (single vs. fractionated bath), sucrose addition and the number of axenization iterations. To address the influence of predictor variables on the sterility response, analyses were restricted to data from surviving explants. For each model, the significance of the predictors was evaluated using Wald's test based on the z-values and associated p-values. To interpret the influence of each parameter on the outcome, we calculated the odds ratios (OR) for each significant variable, which represent the multiplicative changes in the odds of survival or sterility for each unit change in the

corresponding predictor. The odds ratios were then used to quantify the direction and magnitude of the effect of each parameter on the response variable, and to estimate the associated changes in the probabilities of survival or sterility. All statistical analyses were performed using R (version 4.4.2).

Results

Sensitivity of aquatic plants to calcium hypochlorite axenisation

To assess the sensitivity of aquatic plants to the axenisation process, multiple assays were conducted by varying calcium hypochlorite ($\text{Ca}(\text{ClO})_2$) concentration (from 0.1 to 3%) and exposure time (from 1 to 8 min) (Fig. 1a, b). The results and the condition of the explants post-axenisation for each combination tested are presented in Table 2, with the number of trials per combination indicated in each cell.

For *L. minor*, only two trials with 1% $\text{Ca}(\text{ClO})_2$ for 6 min resulted in positive outcomes, with a sterility rate of 100% each time, and the survival of half of the fronds during the 8 trials carried out. For *M. spicatum* and *C. demersum*, the results were more contrasted. Contamination of the explants, by microalgae or non-photosynthetic microorganisms (a mixture of yeasts, bacteria and fungi) quickly appeared after sterilisation. However, an exposure time between 2 and 3 min, with 1% $\text{Ca}(\text{ClO})_2$ yielded the most promising results. This sterility criteria was jointly considered with the survival of the explants, which was highly variable. For *M. spicatum*, increasing the exposure time was clearly the limiting factor for survival (60% survival rate after 5 min, 25% after 6 min, and 0% after 7 min). Shorter exposure (1 min) gave similar results to a 5 min exposure (about 60% survival). For *C. demersum*, the results were more disparate with a variable survival rate depending on the duration of exposure, without any visible trend. Nevertheless, the lowest survival rates were mainly obtained for low exposure duration and/or concentration.

After the first axenization attempt, survival analysis revealed significant differences among species (deviance = 15.02, $\text{df} = 6$, $p = 0.0103$), while neither hypochlorite concentration nor exposure time had a significant effect ($p > 0.1$). No explant survived for *R. fluitans*. Among the remaining species, *L. minor* exhibited the lowest predicted survival probability, whereas *M. spicatum* displayed substantially higher odds of survival (estimate = 2.726, odds ratio = 15.27, $p = 0.0836$), corresponding in the present case to an approximate threefold increase in the predicted survival probability.

Sterility success at this stage was 100% in *L. minor*, while no sterile individuals were obtained in any other

Fig. 1 Post-axenisation survival and sterility rates for several aquatic plant species. Various combinations of calcium hypochlorite $\text{Ca}(\text{ClO})_2$ concentrations and exposure times (one-shot application) were tested, the number of tests is indicated in each corresponding box (**a** and **b**). (**a**) survival rates after axenisation for 3 species of interest (*L. minor*, *M. spicatum* and *C. demersum* respectively). The green color shows a high survival rate. (**b**) sterility rate for the same 3 species, with the pink color indicating a high sterility rate. Both rates are measured 7 days after the axenisation trial, by observation of the plant sample (survival) and agar tests for sterility. (**c**) summarised response of six aquatic plants to calcium hypochlorite axenisation (i.e. ability of the plant to be efficiently axenised), allowing to place six plants in a matrix sterility / survival rates. Four zones correspond to different responses and condition the possible success of axenisation and the effort required to achieve it (see Results for more information) CD: *C. demersum*; ED: *Egeria densa*; LM: *L. minor*; MS: *M. spicatum*; RF: *Ranunculus fluitans*; VS: *Vallisneria spiralis*

species. This extreme pattern led to quasi-complete separation in the logistic model, preventing reliable estimation of odds ratios or inclusion of treatment variables (e.g., hypochlorite concentration or exposure time). A model including only the species factor revealed a highly significant effect (deviance = 20.11, $\text{df} = 4$, $p = 0.0005$), confirming marked interspecific differences in survival rate during the initial axenization trial.

Based on the results of the six species tested, a survival/sterility response table could be constructed, representing the sensitivity of each plant obtained on our axenisation trials with $\text{Ca}(\text{ClO})_2$. Four zones can be distinguished. In green, a zone where plants appear to be most tolerant to axenisation, with rates over 50% for both survival and sterility. In red, an opposite zone where the plants seem to be the least tolerant to axenisation, with rates less than 50% for both survival and sterility. Two intermediate zones are defined in orange (high survival but low sterility rates) and yellow (high sterility but low survival rates).

Except for *L. minor*, which is present at the limit between the yellow and green zones, the other species are distributed at the bottom of the figure, *Ranunculus fluitans* and *Egeria densa* in the red zone and the three others (*Vallisneria spiralis*, *M. spicatum* and *C. demersum*) in the orange one. Among the latter three, *M. spicatum* has the highest sterility rate obtained during the tests, raising its position on the y-axis. It appears that the aquatic plants submitted to a single axenisation have a very low sterility rate overall, and that their scattering on the graph is mainly discriminated by their survival rate.

Iterative strategy allows better axenisation success for *M. spicatum* and *C. demersum*

For species with a sufficient survival rate (*M. spicatum* and *C. demersum*), an iterative axenisation trial was set up (Fig. 2), aiming to progressively reduce the microorganism charge and reach better sterility. Up to 6 and 8 successive

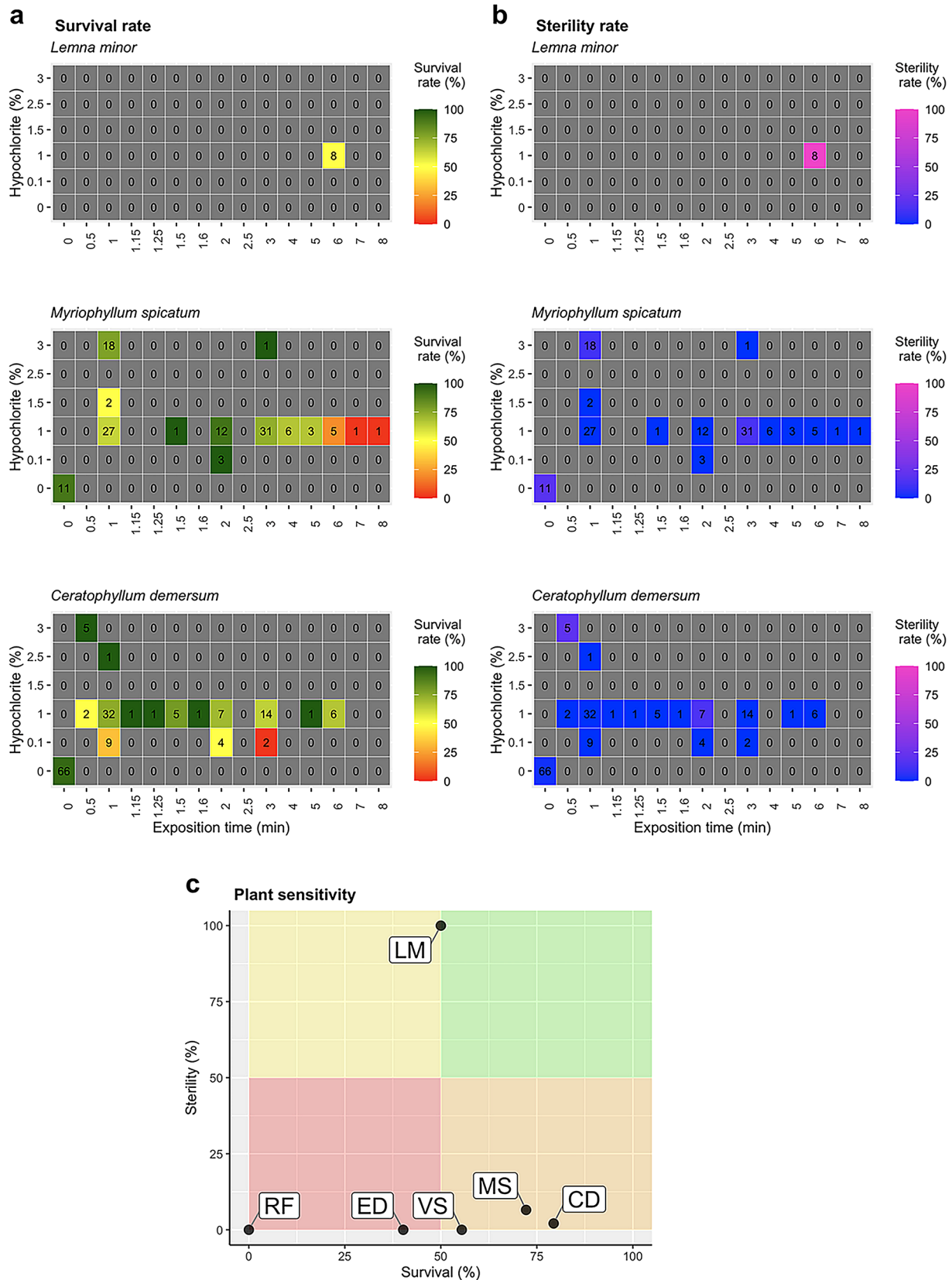


Table 2 Additional data on survival and sterility rates on axenisation trials with protocol variations in *C. demersum* and *M. spicatum*

	<i>Ceratophyllum demersum</i>			<i>Myriophyllum spicatum</i>		
	n	Survival rate	Sterility rate	n	Survival rate	Sterility rate
Exposure mode						
1+1+1 min				6	83.3%	0.0%
1+1+2 min				3	100.0%	0.0%
Simple	91	68.1%	2.2%	102	68.6%	5.9%
Sucrose (g/L)						
0	44	56.8%	0.0%	103	70.9%	5.8%
0.3	23	82.6%	0.0%			
1	3	0.0%	0.0%			
3	21	85.7%	9.5%	6	50.0%	0.0%
10				2	100.0%	0.0%

Exposure mode: 1 + 1 + 1 min means 3 min exposure to $\text{Ca}(\text{ClO})_2$ in total, but carried out in $3 \times 1'$ intervals, interspersed with 2 rinses with distilled water. The “simple” mode is 3 min of exposure in one single treatment without rinsing. Sucrose: concentration in the culture medium receiving the explant after soaking in $\text{Ca}(\text{ClO})_2$

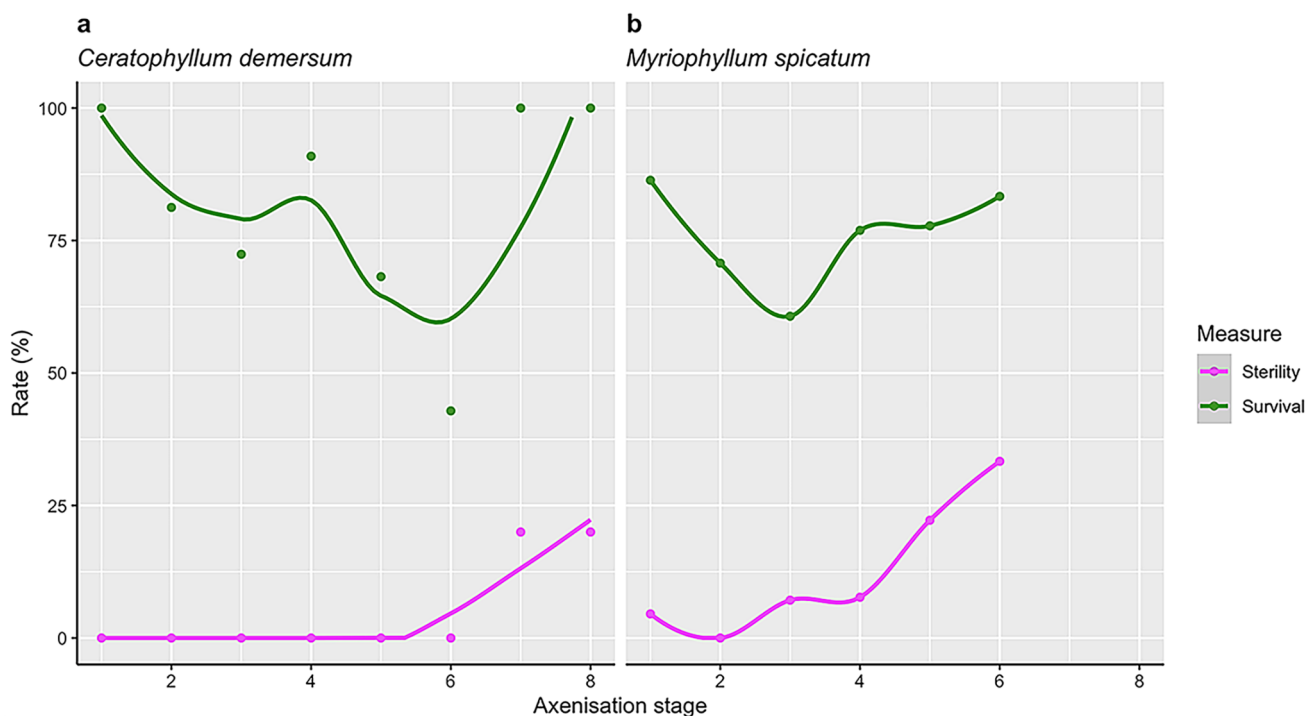


Fig. 2 Effect of iterative axenisation on survival and sterility rates for two aquatic plants. Axenisation stage corresponds to the number of axenisation trials a plant fragment has already been subject to (up to 8

successive axenisation trials). The green curve represents the survival rate, the pink curve the sterility rate; (a) *C. demersum*, (b) *M. spicatum*

attempts on the same explant were tried on *M. spicatum* and *C. demersum* respectively. For each plant, more than 10 explants were subjected to the same test in parallel. The survival rate fluctuated but remained around 75% throughout the trial without difference between species ($p = 0.5501$). However, for both species there was a downward trend in the survival rate in the early stages, before an upward trend at the end of the process, when this was accompanied by an increase in sterility. The latter follows two trends: a regular positive evolution (*M. spicatum*) or a response in 2 phases with a threshold effect from the 6th iterative stage

(*C. demersum*). The number of axenization iterations had no impact on survival rate ($p = 0.7795$). In contrast, along successive axenization trials, survival rate of both species was influenced by hypochlorite exposure time, with a significant negative effect (deviance = 6.71, $df = 1$, $p = 0.0096$). Specifically, each additional minute of exposure to the treatment was associated with a 22% decrease in the odds of explant survival (estimate = -0.247 , odds ratio = 0.78, $p = 0.0338$), which translates into a substantial reduction in survival probability (from 85.5% at 1 min. to 67.8% at 6 min, on average, based on model estimates). The effect of

hypochlorite concentration was only marginally significant ($p = 0.0850$).

Sterility success was positively related both to hypochlorite concentration and exposure duration (model estimates respectively equal to 2.026 and 1.560, $p = 0.0160$ and 0.0236, respectively). However, only hypochlorite concentration had a (marginally) significant effect in the whole model (deviance = 3.41, $df = 1$, $p = 0.0646$). Anyway, the most influencing factor was the number of axenization iterations (deviance = 22.15, $df = 1$, $p < 0.0001$), which had a positive effect on sterility success (estimate = 1.724, odds ratio = 5.61, $p = 0.0025$). This corresponds to a substantial increase in the probability of sterility— from close to 0% after one or two axenization iterations, to 17.6% after seven iterations, on average, based on model estimates.

Effect of sucrose addition and of hypochlorite exposure mode

Supplementary experiments (Table 2) were designed to test the effect of sugar addition (widely reported in the literature (Bowker et al. 1980; Lemon and Posluszny 2000; Nuttens and Gross 2017), as well as the protocol with rinsing between soakings with $\text{Ca}(\text{ClO})_2$ (exposure mode). The addition of sugar (sucrose) appeared to improve survival rate for both species, but this observation should be interpreted with caution due to the limited number of data points (especially for *M. spicatum*) and the dependence of the results on the level of contamination of the explant at the time of transfer to the culture medium. Indeed, the effect of sucrose addition was not significant, either on survival ($p = 0.1676$) or on sterility success ($p = 0.1580$). Regarding the mode of exposure, the limited results for *M. spicatum* suggested a trend towards improved sterility, but this was accompanied by a reduction in the effectiveness of sterilization and a decreasing survival rate. But, again, none of these effects was significant ($p = 0.1090$ and $p = 0.5118$ for survival and sterility rates, respectively).

Furthermore, the addition of extracts from the *C. demersum* native environment at different concentrations did not promote growth after sterilization. At this stage, we cannot draw any conclusion regarding the presence or absence of growth factors.

Discussion

The study aimed to optimize the $\text{Ca}(\text{ClO})_2$ axenisation protocol of aquatic plants. Contrary to other protocols (Barko and Smart 1981; Kane and Gilman 1991; Roshon et al. 1996; Barak et al. 2002; Grossmann 2003; Kittiwongwatana and Vuttipongchaikij 2013), we did not use a non-ionic

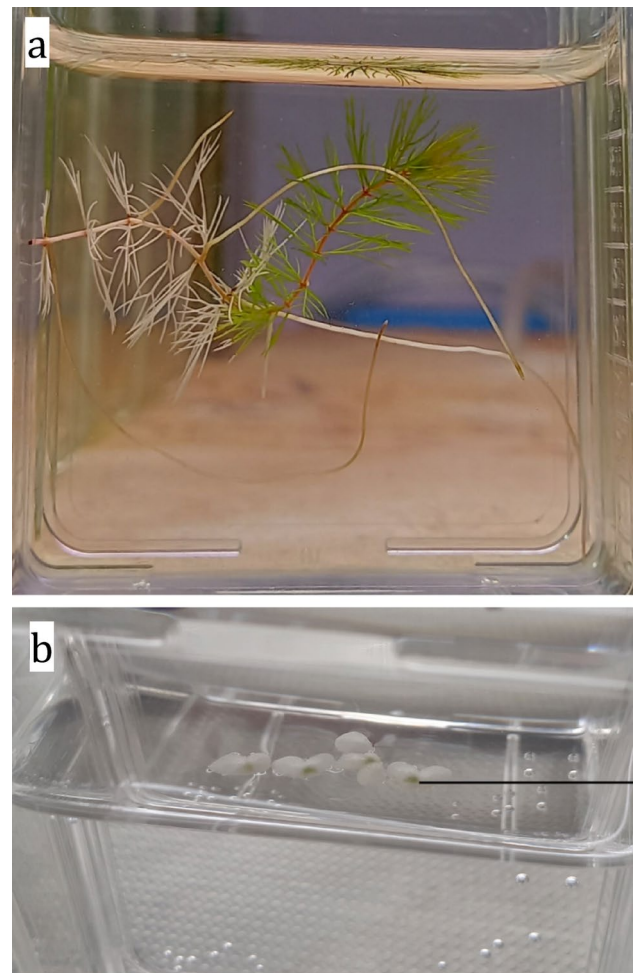


Fig. 3 Effect of axenisation on two aquatic plants. A 4th -stage axenisation (1% $\text{Ca}(\text{ClO})_2$, 3 min) was performed on *M. spicatum* (Garonne strain) four weeks before the photo (a). The bleaching of initial fragment and roots is clearly visible, opposed to new vigorous growing part from survival apex. Axenisation was also performed on *L. minor* (b). 6 min exposition causes bleaching of the fronds except for the central meristematic part (indicated by the black line), allowing the plant to restart

surfactant (Polysorbate 20 / Polyoxyethylene (20) sorbitan monolaurate (*Tween-20*), because it can affect cellular integrity by destabilising cell walls, membranes and proteins (Dresser et al. 2022), and used $\text{Ca}(\text{ClO})_2$ alone. Then, we evaluated the best combination of $\text{Ca}(\text{ClO})_2$ concentration and exposure time.

The results showed that *L. minor* was the easiest aquatic plant to axenize due to its reduced and simple leaf surface and the presence of a waxy cuticle, which reduces the areas of adhesion for microorganisms and improves the plant's resistance to $\text{Ca}(\text{ClO})_2$ penetration. The root insertion was not problematic, as the root systematically falls off during the axenisation process but without impacting plant recovery as long as the central zone of the fronds is still green (Fig. 3b). With 6 min of exposure, the fronds larger than

3 mm become entirely white except for this central zone, which is sufficient for restarting via lateral sprouting.

Among the other plants tested, *M. spicatum* appeared to be fragile to long exposure times due to excessive penetration of the disinfectant into the tissues. Rapid bleaching of leaves exposed to hypochlorite was observed (Fig. 2a), but the explant was still viable as long as the stem and the apical bud, protected by the constriction of the young leaves, are preserved. Regrowth was then possible from this bud (Fig. 2a). However, short exposure times were also problematic as *M. spicatum* and *C. demersum* were affected by a strong contamination by the microorganisms, which induced high explant mortality. For intermediate exposure durations, the high survival rate of these two plant species allowed the explants to be rapidly re-exposed to new hypochlorite treatment. Considering the sensitivity to the exposure time of *M. spicatum*, a combination of 1% hypochlorite during 1.5 to 2 min was appropriated.

For the other aquatic plants tested, fragility due to the absence of reduction of the cuticle was clearly observable. At the same time, sterility was difficult to achieve because of the aqueous tissues, with many small cavities, asperities, and areas for microorganisms to adhere. The fragility gradient according to the robustness of the tissues was clearly demonstrated. In conclusion, we propose a classification into four groups (Fig. 1c). In green, a group of plants easy to axenize. In red, a group of plants not adapted to axenization with $\text{Ca}(\text{ClO})_2$. Two intermediate groups are defined in orange (high survival but low sterility rate, which needs numerous trials with the same plant fragment) and yellow (high sterility but low survival rates, which needs many samples to succeed).

With a survival rate that remains correct (almost always above 60%), the iterative trials on *M. spicatum* and *C. demersum* seem to be an alternative for the species in the orange zone. It allowed for the improvement of sterility through several rounds of sterilisation. However, two response patterns were observed: a progressive improvement in sterility for *M. spicatum*, and a threshold effect around the 6th stage for *C. demersum*. As soon as sterility is improved, the survival rate logically goes up again (by decreasing the microbial pressure). The iterative mode may therefore be suitable for both species (and by extension, for species in the orange zone), although with a larger number of explants needed at the beginning for *C. demersum* (there should be enough explants left after 6 trials with a 60–80% survival rate). Taking an average survival rate of 75%, 42 explants were needed at the beginning to obtain 10 at stage 6, which was the minimum recommended to have results on the last 2 stages with a sterility rate of 25%.

The additional experiments conducted to test the effect of sugar addition and rinsing between $\text{Ca}(\text{ClO})_2$ baths aimed to

assess the necessity of these protocol modifications, which are commonly reported in the literature, particularly the use of sucrose. However, the results are inconclusive due to the samples size and lack of statistical significance. Although the limited data suggest a trend towards improved survival rates for both species with the addition of sucrose, this observation must be interpreted with caution as it is highly dependent on the level of contamination of the explant at the time of transfer to the culture medium.

It is important to note that the explants used in these experiments were already at an advanced stage of axenization. Trials using fresh explants (data not shown) revealed increased mortality associated with significant microorganism growth in the presence of sucrose. This rapid attack of the fragment, resulting in necrosis and a very high mortality rate within just a few days, can be attributed to the explants being weakened by hypochlorite. While fungal development was more localized and could be quickly treated before further axenization, no particular negative effects were observed for algae and yeast.

The exposure mode with successive rinses, tested on *M. spicatum*, reduced exposure of plant cells to calcium hypochlorite between baths and improved survival rate but decreased the sterility. This approach, involving a more complex and time-consuming protocol, and more risks for the plant (increased handling, thus risk of injury and recontamination; successive passages out of the water) is not recommended and the results obtained with the standard protocol are sufficient for axenisation (Fig. 3a).

The axenisation assays were carried out on plant apices. It is essential to choose apices that are alive and actively growing (or restarting after winter dormancy). For fragile and complex species to axenize, the use of seeds, rhizomes or turions (overwintering benthic forms) could be considered, but there is a trade-off between the fragment's resistance to sterilisation and the ability to reach the microorganisms to axenize correctly.

The use of antibiotics could be used in complement of the other methods of axenisation, following the identification of bacteria genus (identification on agar, using then for example a Gram stain (Smith and Hussey 2005)). Compared to the method using TSA and Sabouraud agar, the use of the CytoFLEX flow cytometer was deliberately avoided due to the technical cost and the non-reusable materials (in particular the SYTO 9™ green-fluorescent counterstain). While this technique can ensure that no contamination is missed, false positives are still possible. The use of wide-spectrum agars is recommended in routine to quickly check sterility at a lower cost and identify the microorganisms present.

Conclusions

In conclusion, the $\text{Ca}(\text{ClO})_2$ axenisation method can be effectively applied to certain aquatic plants, with attention given to the plant's shape and the extent of its protective tissue. However, this protocol is not recommended for more fragile plants such as *R. fluitans*, *E. densa* or *E. canadensis*. For other species, the optimal concentration and exposure duration combinations can be derived from our results. For instance, a 6 min treatment at 1% $\text{Ca}(\text{ClO})_2$ is optimal for *L. minor* and may be applicable to other duckweeds. Submerged species, with a more complex shape and primary protective tissues such as *V. spiralis*, *M. spicatum*, *C. demersum* can also be axenized with 1.5 to 2 min at 1% $\text{Ca}(\text{ClO})_2$. For these species, the iterative method with a minimum of 6 consecutive trials on each explant can be effective, but it is important to note the extended duration (minimum 2 months) and the large amount of plant material required at the start of the trial.

Weekly transplanting is recommended to limit excessive development of algae and non-photosynthetic microorganisms that would damage the fragile explant. It may also be necessary to adapt the concentration and duration of exposure depending on the condition of the plant. For sensitive species almost daily monitoring of the explants is required to maintain a balance.

The use of sugar in the axenization process should be limited to explants that have already undergone multiple $\text{Ca}(\text{ClO})_2$ exposures and have a significantly reduced pool of microorganisms. The use of sucrose may help explants that are already weakened by the axenization process due to the supposed mixotrophy of aquatic plants (Frick 1994; Vidakovi-Cifrek et al. 2013; Sun et al. 2020; Firmin et al. 2022).

Possible improvements of the protocol include avoiding the use of intercalated rinses between calcium hypochlorite exposures and reserving the use of sucrose in the culture medium for fragile explants with considerably reduced levels of contamination. Further research is needed to expand the synthesis table of aquatic plant sensitivity and to explore the use of antibiotics, other molecules, or combinations with Tween-20™.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11240-025-03092-5>.

Acknowledgements The authors are thankful to the Paul Sabatier-Toulouse University, the Toulouse INP and the Centre National de la Recherche Scientifique (CNRS) for granting their work. AF is the recipient of a fellowship from the French Ministry of Higher Education, Research and Innovation.

Author contributions All authors contributed to the study conception

and design. Material preparation, data collection were performed by A. Firmin. The first draft of the manuscript was written by A. Firmin and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Open access funding provided by Institut National Polytechnique de Toulouse. Open access funding provided by Institut National Polytechnique de Toulouse. Open access funding provided by Institut National Polytechnique de Toulouse. Open access funding provided by Institut National Polytechnique de Toulouse. Open access funding provided by Institut National Polytechnique de Toulouse. The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data availability The datasets generated during this study are available from the corresponding authors upon request.

Declarations

Competing Interests The authors have no competing interests to declare that are relevant to the content of this article.

Ethical statement Our research did not involved neither human participant nor animals.

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