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Developmental stages of attachment of *in vitro* protoplasts in two Mediterranean *Valonia* species (Siphonocladales, Chlorophyta)

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Abstract

In vitro growing protoplasts of two coenocytic green algae, *Valonia aegagropila* and *V. utricularis*, developed into small spherules, which behaved as adventitious germlings attached to the substrate. Their developmental patterns are compared, considering that the Mediterranean *V. aegagropila* is an unattached (pleustophytic) form present in lagoons, whereas *V. utricularis* is an attached (haptophytic) species of rocky shores. In both species, thin terete branches, able to attach the thallus to the substrate arose from lenticular cells which marked the lower pole of the spherules. Some attachment branches grew as long stolon-like aseptate axes, becoming uniformly green and swelling into secondary vesicular expansions. *V. utricularis* plantlets usually formed numerous attachment branches, some of which produced other peripheral self-attaching vesicles. On the contrary, *V. aegagropila* plantlets were fixed by few, weak attachment branches; stolon-like growth was poor and ineffective in extending the attached system. This developmental pattern suggests that, although the Mediterranean *V. aegagropila* was able to generate attachment branches, these could be easily detached from the substrate by water movement. Therefore, this free floating form typical of lagoons does not originate attached populations as reported for other seas of the world. Some considerations on the taxonomy of Valoniaceae are also made.

Abbreviations: PI: photon irradiance (μmol m^{-2} s^{-1})

Key words: *Attachment, protoplasts, regeneration, Valonia*

Introduction

Previous research has described how, after mechanical fragmentation, the protoplasm of *Valonia aegagropila* C. Agardh and *V. utricularis* (Roth) C. Agardh (Chlorophyta: Cladophorophyceae) was able to reassemble into spherical protoplasts inside the uninjured parent cell wall. These endogenous protoplasts grew by secreting their own cell wall, and a multicellular condition was simulated by their mutual compression (Felicini & Perrone, 1994; Felicini et al., 1997). The process was defined as "pseudo-segregative division" in order to distinguish it from the well-known segregative division described in some members of the Siphonocladales (e.g. *Siphonocladus* and *Dictyosphaeria*; Børgesen, 1905, 1912, 1913; Egerod, 1952; Enomoto & Okuda, 1981; Enomoto et al., 1982; Okuda et al., 1997). This regenerative phenomenon is a manifestation of the contractile properties of the coenocytic cytoplasm (La Claire II, 1987, 1989, 1992; Menzel, 1988), and also represents a repairing strategy induced by occasional traumas in other siphonocladalean algae, such as *Boodlea*, *Cladophoropsis*, *Ernodesmis*, *Microdictyon* and *Phyllodictyon*, in which cell wounding induces a reaction closely resembling segregative cell division (La Claire II, 1982; O'Neil & La Claire II, 1984; Kim et al., 2002; Kim & Klotchkova, 2004). In *Ventricaria* and *Boergesenia*, a "modified segregative division", in which cytoplasmic spheres are released from the parent cell and grow into new individuals, has been described by Olsen-Stojkovich (1986) and Olsen and West (1988).

Protoplast formation from extruded protoplasm is also a common feature in some coenocytic and siphonous green algae (e.g. Tatewaki & Nagata, 1970; La Claire II, 1982; Kobayashi & Kanaizuka, 1985; Pak et al., 1991; Kim et al., 2001, 2002; Klotchkova et al., 2003; Kim & Klotchkova, 2004). Successful production of exogenous protoplasts has been obtained by Tatewaki and Nagata (1970) from the siphonous green alga *Bryopsis plumosa* (Hudson)

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C. Agardh, in which Kim et al. (2001) studied in detail the stages of protoplast formation. Exogenous protoplasts were also obtained from *V. utricularis* by Wang et al. (1997) and from *V. utricularis* and *V. aegagropila* by Felicini et al. (1997).

In a few species of the Valoniaceae family, protoplasmic spherical bodies have been observed developing into new plantlets when released from the parent vesicle (Ishizawa et al., 1979; La Claire II, 1982); they have sometimes been described with the improper terms "cysts" (Fritsch, 1935) or "aplanospores" (Enomoto & Hirose, 1972).

The present paper concerns the development and attachment to the substrate of cultured exogenous protoplasts obtained from Mediterranean specimens of *V. aegagropila* and *V. utricularis*.

Although very few studies concern *Valonia* species from an applied point of view (Misra & Sinha, 1979; Reichelt & Borowitzka, 1984; Ballentine et al., 1987), these green algae represent a good experimental material for biochemical, physiological and ultrastructural research (Itoh & Brown, 1988; Heidecker et al., 2003; Bisson et al., 2006). In addition, a possible field of application of *Valonia* cultivation could be the remediation of eutrophic environments, thanks to both its high photosynthetic ability (Eggert et al., 2006) and well-known aptitude for nitrate accumulation (Chapman & Chapman, 1973; De Boer, 1981).

Many researchers have commented on the vague boundaries within species of the genus *Valonia* (e.g. Fritsch, 1935, 1947; Egerod, 1952; Chapman, 1954; Olsen & West, 1988). *V. aegagropila* was described for the first time by C. Agardh (1822–1823) who examined herbarium specimens collected from the Venice lagoon (type locality) (Egerod, 1952). The name of this species was suggested by its aegagropilous aspect (cf. Norton & Mathieson, 1983) because the thallus is an unattached ball-shaped bush consisting of radially arranged axes (4–6 cm long) composed of apically branched subclavate vesicles. Both this habit and the lagoon habitat are characteristic of Mediterranean specimens (Giaccone, 1974; Fradà-Orestano & Calvo, 1985; Ben Maiz et al., 1987; Giaccone et al., 1994; Nonnis Marzano et al., 2003), whereas in other seas *V. aegagropila* has been described as having an attached thallus (Isaac & Chamberlain, 1958; Chapman, 1961; Jaasund, 1976; Magruder & Hunt, 1979; Littler & Littler, 2000, 2003). Egerod (1952), while re-examining the Siphonocladales from Hawaii, reported *V. aegagropila* as forming "mats or cushions"; nevertheless, he raised doubts about the classification of the Hawaiian specimens, emphasizing the need for a taxonomic revision, because they did not fit Agardh's description. From the literature of the early 1900s, Taylor (1960) reported the statement that *V. aegagropila* thallus is "at first attached, later free".

In contrast, *V. utricularis*, an infralittoral species of exposed reefs widespread in tropical and warm temperate seas, is always described as an attached epilithic or epiphytic form. Its thallus is usually an entangled cushion of irregularly branched, cylindrical clavate vesicular coenocytes (3 mm in diameter and 4–5 cm long). Occasionally, some specimens are found as free floating bushes, but accurate observation shows that they are always attached to very small fragments of rock, shell, seaweed or sea-grass.

In the current study exogenous protoplasts have been used as a readily available culture inoculum in order (i) to verify the ability of protoplasm spherules of both *V. aegagropila* and *V. utricularis* to develop into attached plantlets; (ii) to examine and compare the attachment systems of the two species; and (iii) to find out whether, when and why attached *V. aegagropila* plantlets could become free floating.

Materials and methods

V. aegagropila specimens were freshly collected from the Lesina lagoon (Italy, Adriatic Sea, 41°53′N, 15°30′E) during late spring; *V. utricularis* was collected from Otranto (Italy, Adriatic Sea, 40°08′N, 18°30′E) in summer.

Cultures were kept in a greenhouse at 17° ± 1°C, in vessels arranged on thermo-regulated stainless steel plates. They were exposed to indirect sunlight, the PI, measured at noon by a photometer (HANSATECH, UK), reaching about 50 μmol m^{-2} s^{-1} in the spring–summer period.

Pyrex flasks were used for stock cultures. The material/medium ratio in each flask was about 400 g of algal material per 4 l of filtered seawater, enriched with 0.5 mM $NaNO_3$. The medium was changed every two weeks. Flasks were equipped with continuous air bubbling; air enriched with 20 mg h^{-1} ozone was used for 2 h after each medium replacement. PI_{max} was reduced to 10–15 μmol m^{-2} s^{-1} by neutral density filters.

Vesicles of both species, selected from stock cultures, underwent the following decontamination protocol:

1. cleaning of the external surface under a stereomicroscope;
2. washing with heat-sterilized seawater;
3. individual drying by wiping the external surface with soft paper;
4. further drying in an ozonated air flux for 30 min in order to disinfect the cell surface and, at the same time, to weakly reduce vesicle turgidity.

Each cleaned vesicle was subjected to percussion by means of an electromagnetic vibrator (modified metal-engraver) (Felicini et al., 1997). The vibrating

rubber point was kept in contact with the vesicle for 1 min under moderate pressure, until the protoplasm was detached from the cell wall and mixed with the vacuolar sap. The protoplasm was squeezed through an incision at the distal end of the vesicles, and dropped into vessels containing culture medium. Two substrates were used for attachment: (1) calcareous sandstone slabs, 11 × 8 × 1 cm, lying on the bottom of rectangular glass vessels containing 200 ml of medium and (2) methacrylic Petri dishes, 10-cm diameter, containing 30 ml of medium. After the formation and attachment of the spherical protoplasm bodies, the lower half of each dish was perforated with a few holes (2-mm diameter) and sunk into a cylindrical glass vessel, 11-cm diameter, containing 200 ml of medium. The use of such a transparent substrate allowed both easier observation and photography from the underside.

During the first 8 weeks, the culture medium consisted of filtered, heat-sterilized (up to 80–90°C) seawater, enriched with 0.5 mM $NaNO_3$. GeO_2 (6 ppm) was added in order to control diatom contamination (Lewin, 1966). To prevent trouble with the attachment to the substrate, the medium was replaced every week, carefully using a syringe and striving to avoid moving the vessels. PES medium (McLachlan, 1973), replaced every two weeks, was used during the subsequent period. The culture was maintained for 22 weeks, from February to July.

Results

In stock cultures, both bushes of *V. utricularis* and balls of *V. aegagropila* grew very slowly, forming some upright branches from "lenticular cells" (cf. Fritsch, 1935; Olsen & West, 1988; Okuda et al., 1997). Adult vesicles never attached secondarily to the substrate. Occasionally, in both species vesicles produced swarmers and some germlings were found attached to the glass of the vessel. However, all attempts to induce swarmer production by changing experimental conditions (irradiance, photo-period, temperature, nutrient quality and concentration) failed.

The production of spherules from extruded protoplasm occurred with equal behaviour in both species. Microscope observation showed that amorphous protoplasm fragments became spherical protoplasts within 2–5 min after squeezing (Figure 1). The day after, the average diameter of these spherules ($n = 200$ from 4 vesicles) was 23.9 μm ($\sigma = 14.9$) in *V. aegagropila* and 21.5 μm ($\sigma = 12.2$) in *V. utricularis*. Student's *t*-test for unpaired samples did not show significant differences between the two species ($p = 0.0836$).

Under polarized light, the presence of a refractive cell wall around spherules of both species was evident after about one week (Figure 2). Within 3–4 weeks, most spherules produced one small basal lenticular cell (Figure 3) from which a thin (20–25 μm in diameter) terete stolon-like aseptate axis developed with a granular content including few and sparse chloroplasts (Figure 4). A dark green aggregate of chloroplasts, like a plug, was usually present at the proximal end of the axis (Figure 5). At this stage spherules could be considered as germlings in every respect. These adventitious germlings, however, proved to be different from those originated from reproductive swarmers. Germlings from reproductive events produced almost colourless rhizoids, which remained in open connection with their mother vesicles (Figure 6). Their further development was not observed due to their low viability in culture.

Attachment to the substrate of germlings from protoplasts only occurred at the apices of the attachment branches by means of lobed protrusions (Figure 7), which sometimes formed an irregular disk (Figure 8). The initial lobes were often formed regardless of contact with the substrate (Figure 9). Adhesion to the rough stone surface was, of course, more tenacious than that to the smooth methacrylate. Germlings which had been experimentally detached from the substrate were unable to reattach themselves. Unattached stolon-like branches grew in length up to about 500 μm (Figure 10), whereas attachment stopped their lengthening. Adjacent germlings could also attach to each other by one or more attachment branches of varying lengths, depending on the distance (Figure 11). The production of further attachment branches by the same germling was subsequent to the first one, usually when the latter was already attached. Two attachment branches sometimes arose from the same lenticular cell (Figure 12).

Further development of germlings from protoplasts was distinct in the two species. In *V. aegagropila*, the vesicular part became obovate or pear-shaped and rarely produced more than two stolon-like attachment branches (3–4) (Figure 13). Within about 8 weeks, the scattered granular content of these branches became a homogeneous subparietal chloroplast layer. The sub-terminal end of these branches often swelled into a single conical vesicle (up to about 100 μm in diameter) (Figure 14). Attachment was effected by the terminal lobes, which grew out into very thin, colourless rhizoids (Figure 15). Some attachment branches grew horizontally as stolons, either swelling all along their length or producing a series of fusiform vesicles from the basal side of which a few very thin rhizoids arose (Figure 16). These vesicles were not able to develop further during the culture period. The small (1–3 mm in diameter) vesicular part of *V. aegagropila* plantlets was able, early on, to form upright first-order branches (Figures 17–19), and became a small bush (Figure 20). Therefore, in the culture vessel,

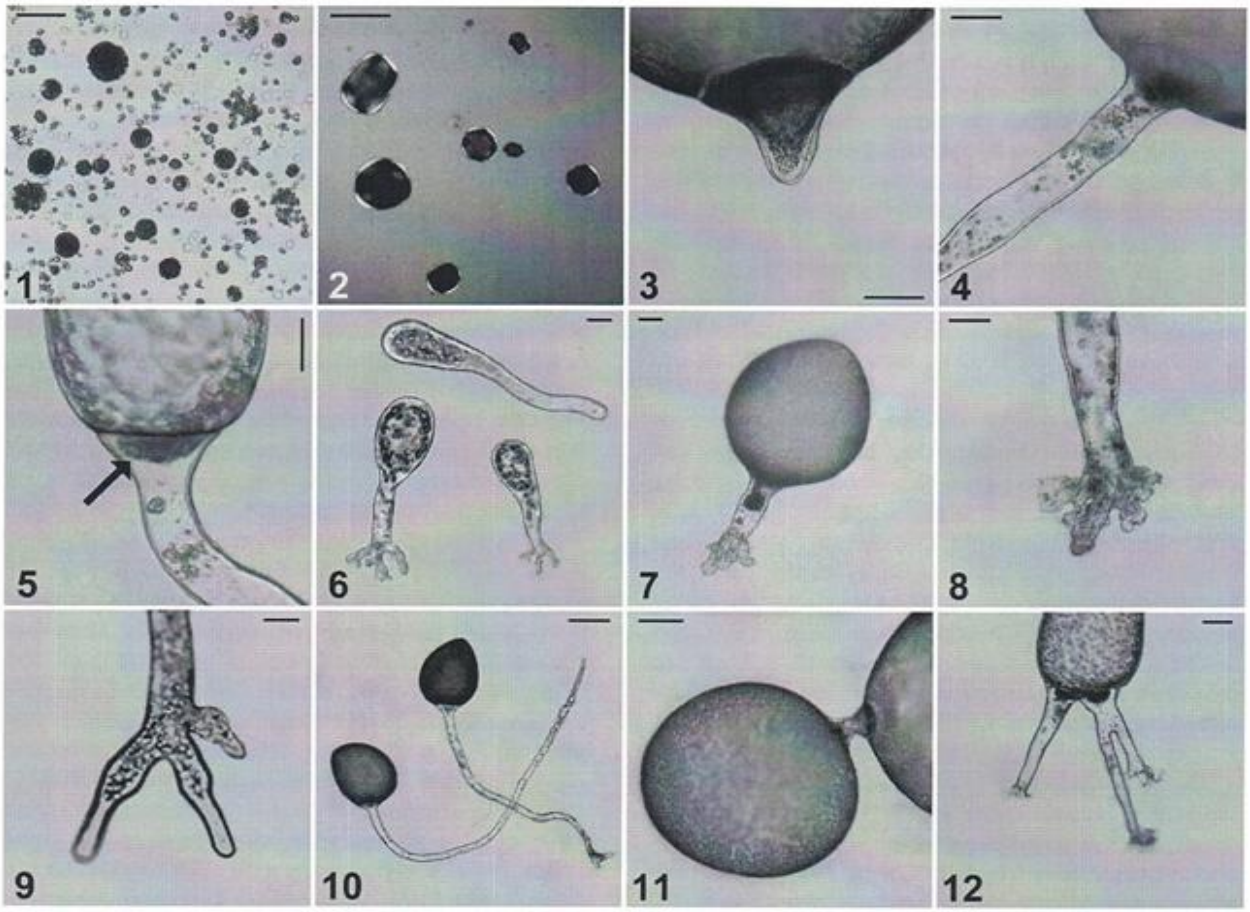
branched plantlets weakly attached to the substrate very often became free floating just because of accidental water oscillations.

By contrast, within 10–12 weeks, *V. utricularis* germlings produced up to a dozen basal lenticular cells developing as attachment branches (Figures 21 and 22). Some of these branches grew as long stolon-like axes, increasing their thickness, becoming green, and producing single or seriate expansions (Figure 23). These expansions developed into new secondary vesicles which, in turn, produced their own rhizoids. Therefore, the substrate was gradually colonized by numerous satellite plantlets attached to the substrate by an extensive attachment system. Plantlets remained almost unbranched until the end of the culture period.

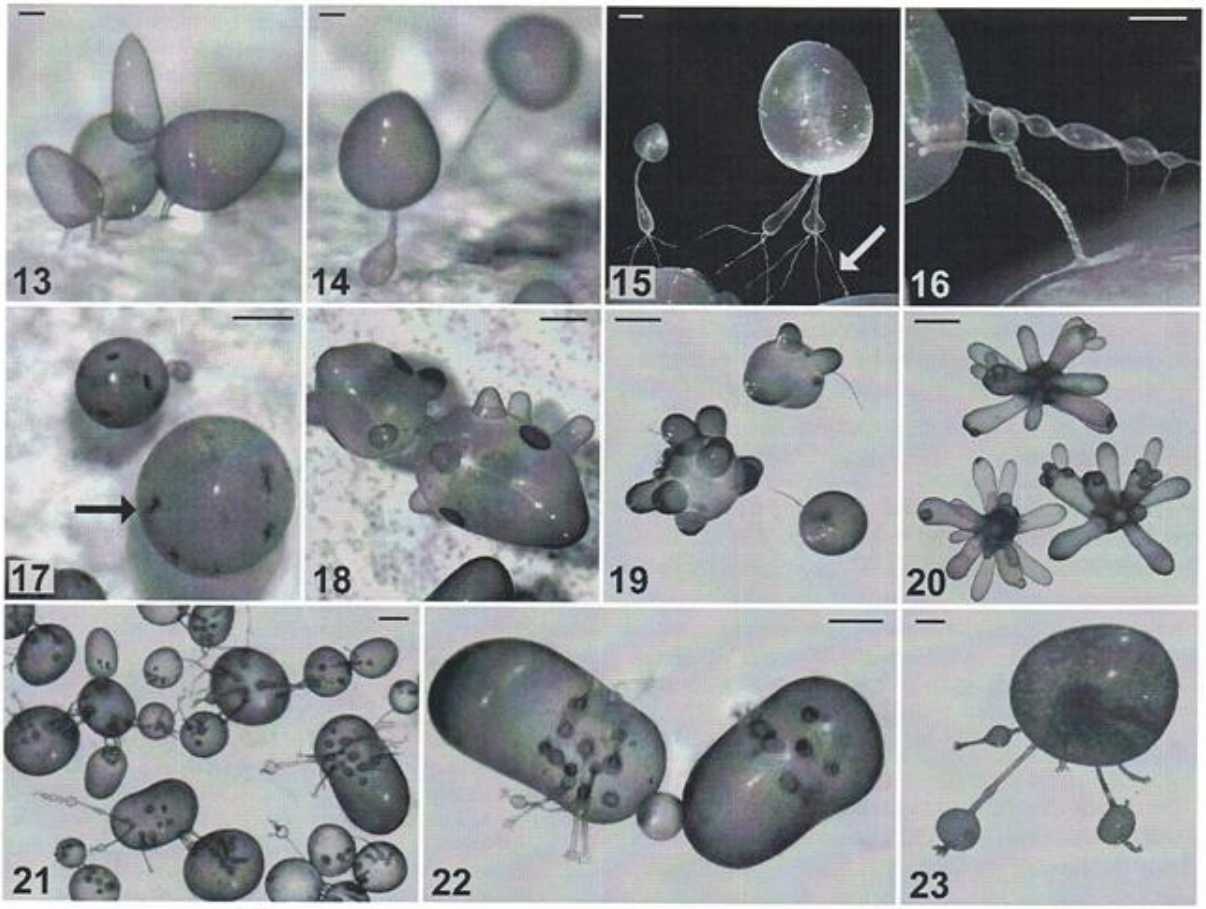
Discussion

Our observations confirm the ability of free protoplasts of both *V. utricularis* and *V. aegagropila* to develop into adventitious germlings, which attach themselves to the substrate. Attachment occurs by means of attachment branches originated from small basal lenticular cells, the so-called "rhizoidal cells" or "rhizoidal attachment cells" of Valoniaceae (Fritsch, 1935; Olsen & West, 1988).

Comparing the development of germlings from exogenous protoplasts of *V. aegagropila* and *V. utricularis*, morphological differences are evident in both the primary vesicles and attachment system. In culture, the young vesicle of *V. aegagropila* usually has a pear-shaped distal end, and quickly undergoes



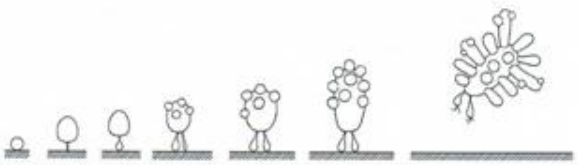
Figures 1–12. Development of protoplasts from *V. aegagropila* and *V. utricularis*. 1: *V. aegagropila* protoplasts observed 4 min after the vesicle squeezing; 2: 7 days cultured protoplasm spherules of *V. utricularis*; under polarized light cell wall appears as a bright border; 3: protoplast from *V. utricularis*; a basal lenticular cell is producing a thin long aseptate axis; 4: stolon-like axis showing a sparse granular content; 5: protoplast from *V. aegagropila*; note the chloroplast plug at the origin of the stolon-like axis (arrow); 6: *V. utricularis* germlings from reproductive swarmers; note the open connection between rhizoids and their mother vesicles; 7: germling from protoplast of *V. aegagropila* showing lobed protrusions at the apex of the attachment branch; 8: lobed protrusions forming an irregular attachment disk; 9: germling from protoplast of *V. aegagropila*; sharpened lobed protrusions forming thin colourless rhizoids; 10: unattached germlings from protoplasts of *V. aegagropila* with long stolon-like branches; 11: very short attachment branch joining two *V. utricularis* spherules; 12: germling from protoplast of *V. aegagropila* showing a forked attachment branch. Scale bars are as follows: for 1, 2 and 10-12: 50 μm; for 3-9: 20 μm.



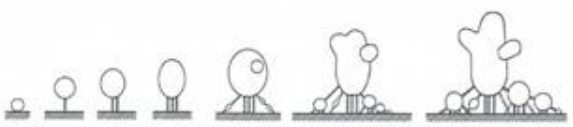
Figures 13–23. Plantlet attachment in *V. aegagropila* and *V. utricularis*. 13: plantlets of *V. aegagropila* attached to sandstone; note the characteristic pear-shaped distal end of the vesicles; 14: *V. aegagropila* attached plantlet showing the basal swelling of the stolon-like branch; 15: *V. aegagropila* unattached plantlets with stolon branch basal swellings and thin rhizoids (arrow); 16: in series swellings along a stolon-like axis; 17: appearance of sub-parietal dark spots (arrow) giving origin to lenticular cells on very young *V. aegagropila* plantlets; 18: lenticular cells and upright branch primordia on *V. aegagropila* plantlets; 19: precocious production of upright branches on very small *V. aegagropila* plantlets; 20: detached plantlets of *V. aegagropila* which become spherical bushes; 21: dense population of *V. utricularis* established on plastic Petri's dish (observed underneath); 22: *V. utricularis* plantlets attached to plastic Petri's dishes by numerous attachment branches; 23: *V. utricularis* plantlet with stolon-like branches forming secondary vesicles. Scale bars are as follows: for 13-16 and 23: 100 μm; for 17-19: 1 mm; for 20: 3 mm; for 21 and 22: 300 μm.

diffuse branching. On the contrary, in *V. utricularis* the young primary vesicle is spherical or ovate, and shows a poor tendency to the early production of upright branches. *V. aegagropila* plantlets are fixed to the substrate by means of one or very few attachment branches that do not show significant colonization ability. On the contrary, plantlets of *V. utricularis* produce numerous attachment branches some of which become stolons producing other self-attaching vesicles, so that an attached clonal population can be easily established. This difference in the developmental pattern of the two species is summarized in Figure 24.

In conclusion, the scanty and weak attachment system of *V. aegagropila* as well as the extensive upright system which confer buoyancy to the small



V. aegagropila



V. utricularis

Figure 24. Synoptic scheme of the different attachment system in the two *Valonia* species.

bushes by entrapping gas bubbles in the gaps between vesicles, make the plantlets easily influenced by hydrodynamic forces. As a consequence, plantlets quickly detach, becoming free-floating bushes, and making it impossible for attached mats or cushions of adult plants to be formed. Therefore, the ancient statement "at first attached, later free", concerning the thallus of *V. aegagropila*, has been confirmed. Outside sheltered lagoons, even if floating bushes might be carried by streams and wave motion towards rocky shores, and there become attached, established populations belonging to the same species have never been found in the Mediterranean.

The following taxonomic enigma appears obvious: are the unattached form of the Mediterranean *V. aegagropila* and the attached one living in other seas really conspecific? Could they be ecads of the same species? Evidence of an initial attachment ability to the substrate, followed by the inability to develop and maintain the attached form, could represent a vestigial character of the species which is no longer used in the lagoon habitat. On the other hand, this culture study clearly demonstrates that the inability of the Mediterranean *V. aegagropila* to attach to a substrate is not only caused by environmental conditions (i.e. the unavailability of suitable substrates), but is an inherent character of this species. Only molecular phylogenetic studies could give a decisive answer to the question of whether or not attached and unattached forms of *V. aegagropila* are distinct species.

Another important taxonomic conclusion can be drawn from this study. Both in *V. aegagropila* and *V. utricularis* the formation of spherical bodies of cytoplasm, generating their own cell wall, has been demonstrated. Based on immunological distance analyses, and the presumption that a "modified segregative cell division" typically occurs in *Valonia ventricosa* while all other *Valonia* species can only divide by lenticular cell formation, Olsen and West (1988) transferred this species to a new genus, *Ventricaria*. Results from the present study confirm that this mode of cell division does not only occur in *Ventricaria*, but that it can also be induced by mechanical fragmentation of the protoplast in *V. utricularis* and *V. aegagropila*. This is also in agreement with recent bio-molecular studies (Leliaert et al., 2003, 2007), which have confirmed that *Ventricaria* belongs to the genus *Valonia*.

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