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Tolerance of apexes of coral *Pocillopora damicornis* L. to cryoprotectant solutions ☆

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ABSTRACT

In this study, we investigated the tolerance of *Pocillopora damicornis* apexes to treatments with solutions containing penetrating and non-penetrating cryoprotective agents (CPAs). CPAs were employed individually or in binary, tertiary or quaternary solutions. In some experiments apexes were treated successively with two CPA solutions with increasing total concentration. *P. damicornis* apexes withstood exposure for up to 30 min to solutions containing 0.6–0.8 M sucrose (Suc) or trehalose (Tre). When apexes were treated with binary cryoprotectant solutions containing Suc and ethylene glycol (EG), methanol (Meth), dimethyl sulfoxide (Me_2SO) or glycerol (Gly), the CPAs employed in combination with Suc could be ranked in the following order of decreasing tolerance: EG > Meth > Me_2SO > Gly. *P. damicornis* apexes tolerated exposure to complex CPA solutions containing Suc, Me_2SO, EG and/or Meth with a total molarity of 2.45 M. In experiments where two successive CPA solutions were employed, apexes withstood treatment with the second, more concentrated solution at 0 °C for up to 10 min. These preliminary results pave the way to the development of a cryopreservation protocol for *P. damicornis* apexes.

Introduction

During the 12th International Coral Reef Symposium in Cairns, Australia, an emergency call was launched by the scientific community, aiming at urgently safeguarding coral ecosystems. Indeed, human activities such as pollution (pesticides, herbicides, fertilizers, oils), ore mining, degradations, sampling, over-fishing, tourism and natural aggressions including storms, earthquakes, predation from the crown-of-thorns starfish (COTS) *Acanthaster planci*, global warming and ocean acidification have led to an important reduction of the world's coral cover [5,9,23]. This loss of coral abundance has also had a negative impact on vertebrate and invertebrate species restricted to coral ecosystems. Corals constitute food zones, refuges, reproduction areas and nurseries for numerous marine species [14,25,27]. For local human populations, coral reefs have a high socio-economic importance and they also provide a natural protection against insular erosion.

The inclusion, in 1981, of the Great Coral Reef in the UNESCO World Heritage raised awareness of human populations on the fragility of coral reefs. However, the measures taken to date to protect and restore coral ecosystems have proved insufficient to stop reef degradation, which still takes place at an alarming rate, thus threatening the sustainability of coral ecosystems [23,31,32].

Cryopreservation provides a safe and cost-effective option for long-term conservation of animal, plant and micro-organism biodiversity [12,22]. For marine invertebrate organisms, cryopreservation was first employed to manage the production of commercially important Mollusc and Arthropod species, using larvae and reproduction products [11,33,48]. In a second stage, it was targeted towards marine biodiversity conservation purposes and was applied to various Sponge, Echinoderm and Arthropod species [33,47]. Compared to other phyla of marine invertebrates, cryopreservation of biological forms of Anthozoa species has been little studied.

For a cryopreservation protocol to be successful, the parameters of all its successive steps including conditioning of explants, application of cryoprotective agents (CPAs), cooling and warming rates, removal of CPAs, must be optimised. Another parameter of paramount importance is the selection of the developmental stage of the organisms to be cryopreserved [6,12,34,47].

A broad range of CPAs, including penetrating and non-penetrating molecules, are available for cryopreservation. In

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cryopreservation studies of marine invertebrates, permeating CPAs such as glycerol (Gly), dimethyl sulfoxide (Me_2SO) and propylene glycol (PG) have been the most commonly employed [33,45,47]. These compounds can be used separately [2,38] or in combination with non permeating CPAs including sugars such as trehalose (Tre) and glucose [1,24,29,35,37] or Heat Inactivated Fetal Bovine Serum (HI-FBS) [19].

In most cases, gametes, embryos and larvae from marine invertebrates have been cryopreserved using a slow cooling procedure [33,45]. However, some authors suggested using the vitrification technique for cryopreservation of sensitive species [15,21]. This technique requires high concentrations of CPAs (up to 7 M) to induce the vitrification (formation of an amorphous glassy state) of intracellular solutes during cooling of explants. A vitrification procedure comprises the following successive steps. During the first step, termed loading treatment, partial cell dehydration is induced by exposing cells to a moderately concentrated CPA solution, the loading solution (LS). The application of a concentrated vitrification solution (VS) is the second step of the protocol. Exposure of explants to VSs leads to the removal of most or all intracellular crystallisable water. As a result, intracellular vitrification is achieved during rapid cooling of explants in liquid nitrogen (LN). After rapid rewarming, explants are partially rehydrated in an unloading solution (ULS), which contains an intermediate CPA concentration, before their transfer to standard culture conditions.

The first report of successful cryopreservation for corals was published by Franks et al. [13] who froze dissociated cells from 10 Anthozoan taxa. Two cryoprotectant solutions were used in this work, including a combination of 80% phosphate buffered saline (PBS adjusted to the pH of the seawater) with 2.5 M Me_2SO and a mixture of 80% Leibovitz cell medium (L15), 10% HIFBS and 1 M Gly. Cells suspended in the CPA medium were placed for 2 h at 4 °C, transferred at −70 °C for 24 h and then immersed in LN. After rewarming, cells were resuspended in culture medium according to Franks et al. (1994) and their proliferation started within 7 days.

More recently, Hagedorn et al. [18,19] cryopreserved sperm cells of *Fungia scutaria*, *Acropora tenuis*, *Acropora palmata* and *Acropora milleopora* and dissociated embryonic cells of *F. scutaria*, *A. tenuis* and *Acropora millepora* using a slow cooling protocol. With *F. scutaria* and *A. palmata* sperm cells, the highest survival was achieved using a CPA solution containing 1.5 M Me_2SO and cooling rates of 20–30 °C/min [18]. The same protocol was used for *A. tenuis* sperm cells with an exposure to 1.5 M Me_2SO for 20 min before cooling (18 °C/min from 25 to −80 °C) [19]. The highest post-rewarming viability of *F. scutaria* embryonic cells was obtained following treatment with 1.5 M Me_2SO for 20 min and cooling at 0.5 °C/min. For *A. tenuis* and *A. millepora*, embryonic cells were exposed to 1.5 M Me_2SO with 1% Bovine Serum Albumin (BSA) for 20 min and then cooled at 0.5 °C/min from 25 to −80 °C [18,19]. Samples were rewarmed at 30 °C.

In case of *Pocillopora damicornis*, preliminary studies have been performed using planulae and nubbins [15–17]. These studies evaluated the toxicity of CPA solutions, the protective effect of CPA solutions against chilling and the chilling sensitivity of zooxanthellae. Symbionts appeared very sensitive to cryopreservation, which may increase the difficulty to cryopreserve coral planulae. Slow cooling (0.1–4 °C/min) was not efficient for cryopreservation of *P. damicornis* and *F. scutaria* larvae. Using the vitrification technique may therefore offer an interesting alternative [16].

In this study, we investigated the tolerance of *P. damicornis* apexes to exposure to solutions containing mixtures of penetrating and non-penetrating CPAs, which have been employed for cryopreservation of plant [12] and animal [34] tissues and organs. Apexes were chosen for the following reasons: they could be easily and regularly sampled from coral branches raised in captivity, thus avoiding the dependence on random production of reproductive material; apexes represented an homogeneous material in terms of size, developmental stage and genetic makeup; finally, apexes could regrow directly after the treatments, without passing through a larval settlement stage. After exposure to the different CPA solutions, apex survival was evaluated by polyp redeployment, tissue necrosis and expulsion of zooxanthellae.

Materials and methods

Biological material

Experiments were performed using the Scleractinian species *P. damicornis* (Linaeus 1758) from Océanopolis, Brest Aquarium, France (branches resulting from spontaneous reproduction on CITES n IOX 01448/01450). The colonies were stalled for 2 months before the start of experiments at the Oceanological Observatory Arago, Pierre and Marie Curie University, Banyuls/mer, France.

Branch tips were cut in small fragments (maximal length: 0.5–1.0 cm) with a scalpel blade (cleaned with 96% alcohol) and immersed in Artificial Sea Water (ASW: temperature 25 °C, salinity: 35, pH: 8.4). The size of apexes was selected to allow their introduction in 2 ml cryovials. Apexes were placed equidistantly in square sterile plastic Petri dishes ($N = 16$ apexes/Petri dish) to avoid disrupting healing of apexes. To prevent their adhesion on the Petri dishes, these small coral pieces were moved weekly and the plastic dishes were cleaned. Apexes were maintained in these conditions for 2–3 weeks prior to trials to allow tissue colonisation on the exposed skeleton and fresh cuts [43]. Only healthy apexes were used for experiments.

Captivity conditions

Corals branches and apexes were maintained in the same culture conditions (water temperature: 25 °C; salinity: 35; pH 8.3; water exchange rate: 90 L/week) in a 230 L aquarium with ASW mixed with osmolated water (Reef Crystal salt, Aquarium System label; Ca: 450 ppm; Mg: 1300 ppm; KH ≈ 7). Corals were illuminated with a constant irradiance of 170 μmol m^{-2} s^{-1} with two 10,000 K metal halide lamps (VENTURE®LIGHTING, 250 W) with a 12 h light: 12 h dark photoperiod and a natural radiance from windows in the roof. The aquarium filter system was composed of a biological filter, UV-C 11 W clarifier (BOYU®) to sterilise the water and a protein skimmer (Aquarium systems NEWA400). Water circulation was maintained with two circulation pumps (Aquarium systems NEWA2000 – 2200 L/h). On a daily basis, a large quantity of *Artemia salina* (EG type) nauplii was distributed to corals and apexes to ensure a permanent state of satiety.

Treatments with cryoprotectant solutions

Apexes were treated with different CPA solutions following various experimental procedures (see below – Experiments 1–6). Biological samples ($N = 16$ apexes/experimental condition) were placed in a sterile plastic Petri dish and washed several times with ASW. Apexes were placed in a 200 μm cell-straining basket to facilitate their manipulation. After treatment with a CPA solution, apexes were drained on absorbent paper to eliminate any remaining solution. Apexes were rinsed five times for 1 min each with ASW (25 °C – Salinity: 35), then placed equidistantly on a new sterile Petri dish. Apexes were maintained for 48 h in a 2 L ASW aquarium at 25 °C. During stalling, apexes were not illuminated to avoid stressing the animals and to prevent coral bleaching [39].