Endogenous cryoprotectants in *Porphyra* C. Agardh (Bangiales, Rhodophyta) collected in the Straits of Messina (Italy)

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Abstract. In the present study we observed that thalli of *Porphyra elongata* from the Straits of Messina, dehydrated and freezed at -20°C up to 31 months, were able to resist in a viable state probably due to the synthesis of endogenous cryoprotectants.

Keywords: cryopreservation; freezing stress; nori; *Porphyra*; seaweed mariculture.

INTRODUCTION

The red algal genus *Porphyra* is the most valuable seaweed in the world, maricultured and traded with the Japanese name of nori (Yarish et al., 1999; Tseng, 2001; Andersen, 2005). The exact date of beginning of the nori culture is not known, but the practice is quite old (Kyōhō Period 1716-1736) and probably began with *Porphyra* species grown naturally on fixed bundles of bamboo or oak brushwood (hibi) into the mud of the Sumidagawa river. After gathering by hands nori was washed in barrels of fresh water in order to remove sand and mud and dried in open air on bamboo mats obtaining very thin sheets of uniform size (Miyata & Tomizuka, 1997).

The nori sheets are obtained from the foliose phase of *Porphyra.* The genus is characterized by an heteromorphic life cycle with an alternation between a macroscopic foliose gamethophytic phase and a filamentous sporophytic phase called conchocelis (Fig. 1). *Porphyra* thalli, likewise for other taxa, are propagated from spores and the modern cultivation techniques are basically similar in all countries and involve four major steps: culture of conchocelis, seeding of culture nets with conchospores, nursery rearing of sporeling and harvesting.

Frozen-storage of nets is commonly used to have continuous cultures throughout the season and to replace lost or damaged nets. The introduction of this technique was devised around 1969 and stabilized the Japanese yield of nori (Andersen, 2005).

This method involves the collection of nets from the field and their storage at freezing temperatures (Miura, 1975; Tseng, 1981). When *Porphyra* thalli reach 1 to 3 cm in length on the nets, they are placed in vinyl or polyethylene bags and stored at -20/-30°C. As reported in the literature (Kurakake & Hori, 1966; Migita, 1966; Fujiyoshi, 1997) blades can survive up to 10 months in such conditions. The water content of the thalli has pronounced effect on survival. Half-dried thalli are much more tolerant to freezing than fully hydrated thalli, perhaps because there is less mechanical damage by intracellular ice.

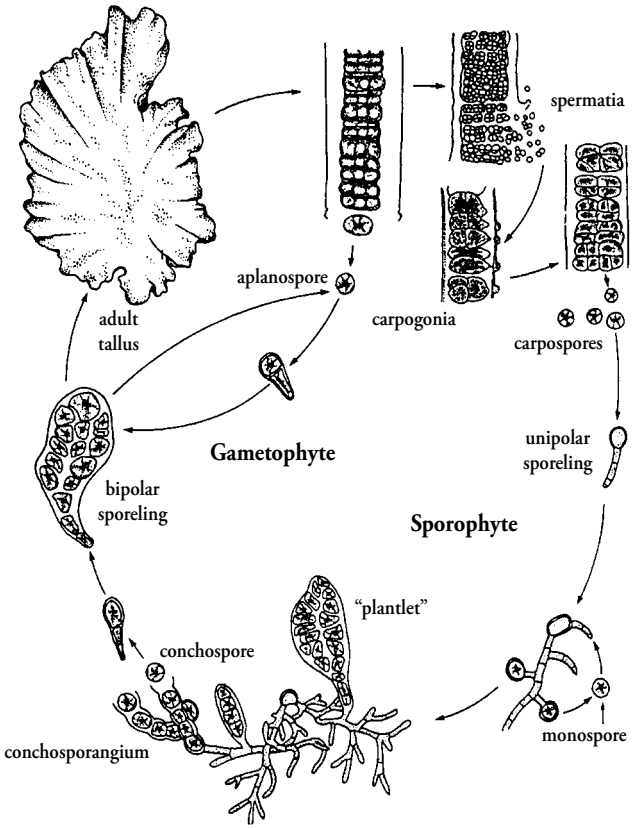

Fig. 1 – Life history pattern of the genus *Porphyra*.

Cryopreservation is a process that preserve organisms without changes in their morphological, physiological, biochemical characteristics, ensuring their long-term genetic stability (Saks, 1978; Grout, 1995). It has been successfully applied to the preservations of animals, vascular plants and algae (Taylor & Fletcher, 1999). In recent years, the cryopreservation of macroalgae has been increased (Arbault et al., 1990; Ginsburger-Vogel et al., 1992; Day et al., 1998; Kuwano & Saga, 2000) and used in aquaculture practice to allow a long-term storing material (Saks, 1978; Saga, 1990; Sakai, 1992).

The cryopreserved living cells, in fact, have the ability to slow their metabolism if processed to particular conditions. During freezing the cell keeps most of the biochemical activities to the minimum levels avoiding lethal intracellular freezing.

In the cryopreservation process developed for mariculture of *Porphyra* nets several cryoprotectants were tested in order to increase the survival rate (Kuwano et al., 1994; Fujiyoshi, 1997; Fujiyoshi & Umezawa, 1997; Kunimoto & Kito, 2002; Liu et al., 2004; Zhou et al., 2007). Liquid nitrogen (N_2) and a very low temperature are considered to be the most important tools for long term preservation of biological material. It is necessary to adequately dehydrate cells before they are immersed in N_2 or freezed.

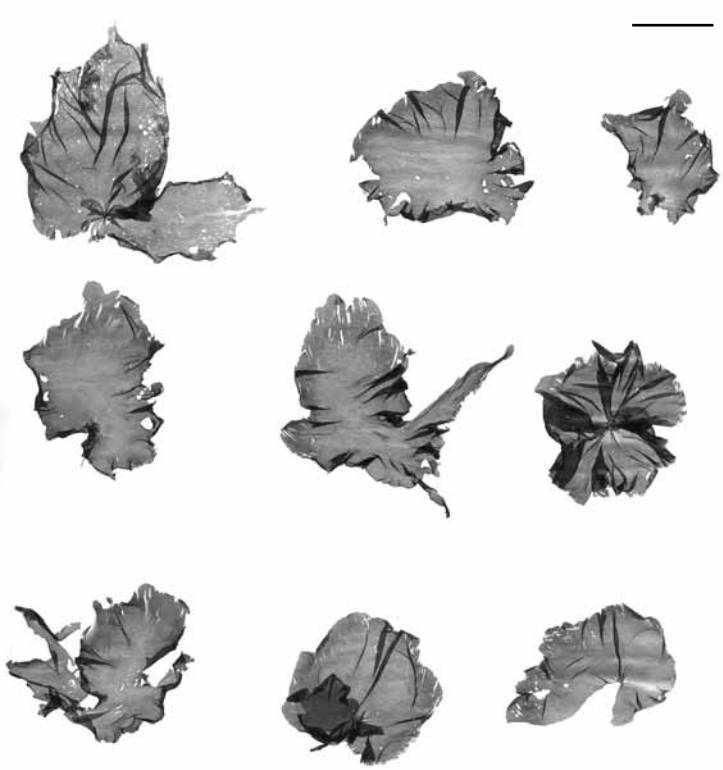

Fig. 2 – Exsiccata of *Porphyra elongata* collected at Torre Faro (Messina, Italy), scale bar: 1 cm.

The preventive dehydration avoids the freezing of cytoplasm and allows vitrification. Some materials strongly tolerate freeze-dehydration if immersed in pretreating liquids (dimethil-sulfoxide - DMSO, sorbitol or sucrose) to allow the extracellular freezing (pre-freezing method) (Kuwano et al., 1994; Fujiyoshi, 1997; Fujiyoshi & Umezawa, 1997; Kunimoto & Kito, 2002). Other dehydration methods are the osmotic dehydration, that involves the use of highly concentrated solutions (osmotic dehydration o vitrification method) (Fujiyoshi, 1997) and desiccation method by air drying (Sakai, 1993; Fujiyoshi, 1997).

The present study aims to verify the survival potential of *Porphyra elongata* (Areschoug) Kylin thalli collected in the Straits of Messina (Italy), after treatments under freezing conditions (cryopreservation) during two years.

MATERIALS AND METHODS

Porphyra elongata (Areschoug) Kylin plants (Fig. 2) were collected in April on intertidal rocks in the Straits of Messina, Italy, at Torre Faro (Messina, 38° 15' N, 15° 37' E) (Fig. 3).

Plants were gently brushed, washed in sterile seawater to remove as many epiphytes as possible and dehydrated at room temperature up to 20-30% of their water content. Then thalli were frozen at -20°C in Petri dishes sealed with Parafilm®.

Fragments lacking the basal portion to limit contamination were excised from frozen

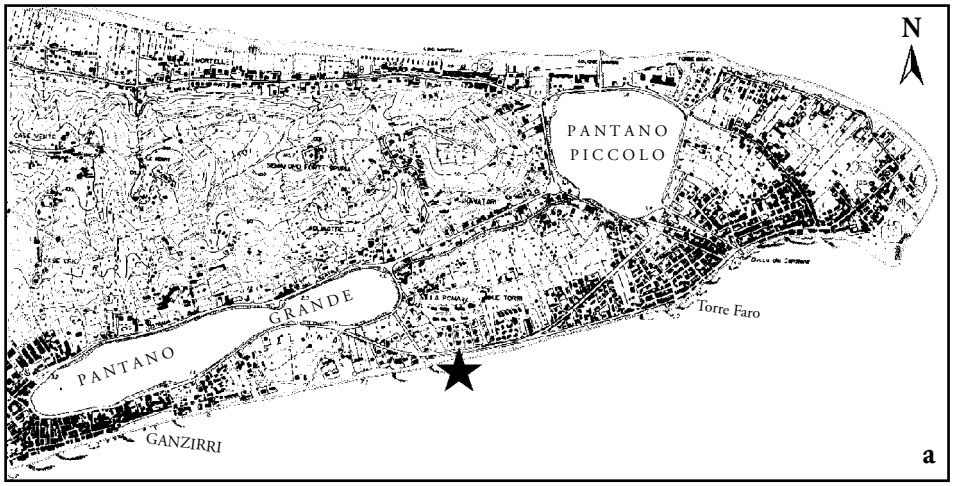

Fig. 3 – Collection site of *Porphyra elongata*: a, Cape Peloro area; b, exact site.

thalli after 1, 7, 13, 19, 25, 31 months respectively. Frozen fragments were put in Petri dishes (50mm ∅) with culture medium. Four different culture media were used: modified von Stosch at three different concentrations (full strength-VS20, half strength-VS10, quarter strenght-VS5) (Guiry & Cunningham, 1984) and sterile sea water (SW2). To limit the growth of diatoms and bacteria Germanium oxide (5 mg/l) and Penicillin G sodium salt (100˙000 U/l) were added to the medium. Two replicates with one fragment were prepared for each culture medium in each experiment. Petri dishes were incubated at 19°C under natural light conditions and daily checked for spore release.

After release, spores were isolated and transferred in a new dish with fresh medium.

Spores were observed daily to record germination and growth. Medium was changed weekly. Observations were made on a Diavert Leitz and a Diaplan microscopes and photomicrographs were taken by Leica DFC280 digital camera connected to the microscopes.

RESULTS

In all cultured dishes, regardless the length of freezing period, blades continuously released spores for 4-5 days; release occurred after two days from rehydration in VS20 medium, after 3-5 days in VS5 e VS10 media and after 5-10 days in SW2 medium. The amount of re-

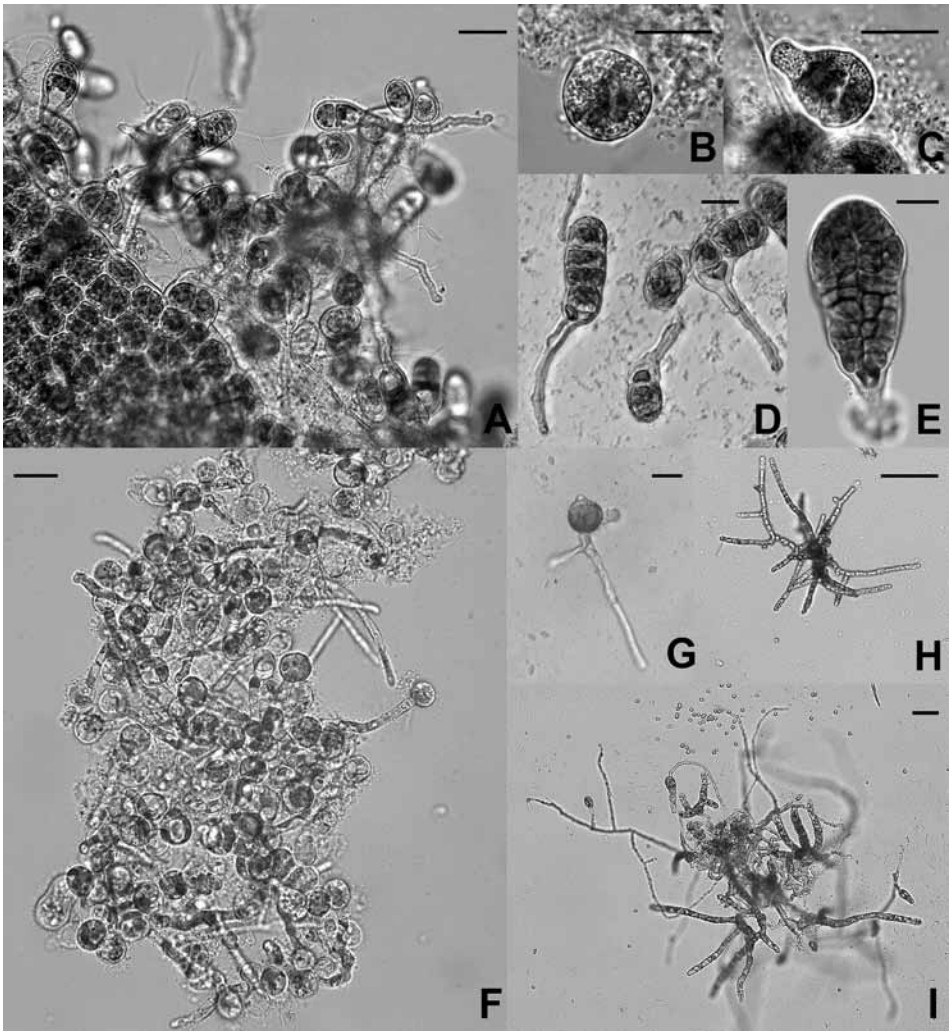

Fig. 4 – *Porphyra elongata* cultures after thawing of cryopreserved thalli: a-d, release and bipolar germinations of monospores; e, young gametophytic blade; f-g, unipolar germinations of carpospores; h, young conchocelis; i, conchocelis with differentiated conchosporagia (arrows). Scale bars: a, 20 µm; b-c, 10 µm; d-g, 20 µm; h-i, 100 µm.

leased spores was proportional to the medium concentration. In fact, a low number of spores was observed in SW2 and VS5 media, whereas spore release was more copious in VS10 e VS20 media. The release was continuous during 4-5 days.

Monospores and carpospores germinated according to bipolar and unipolar models, respectively (Fig. 4). Bipolar germlings developed in gametophytic blades, while unipolar germlings developed in conchocelis filaments, which produced viable conchospores at maturity. The released conchospores germinated bipolarly producing new blades. The growth rate of thalli cultured in the VS20 medium decreased after 10-12 days in culture, and thalli appeared discoloured in a few days.

DISCUSSION

In the present study we observed that *Porphyra elongata* thalli survive after dehydration and freezing at -20°C up to 31 months, without addition of cryoprotectants. These results are relevant in order to improve aquaculture and long term storage of nets of *Porphyra*, limiting or avoiding the use of cryoprotectants and simplifying pretreatments in culture.

The thawed plants maintained their potentiality after a long freezing process. In fact they released vital spores (monospores and carpospores) producing gametophytic foliose thalli and conchocelis filaments able to produce viable conchospores. *Porphyra* thalli were able to close the life cycle under the experimental conditions.

Our hypothesis is that the deep dehydration of thalli (about 30% of initial water content) stimulates the synthesis of molecules that worked as endogenous cryoprotectants causing the vitrification of cytoplasm (Fig. 5).

The decrease of water induces the increase of salinity in extracellular medium; consequently cells should respond modifying their inner water potential. Capability of algal cells to tolerate high salinity depends on their ability to regulate osmotic potential and the flexibility of their cell walls avoiding plasmolysis (Lobban & Harrison, 1994).

Minimal increases of the external salinity stimulate the increase of K ions or other inorganic ions inside cells. When the external salinity is very high, the K ions level should be very

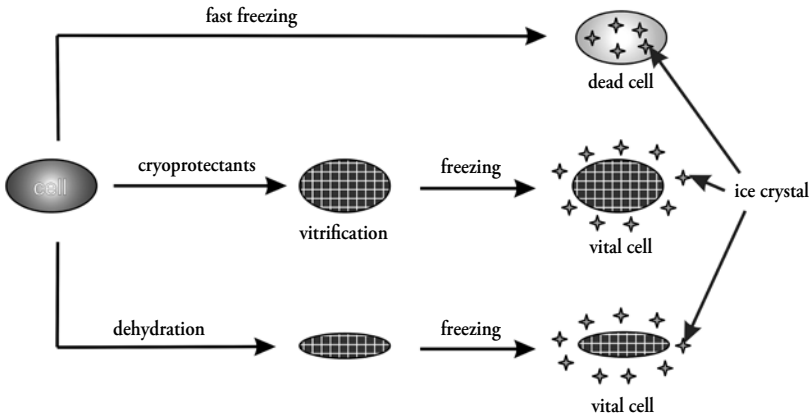

Fig. 5 – Different responses of living cells to freezing.

high to osmoregulate but this could determine inhibition of enzymatic activity. In this case the production of polyols is carried out as alternative strategy in relation to the increase of the external molarity.

Osmotic balance occurs by the synthesis of compatible solutes, that is compounds without net charges, unable to cross plasmalemma and tonoplast and that are not intermediates in any major biochemical pathways (Blunden & Gordon, 1986; Borowitzka, 1986). However, they do more than merely serve as particles in the osmotic equation; they interact with enzymes to stabilize them against conformation changes due to water loss (Borowitzka, 1986). Compounds involved in those responses belong to three categories (Lobban & Harrison, 1994): low molecular weight carbohydrates, in which hydroxyl groups blend well with the structure of cellular water (Borowitzka, 1986), amino acids, especially proline (Edwards et al., 1987), and quaternary ammonium compounds and their tertiary sulfonium analogues (Blunden & Gordon, 1986; Borowitzka, 1986).

In algae carbon may be stored in monomeric or polymeric compounds. One advantage of polymers for storage is that they have smaller effects on the osmotic potential than the same amount of carbon in monomeric forms (Lobban & Harrison, 1994). Rhodophyta produce low molecular weight storage products (Kremer, 1981; Karsten et al., 2003): digeneaside (O-α-D-mannopiranosil-(1-2)-glycerate), D-sorbitol, D-dulcitol, floridoside (O-α-D-galattopiranosil-(1-2)- glycerate) and the isofloridoside (O-α-D-galattopiranosil-(1-1)-glycerol). The floridoside is widely distributed in the Rhodophyta excluding the Ceramiales while the isofloridoside is present in many Bangiales, including *Porphyra* (Kremer, 1981; Karsten et al., 2003). In Bangiales the floridoside concentration increases proportionally to the external salinity (Reed, 1985).

The presence of these compounds in the cells might be correlated to the ability to survive for long periods at low temperatures. In *Porphyra* the osmotic regulation is mainly due to interconversion between storage polysaccharides and low molecular weight carbohydrates, such as floridoside and isofloridoside, while other species synthesize glycerol (Falkowski & Raven, 2007).

During the dehydration/freezing treatment (cryopreservation) cells likely avoid lethal intracellular freezing by the preventive dehydration of cytoplasm that vitrifies by rapid temperature change. Low molecular weight carbohydrates and polyols (polyhydric alcohols or sugar alcohols) might be involved in the process similarly to what happens in the response to osmotic and hydric stresses in nature.

The glycerol and sucrose lower intracellular water potential and avoid the formation of ice crystals. Damage of the membrane would be avoided by the interaction of polysaccharides with water molecules. This could stabilize the membrane proteins allowing dehydration and freezing without altering the cellular order.

The polyols, occurring in Rhodophyta as products of photosynthesis, form hydrogen bridges with phospholipids of the membrane, making it stable during the dehydration.

Furthermore, the presence of solutes in water lowers its freezing point, and the high concentration of salts in cytoplasm provides some protection against freezing for intracellular water. In addition, the crystallization temperature typically is lower than the freezing point (Lobban & Harrison, 1994). During the progressive cooling, ice crystals form on the outsides of the cells first. This tends to draw water out the protoplasts causing dehydration, unless cooling

is very rapid, in which case the protoplasts may freeze. Damage is caused by mechanical disruption of cell components by ice crystal formation (Bidwell, 1979).

We observed that dried and frozen thalli of *Porphyra elongata* collected from the Straits of Messina, were vital up to 31 months, even without any pretreatment. Therefore, we hypothesize that in *Porphyra* resistance to freezing temperatures is mainly due to the interconversion between storage polysaccharides and low molecular weight carbohydrates, such as floridoside and isofloridoside. Deep dehydration of thalli may stimulate the synthesis of endogenous cryoprotectants which causes the vitrification of cytoplasm. Low molecular weight carbohydrates and polyols may be involved in the process similarly to what happens in the response to osmotic and hydric stresses. Therefore, further study could be necessary to verify which methabolites increase during dehydration and their actual role as endogenous cryoprotectants.

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RIASSUNTO

Crioprotettivi endogeni in *Porphyra* c. Agardh (Bangiales, Rhodophyta) raccolta nello Stretto di Messina (Italia)

Il genere *Porphyra*, appartenente alle Rhodophyta, comprende specie particolarmente importanti dal punto di vista economico che vengono coltivate largamente per uso alimentare e commercializzate col termine giapponese nori. Le tecniche di coltivazione su larga scala prevedono l'uso di reti che, per garantire raccolte continuative lungo l'arco dell'anno, vengono congelate. Le lamine vengono preventivamente disidratate per circa 70-80% e poi conservate a -20° C in sacchi di polietilene, in cui le lamine possono sopravvivere fino a 10 mesi. La conservazione dei talli a bassa temperatura si basa sulla capacità delle cellule di rallentare il proprio metabolismo. L'alga, infatti, è in grado di sopravvivere a condizioni estreme e riprendere la vita vegetativa anche dopo lunghi periodi. Durante il congelamento si attuano una serie di cambiamenti fisiologici che consentono alle cellule di mantenere la maggior parte delle attività biochimiche a livelli minimi. Nel presente studio viene osservata la capacità dei talli di *Porphyra elongata*, raccolta nello Stretto di Messina, di resistere a -20°C fino a 31 mesi, purchè venga effettuata una preventiva disidratazione dei talli. Questo passaggio evita il congelamento letale della cellula e consente la vitrificazione del citoplasma. In tali condizioni la cellula probabilmente riesce a conservare i meccanismi metabolici per lunghi periodi effettuando l'interconversione tra polisaccaridi di riserva e carboidrati a basso peso molecolare, come floridoside e isofloridoside, glicosidi caratteristici delle alghe rosse. La nostra ipotesi è che la fase di disidratazione profonda, preliminare al congelamento, sia determinante per la sintesi di tali molecole capaci di proteggere la cellula dagli effetti dannosi del congelamento. I fattori che inducono la produzione cellulare di queste sostanze sono legati soprattutto alle condizioni di stress, come l'elevata salinità del mezzo, che non consente un normale svolgimento della fotosintesi. Le alghe che crescono a livelli di salinità inferiori o uguali a quelli dell'acqua di mare mantengono il loro bilanciamento osmotico con gli ioni inorganici. Ad un lieve aumento della salinità esterna si ha un crescita quantitativa di ioni potassio all'interno della cellula. Quando la strategia di compensazione si rende difficoltosa per elevati incrementi di salinità nell'ambiente extracellulare (come durante la disidratazione) e la quantità di ioni inorganici

necessari alla osmoregolazione inibirebbe l'attività di molti enzimi, si attua allora una via alternativa che prevede la produzione di grandi quantità di polialcool (alcol-zuccheri o polioli). Ne consegue che dall'interazione di questi oligosaccaridi con le molecole d'acqua si ottiene la stabilizzazione delle proteine con conseguente mantenimento dell'integrità delle membrane e quindi del livello di ordine dell'intero sistema. Infatti, tutti i composti appartenenti alla categoria dei polioli hanno gruppi ossidrilici che tendono a formare ponti idrogeno con i fosfolipidi delle membrane e ciò conferisce stabilità alle membrane stesse durante la disidratazione. Ulteriori studi potrebbero consentire di accertare l'effettiva correlazione tra la presenza dei glicosidi e la resistenza prolungata alle temperature di congelamento.

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