Cysted forms of halophilic microalga *Dunaliella salina* under different stress conditions

Dunaliella salina is a chlorophycean, cell wall-less microalga ubiquitous in solar salterns worldwide. It is commercially exploited as a source of natural β-carotene with a high proportion of bioabsorbable isomer of the pigment[1,2].

It has been reported that *D. salina* forms cysts in adverse conditions[3–8]. Cysts constitute a vital morphological form to tide over conditions unsuitable for living in an active vegetative condition. In *D. salina*, palmelloids (vegetative stage), aplanospores (resting spores) and zygospores (zygotes) have been described to be in the form of a cyst[4–11]. Illustrative unambiguous representations of these cysted forms are, however, not available. The fate of the palmelloid cysts in nutrient-sufficient and nutrient-poor conditions, and the precise conditions in which they are formed are not clearly documented. Here we provide a description of these different cysted forms and their development supported by photomicrographs.

We observed palmelloids along with free *D. salina* cells in water collected from salterns (in the suburbs of the coastal city of Visakhapatnam, India) during monsoon months of September and October (32 ± 2°C), three days after heavy showers, when salt in the water is diluted (60–70 ppt (9%) salinity; as measured with a salinometer). We observed them consecutively for three years (2008–2011) in saltern waters collected after heavy rainfall. When the cultures were set up from such water samples in De Walne's medium (with 12.5% NaCl – optimal for its vegetative growth[12]) at 25 ± 1°C, 100 μmol photons $m^{-2} s^{-1}$ light on a 12/12 h light/dark cycle, the palmelloid cysts grew bigger in size, were oval in shape and thickness of the envelope increased several fold (Figure 1 *a*). The surrounding envelope was two-layered (Figure 1 *a*). A group of green cells was found packed inside in a perfect round shape, often at one end of the oval cyst (Figure 1 *a*). They measured 40–42 μm/20–22 μm at 400× magnification. The radius of the inner mass of green cells ranged between 10 and 12 μm. Measurements were made with ProgRes CapturePro® 2.8 software provided with the LCD attached to Lx 400 trinocular research microscope with iVu 3000 camera module system. Occasionally, two round masses of cells each with their own sheath were together found surrounded by a common cyst wall (Figure 1 *a*, inset). When the mass of the green cells increased, the oval-shaped cysts turned spherical and thickness of the outer layer decreased (Figure 1 *b*). Then, the perfectly round shape of the cells in the cysts was distorted (Figure 1 *c*) and finally cells were seen to be released through the broken cyst wall (Figure 1 *d*). The cells released from mature cysts were small and green, and were found to briskly swim away. When these cells were sub-cultured in fresh nutrient medium, they grew into larger green cells (Figure 1 *e*) and finally matured into orange *D. salina* cells (Figure 1 *f*).

Our observations in *D. salina* cultures depicted through photomicrographs in Figure 1 are a recapture of the events that occur in nature. The same sequence of events might happen in nature when the salt concentration increases in the water of the salterns. Borowitzka and Siva[8] reported the occurrence of palmelloids in *D. salina* under low-salinity conditions both in nature and culture, but no photograph has been provided by them. The green cysts that we observed in *D. salina* are similar to the palmelloids of *D. viridis* var. *palmelloides*[8], a species in which the palmelloid stage is dominant in its life cycle. *D. viridis* is not a carotenogenic species of *Dunaliella*. Palmelloid forms of *D. salina* have been described by Lerche[9] as a group of non-motile cells encased in a gelatinous mass of mucus.

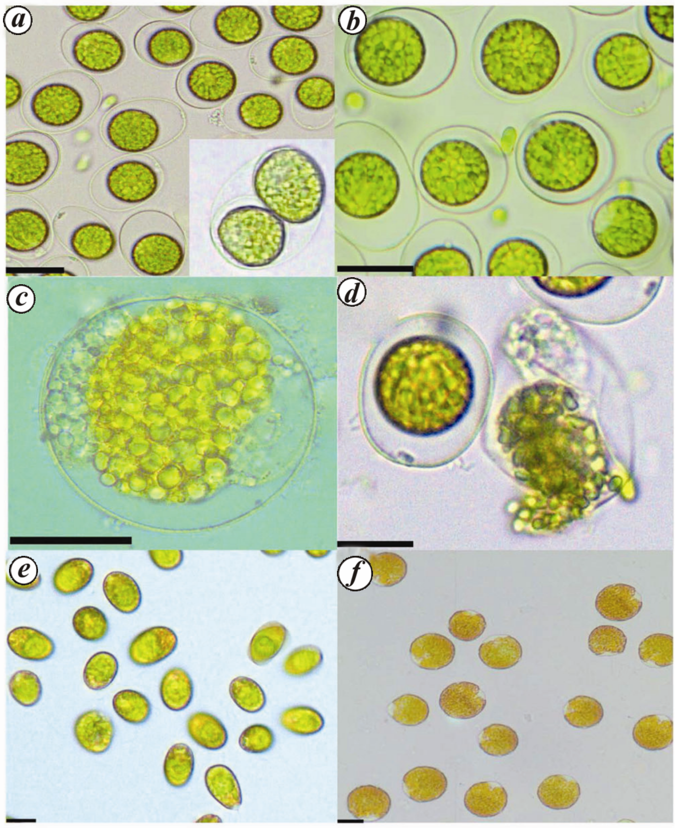

Figure 1. Development of palmelloid cysts of *Dunaliella salina* in laboratory cultures initiated from waters with low (~6%) salinity from solar salterns: ***a***, Palmelloid cysts in 5-day-old laboratory cultures (12.5% NaCl). ***b***, ***c***, Mature cysts in 8-day-old culture magnified 400 and 1000× respectively. ***d***, Release of cells from the palmelloid. ***e***, ***f***, Green and orange cells of *D. salina* in 14 and 35-day-old cultures respectively, developed by sub-culture of cells released from the palmelloid. Bar represents 10 μm.

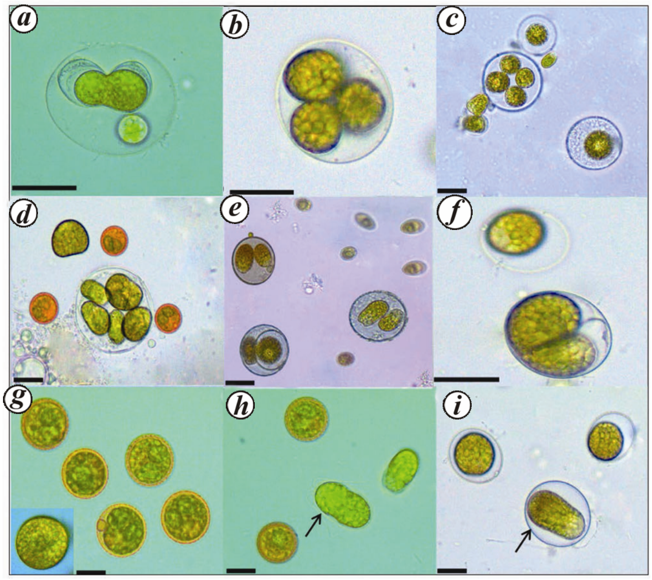

Figure 2. Cysted forms of *D. salina* in nutrient-depleted old culture of the first-generation cultures initiated from low-salinity waters of salterns. ***a***, Palmelloid with enclosed mass of green cells in the process of partitioning. ***b–d***, Palmelloid cysts with multiple masses of green cells. Note the presence of red cysts each enclosing two masses of cells in (***d***). ***e***, ***f***, Palmelloid cysts enclosing two multicellular cysts at 100 and 400× respectively. ***g***, Aplanospores with rugose wall. (Inset) Green mass of cells in the red cysts. ***h***, Aplanospores along with a giant tetra flagellate zygote (→) of *D. salina* enclosing numerous cells. ***i***, Zygospore. Bar represents 10 µm.

Benthic, macroscopic (3 mm diameter), irregular, lobulated clusters of cells in a mineral film found in hypersaline (16.5–35%) pools of the central coast of Peru were described as palmelloid stage of *D. salina* by Montoya and Olivera[4]. Palmelloids have been reported to be formed from single cells in *D. salina*[4,8]. In this process, the cells lose flagella and eyespot, secrete a mucilage layer and then divide[4,8]. Cells in the palmelloids were described to be green in colour by Borowitzka and Siva[8], and red in colour by Montoya and Olivera[4].

In old (60 d) cultures initiated from low-salinity waters of the salterns, when the nutrients were depleted, in the palmelloid that remained dormant and did not release the enclosed cells, the green mass of cells was found to partition into two or more units (Figure 2 *a–d*). Two palmelloid cysts were also found to aggregate into a single envelope (Figure 2 *e* and *f*). In nutrient-starved conditions, the palmelloid cysts of *D. salina* initiated in low-salinity waters were thus triggered to either aggregate or partition the enclosed mass of vegetative cells into smaller units. Such description of the fate of palmelloid forms has not thus far been reported in *D. salina.* In the nutrient-depleted cultures, round red cysts with a mass of green cells enclosed in a double-layered rugose wall were also observed along with the palmelloids with partitioned cell masses (Figure 2 *d*, *g* and *h*). They appear as aplanospores described by Borowitzka and Siva[8]. Aplanospores have been reported to be formed both in low- and high-salinity conditions and under low light and temperature[3,9]. Montoya and Olivera[4] noted that aplanospores were formed when *D. salina* was brought from nature (with high irradiance and temperature) to the laboratory, and they implicated their formation to the lower light intensity and temperature in the laboratory.

Alongside the red cysts, a giant tetra flagellate cell harbouring numerous cells was also observed (Figure 2 *h*). It is a zygote with the flagella of the fused partner cells yet to be lost. Subsequently, the zygote was encysted (Figure 2 *i*). Zygospores in *D. salina* have been reported to be similar in appearance to aplanospores[9], and both are reported to be formed under nutrient-depleted conditions or induced in low salinity[3,4,8–10].

The cysts described here were observed only in cultures initiated from low-salinity saltern waters during monsoon season. When water samples were collected from salterns during March and April, cysts were never observed and they always contained flagellated *D. salina* cells.

Thus, in *D. salina* low salinity induces the formation of palmelloids. Culture of these palmelloids in a medium with optimal salt condition promotes the release of cells from them. Nutrient-deficient conditions trigger the palmelloids to partition the enclosed cell mass into multiple units or the aggregate to be enclosed in a common envelope. Nutrient-deficient conditions also promote the formation of aplanospores and zygospores.

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SUMAN KEERTHI[1,4]
UMA DEVI KODURU[1,*]
SUBBA RAO DURVASULA VENKATA[2]
NITTALA SUBRAHMANYA SARMA[3]

[1]*Department of Botany, and*
[3]*School of Chemistry,*
Andhra University,
Visakhapatnam 530 003, India
[2]*Bedford Institute of Oceanography,*
Dartmouth, NS, Canada
[4]*Centre for Marine Living Resources and Ecology, MoES,*
Kendriya Bhavan,
Kochi 682 037, India
**For correspondence.*
e-mail: umadevikoduru@gmail.com