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**Reproductive effort in the domesticated red alga *Agarophyton chilense*: differences
between farms and natural populations**

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Running title: Trade-off between reproduction and growth

Abstract

Macroalgal domestication and farming can induce significant ecological and biological changes in exploited species. In the red macroalga, *Agarophyton chilense*, marine farming is based on clonal propagation by cuttings of the largest plants. This type of mass selection by farmers can have a considerable impact on the life history characteristics of cultivated strains. In this study, we investigated the potential effect of this type of selection on the reproduction in *A. chilense*, comparing female gametophytes from the natural and farmed populations. Our results showed strong differences between these two types of populations. As expected, individuals were larger in the farm than in the wild population. On the other hand, the number of cystocarps per centimeters of fronds was ten times lower in the farm than in the natural population. These differences suggest that artificial selection and cultivation environment significantly modify life-history traits in this macroalga. Moreover, the positive relationship between female size and number of cystocarps per centimeters of fronds observed in Dichato point out to the possible existence of cost of reproduction in *A. chilense*, with bigger females allocating more resources or energy to reproductive structures in natural populations.

Key index words: Aquaculture, Gracilariales, cystocarp production, reproductive effort, investment, trade-off, vegetative growth.

1. Introduction

New macroalgal cultivation methods, most often adapted from the agricultural practices of terrestrial plants, are being developed in marine aquaculture (Robinson et al. 2013, Hafting et al. 2015). These new cultivation practices in human-controlled

environments are associated with significant ecological changes (Smith 2016), as well as alterations in the biology and reproduction of cultivated species (Zeder 2015; Valero et al. 2017). In macroalgae, these domestication processes are still poorly understood and further research, integrating knowledge on the evolutionary biology and ecology of natural populations and on the selective pressures exerted by cultivators, is therefore necessary to promote sustainable macroalgal aquaculture (for review Valero et al. 2017). In some species of red algae, cultivation is based on the clonal propagation of rapidly growing algal strains on farms. This type of clonal multiplication can generate an important impact in the characteristics of the biological cycle and the genetic diversity of cultivated species (Guillemin et al. 2008, Valero et al. 2017). One example of this is the Rhodophyta *Agarophyton chilense* (C.J.Bird, McLachlan et E.C.Oliveira) Gurgel, J.N.Norris et Fredericq previously referred as *Gracilaria chilensis*. This species has been domesticated and cultivated in Chile since the early 1970s, mainly for agar production (Buschmann et al. 2008). It has been hypothesized that farmers have unconsciously selected for fast growth rate genotypes in farms, potentially simultaneously counter-selecting against high reproductive investment (Guillemin et al. 2008; Usandizaga et al. 2019). Indeed, in controlled laboratory conditions, trade-offs have been reported between growth and reproduction for this species (Guillemin et al. 2013).

Agarophyton chilense presents a typical isomorphic haplodiplontic life cycle with two free-living isomorphic generations: haploid gametophytes (males and females) and diploid tetrasporophyte. When released, the haploid tetraspores attach to the substrate to give new gametophyte individuals consisting of perennial holdfasts from which spaghetti-like thalli grow. Male gametes (spermatia) are released in the water column. Female thalli bear uniformly distributed gametangia, which consist of three cells and can be observed only on

thallus sections under microscope (Kling and Bodard 1987). Fertilization occurs on the female individual and involves complex cytological events, resulting in the formation of a cystocarp on the female (Hommersand and Fredericq 1990). The development of a haploid pericarp is induced, within which the zygote divides mitotically (gonimoblast), protected and nurtured by the female. After a few weeks of maturation, the cystocarp liberates thousands of diploid spores named carpospores (Lefebvre et al. 1987; Fredericq and Hommersand 1989; Kain and Destombe 1995). These carpospores, after their release, give rise to new tetrasporophyte individuals.

Two types of populations, potentially presenting strong difference in reproductive investment (Guillemin et al. 2008), are found in Chile: (1) natural populations formed by individuals growing from a perennial holdfast and fixed to hard substratum and (2) floating thalli that grow and propagate by self-replication in sandy and muddy bays and estuaries (i.e., farms). The natural populations are maintained by sexual reproduction and spores recruitment (Guillemin et al. 2008). In these populations fertile tetrasporophytes, male and female gametophytes are frequently encountered (Meneses 1996; Vieira et al. 2018a). On the other hand, farmed populations - growing on muddy or sandy beds - are composed by a high proportion of genetically identical units (Guillemin et al. 2008), mainly maintained through human-assisted thallus breakage and embedding. Most of the Chilean crops of *A. chilense* are composed of clonal tetrasporophytes, except the farmed population in Lenga (36° 45' S, 73° 11' W), where growing thalli are mainly female gametophytes (Guillemin et al. 2008). Farmed thalli are generally recognized to be poorly reproductive sexually (Prieto et al. 1991). However, even if slightly less reproductive individuals have been observed in farms, no clear significant differences have been reported in terms of frequency of reproductive thalli when compared to natural populations (Guillemin et al. 2008).

In haplodiplontic red macroalgae, the quantification of cystocarps production is simple since these reproductive structures are visible with the naked eye and have hemispherical fruiting bodies which develop on the female gametophyte after fertilization (Richerd et al. 1993, Kamiya and West 2010). In *A. chilense* (as *G. chilensis*) and *Gracilaria domingensis* (Kützinger) Sonder, the vegetative growth of female gametophytes decreases after fertilization during the development of cystocarps (Santelices and Varela 1995; Guimarães et al. 1999). These observations are consistent with resource allocation theory which predicts that reproduction imposes a cost on an organism (for reviews see DeWreede and Klinger 1988; Santelices 1990). The number and size of cystocarps produced by a female has been used to measure red macroalgae reproductive output (Edyvean and Ford 1984). The reproductive effort, classically defined as the relative amount of resources allocated to reproduction over a defined period of time, is much more complex to measure. The ratio of the reproductive biomass to that of the whole plant or the vegetative biomass has been used as a proxy of reproductive effort in Fucales (Cousens 1986; Ang 1992; Åberg 1996) and crustose macroalgae (Edyvean and Ford 1984). In red macroalgae, reproductive output will depend on the female genotype and the number of gametangia it produces (Engel and Destombe 2002), the quantity and quality of male gametes (Engel and Destombe 2002; Engel et al. 2002) and the female investment in cystocarps (Kamiya and Kawai 2002). However, reproductive output and reproductive effort are sometimes difficult to distinguish, and cystocarp production per unit area or frond length has been used as an indicator or proxy for reproductive effort (Krueger-Hadfield et al. 2013). In this study, we examined the relationship between thallus size and reproductive output in natural versus crop populations. We hypothesize that clonal propagation and artificial selection in farmed populations have

induced reproductive costs in *A. chilense* evidenced by the trade-off between thallus size and cystocarps production.

2. Materials and methods

2.1 . Collection and maintenance of algal material

In *A. chilense*, gametophytes and tetrasporophytes share the same gross morphology and the phase and sex can only be recognized during the reproduction period. Even if reproductive individuals could be encountered all year round and reproductive season is not clearly defined for *A. chilense*, the highest percentage of reproductive individuals is encountered during summer (December to April in the southern hemisphere, Guillemin M-L. unpublished data) and the highest spore production occur at the end of summer / beginning of autumn (after February, Vieira et al. 2018b).

Reproductive female gametophytes were collected in March 2009 in the natural population of Dichato (36° 32' S, 72° 56' W) and in the crop of Lenga (36° 45' S, 72° 11' W) separated geographically by about 25 km (see Figure 1). We sampled different genets (i.e. females with distinct holdfasts) in Dichato and different ramets (i.e. thalli developing from an under-ground thallus system) in the farm of Lenga. In Dichato, females with cystocarps were selected directly in the field. In Lenga, a farm where much less reproductive structures are generally encountered (Guillemin et al. 2008), only well-isolated thalli (i.e., a well-defined group of fronds surrounded by at least 10 cm of sand from any other *A. chilense*) were randomly selected (N=100) and the above-ground biomass was collected. In each site, individuals were collected in an area no larger than 20 m². The collected individuals were placed separately in plastic bags and transported in a cooler to the “Calfuco Water Resources

Coastal Laboratory”, in Valdivia. Once at the laboratory, the presence of cystocarps was revised thoroughly under a stereoscope microscope (Zeiss Stemi DV4/DR) and species-specific molecular sex markers were amplified in vegetative individuals from Lenga using the protocol defined by the authors (Guillemin et al. 2012). In total, 20 females were selected in each population for further analyses.

2.2. Biomass and reproductive investment estimators

In the natural population of Dichato, all females were attached to the substratum and primary fronds were defined as thalli directly growing from the holdfast. In the farm of Lenga, after cutting individuals at sand level, the independent thalli forming the clump were defined as primary fronds. In both populations all primary fronds and up to seven secondary fronds per female were measured. For each female, we measured four estimators of biomass: i) the dry weight (g), ii) the volume of the thallus (i.e., the volume of a cylinder of the maximum length and maximum diameter of the thallus in cm^3), and iii) the length of primary fronds (cm) and iv) the length of secondary fronds (cm). As reproductive output estimators, we measured: i) the number of cystocarps observed per centimeter of primary fronds (no. of cystocarps $\cdot \text{cm}^{-1}$) and, ii) the number of cystocarps observed per centimeter of secondary fronds (no. of cystocarps $\cdot \text{cm}^{-1}$). Hereafter, the diameter of the cystocarps was measured under a stereoscope microscope in the lab (Zeiss Stemi DV4/DR). Up to 60 cystocarps were measured per female in Dichato and up to 45 in Lenga. In order to verify for the presence of viable carpospores in the cystocarps, six fragments of fronds carrying cystocarps were taken haphazardly in each population and fixed in 10% formalin-seawater. Material embedded in paraffin was sectioned in a microtome (Leica RM 2035) and stained with dilute methylene

blue or haematoxylin-eosin for observation of general morphology. Photomicrographs were taken with an Olympus BX41 microscope.

2.3. Statistical analysis

Data were tested for homogeneity of variances and normal distribution using Levene's and Shapiro-Wilk tests, respectively. When existence of non-normal residuals and/or heteroscedasticity was detected, data were logarithmically transformed prior analyses. Student T tests were performed to evaluate differences between Dichato and Lenga whenever homogeneity of variances was achieved (i.e., dry weight, volume of the thallus and primary and secondary fronds length), while the non-parametric Mann-Whitney U-tests were used when homogeneity of variances was not fulfilled after data transformations (i.e., number of cystocarps per centimeter of primary and secondary frond). Correlation between length of primary fronds and cystocarps production in females were tested using Spearman rank correlation, performed independently in Dichato and Lenga. All differences were considered significant at p -value < 0.05 and correction for multiple testing was carried out using the Bonferroni method. All analyses were performed in R (3.6.3 version) (R Core Team 2016).

3. Results

In the farm of Lenga, cystocarps were observed in only 80% of the females (16 out of 20), whereas in the natural population of Dichato, all 20 females studied bear cystocarps. Strong differences were observed between Dichato and the crop of Lenga for female size (length of primary and secondary fronds, volume of thallus and dry weight, $p < 0.05$ for all Student T tests, Table 1) and production of cystocarps (number of cystocarps observed per centimetre of primary and secondary frond, $p < 0.05$ for both Mann-Whitney U-tests, Table

1). Females from Dichato were much smaller than the ones from Lenga (dry weight, volume of thallus and length of primary fronds, Table 1). The females sampled in the natural population of Dichato were characterized by a higher production of cystocarps with a number of cystocarps per centimeter of frond ten times higher for primary fronds and twenty times higher for secondary fronds than the females from Lenga (Table 1). No significant difference in cystocarp size was observed between populations (Table 1). The number of cystocarps observed along the primary fronds was positively correlated with their length in Dichato (Figure 2) but not in Lenga (Figure 2). The number of cystocarps along the secondary fronds was not correlated with their length irrespective of the locality ($r_s = -0.234$, $p = 0.306$ and $r_s = -0.137$, $p = 0.575$ in Dichato and Lenga, respectively, data not shown). Finally, carpospores were observed in cross-section of cystocarps in both localities (Figure 3).

4. Discussion

Our results demonstrated that female gametophytes of *Agarophyton chilense* are bigger and less sexually reproductive in farms than in natural populations. These differences suggest that cultivation significantly modified life-history traits of this macro alga. In this species, cultivation techniques have usually relied only on replanting of thallus cuttings. This practice allows farmers to selectively multiply (consciously or unconsciously) the phenotypes with the highest biomass production but also the ones best adapted to these new growing conditions. We propose that the ecological modification of the environment and the clonal selection oriented towards the production of biomass operated by farmers (i.e., the first step in the domestication process of the species; Valero et al. 2017), had the indirect consequence of reduced investment in sexual reproduction leading to a much lower reproductive output. Observations of the effect of domestication on the reproduction on

plants suggest that human selection for increased vegetative propagation led to architectural changes that resulted in reduced sexual fecundity (in cassava, McKey et al. 2010) and fertility (e.g. in potato, Simmonds 1997; and yams, Segnou et al. 1992). In cultivated seaweeds, strong evidence for domestication exist (*Agarophyton*, Guillemin et al. 2008 and *Kappaphycus*, Ask and Azanza 2002). Nevertheless, no clear results have been obtained regarding the diminished reproductive output as a consequence of domestication process in macroalgae yet.

Field studies focused on natural populations of *A. chilense* have shown that the probability of a frond to become fecund is size-dependent (Vieira et al. 2018a and b). In the present study, a positive relationship between the size of the primary fronds and the number of cystocarps produced was observed in the natural population (Dichato). This could be explained by the fact that the external part of this organ (the pericarp) is produced directly by the female. Indeed, in the Florideophyceae, the size of the cystocarp and the number of spores produced by cystocarp are related to the thallus biomass supporting the reproductive structures (in *Antithamnion nipponicum*, *Ceramium boydenii* and *C. Japonicum*; Kamiya and Kawai 2002). In the same way, in brown algae, positive correlations between thallus size and the proportion of biomass allocated to reproductive tissue have been reported in various species (*Ascophyllum nodosum*, Åberg 1996; *Hizikia fusiformis*, Zou et al. 2006; *Sargassum thunbergii*, Zhang et al. 2009) and were related to the higher number of reproductive meristems available for receptacle production present along the thalli of larger individuals (Zhang et al. 2009). On the other hand, the positive relationship between the size of the frond and the production of cystocarps was not observed in females from the farmed population (Lenga). In addition, these females had on average a production of cystocarps ten times lower than in the natural population. As the development of cystocarps occurs after the fertilization

(Hommersand and Fredericq 1990), this result could be linked either to a low number of carpogonium (female reproductive structure) per female or to a general low availability of male gametes in this farm. Indeed, contrary to what is observed in natural populations where the sex ratio is generally balanced (Guillemin et al. 2008), the number of males in Lenga is reduced (data not shown). Farmers seem to avoid males in *A. chilense* crops probably due to their lower vegetative growth rate, when compared to females or tetrasporophytes (Guillemin et al. 2013). Despite these differences between the two types of populations, we assume that the few reproductive males observed in the farmed population can actively participate in fertilization. Furthermore, we cannot rule out the hypothesis that the male gametes could come from neighboring natural populations, maintaining some degree of sexual reproduction in the farm. It has been shown in *Gracilaria gracilis* that spermatia have a lifespan of more than 6 hours and can be carried by currents (Destombe et al. 1990).

According to life-history theory, reproductive cost implies trade-offs in resource distribution, expressed as changes in future growth, fecundity, and/or survival (Stearns 1992; Álvarez-Cansino et al. 2010; Vieira et al. 2018a; Vieira et al. 2018b). In this context, some empirical studies in plants have documented trade-offs in terms of allocation of energy and limited resources, particularly between reproduction and vegetative growth (Ashman 1994; Campbell 2000; Ehrlén and Groenendael 2001; Henriksson 2001; Obeso 2002, 2004; Bañuelos and Obeso 2004; Álvarez-Cansino et al. 2010). In some Rhodophyta, gametophytic females have been shown to present a clear decrease in vegetative growth when reproductive (Santelices and Varela 1995; Guimarães et al. 1999) as resources are diverted from the adjacent thallus to allow the development of cystocarps (Kamiya and Kawai 2002). In *A. chilense*, vegetative fronds show a higher survival and a better growth rate than reproductive ones irrespective of the type of individual analyzed (male gametophyte, female gametophyte,

or tetrasporophyte) (Guillemin et al. 2013). Studies monitoring *A. chilense* in natural populations also support the existence of a trade-off between survival and reproduction (Vieira et al. 2018a and b). A study developed in central Chile focusing on gametophytes of the two intertidal red algae *Ahnfeltia durvillaei* and *Gymnogongrus furcellatus*, suggests the existence of reproductive costs and trade-offs between growth and fecundity (Camus 1992). The study documented a size-independent threshold in reproductive effort for each species. In the same way, field studies in brown algae support the existence of a reproductive cost, evidenced by decrease in growth or survival rates in reproductive individuals (Ang 1992; Åberg 1996).

In this study, despite the lower production of cystocarps in females from the farmed population, these structures seem to be fertile and produce carpospores. However, it is very unlikely that these spores will succeed in germinating in this type of sandy environment, which could explain the low frequency of tetrasporophytes in this farm. The evolution of functionally sterile clonal thalli in farms has been previously hypothesized in *A. chilense* as a result of farming techniques mostly/only based on clonal multiplication of vegetative thalli (Guillemin et al. 2008). The theoretical framework behind this evolutionary hypothesis suggests that organisms maintained only by asexual reproduction for a number of generations could lose their ability of reproduce sexually due to the accumulation of sterility mutations in populations where sexual recruitment is impaired by the environment (“use it or lose it” hypothesis; Eckert 2002). In these cases, sterility arises through the accumulation of mutations in genes necessary for sexual reproduction (i.e. genes involved in meiosis, gamete production or necessary for gametes encounter and fusion), these becoming non-functional. The lack of sexual reproduction during various generations impedes counter selection and the purge of the mutation load accumulated along these genes (Eckert 2002). Our results do

not, however, support the idea of a sterilization of the Lenga farm clonal female thalli. This result may be explained by the relatively recent domestication of *A. chilense* (Buschmann et al. 1995; Valero et al. 2017) and evolutionary pathway to sterilization could still be ongoing in this species. However, measurements made during the present study include only the cystocarps formation while other important steps in the sexual life cycle have not been considered (e.g., the capacity of the carpospores to settle, germinate and grow) (Fletcher and Callow 1992). Complementary common garden experiments and genomic screening are now needed to test for differences in survival and growth of carpospores produced by females from natural and farmed populations and study the potential load of accumulated mutation in genes necessary for sexual reproduction.

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Table 1: Differences in dry weight, thallus volume, length of primary and secondary fronds, number of cystocarps observed per centimetre of primary and secondary fronds and size of cystocarps between females from the natural population of Dichato and the crop of Lenga. Bold characters represent significant values (*Mann-Whitney U Test; \$Student T test) after a Bonferroni correction for multiple tests; 20 individual measured per locality; values are means \pm SE. n.a.: not applicable.

Variables	Natural Population Dichato	Crop Population Lenga	df	t-value	p-value
Dry weight (g)	8.39 \pm 5.82	50.09 \pm 39.54	40	-6.55	(\$) < 0.001
Volume of the thallus (cm ³)	82.84 \pm 70.96	291.01 \pm 145.65	30	-5.84	(\$) < 0.001
Length of primary fronds (cm)	8.93 \pm 4.88	27.54 \pm 7.57	30	-9.89	(\$) < 0.001
Number of cystocarps per centimetre of primary frond	2.09 \pm 1.27	0.26 \pm 0.28	n.a.	n.a.	(*) < 0.001
Length of secondary fronds (cm)	5.29 \pm 2.08	7.94 \pm 2.66	38	-4.00	(\$) = 0.001
Number of cystocarps per centimetre of secondary frond	2.00 \pm 0.93	0.17 \pm 0.22	n.a.	n.a.	(*) < 0.001
Average size of cystocarps (mm)	1.05 \pm 0.34	1.66 \pm 0.57	22	-1.45	(\$) = 0.140

Figure legends

Fig. 1. Map of the Chilean coast (A) and of the Concepción region, where Dichato and Lenga are located (B). Photographs of the natural population of Dichato (C) and of the crop of Lenga, note the *Agarophyton chilense* collected by Lenga residents forming piles on the beach (D). All photographs by C. Destombe.

Figure 2: Relation between the primary frond length (cm) and the number of cystocarps observed per centimetre of primary frond (no. of cystocarps · cm⁻¹) in *Agarophyton chilense* from Dichato (A) and Lenga (B). Each data point represents individual average value; solid line represents the least square linear regression (rs: Spearman rank correlation coefficient, *p*: probability level).

Figure 3. Photograph of female thalli from Dichato - details of cystocarps growing on the thallus are given in insert - (A) and Lenga (B). Cross-section of cystocarps (arrowheads: carpospores) from Dichato (C) and Lenga (D). Black line represents 5 cm in (A) and (B) and 80 µm in (C) and (D). All photographs by M-L. Guillemin. Ca: carposporangium (corresponding to a carpospore not yet released), cc: female cortical cell, cy: cystocarp, fm: female medulla, ft: female thallus, g: gonimoblast, p: pericarp.

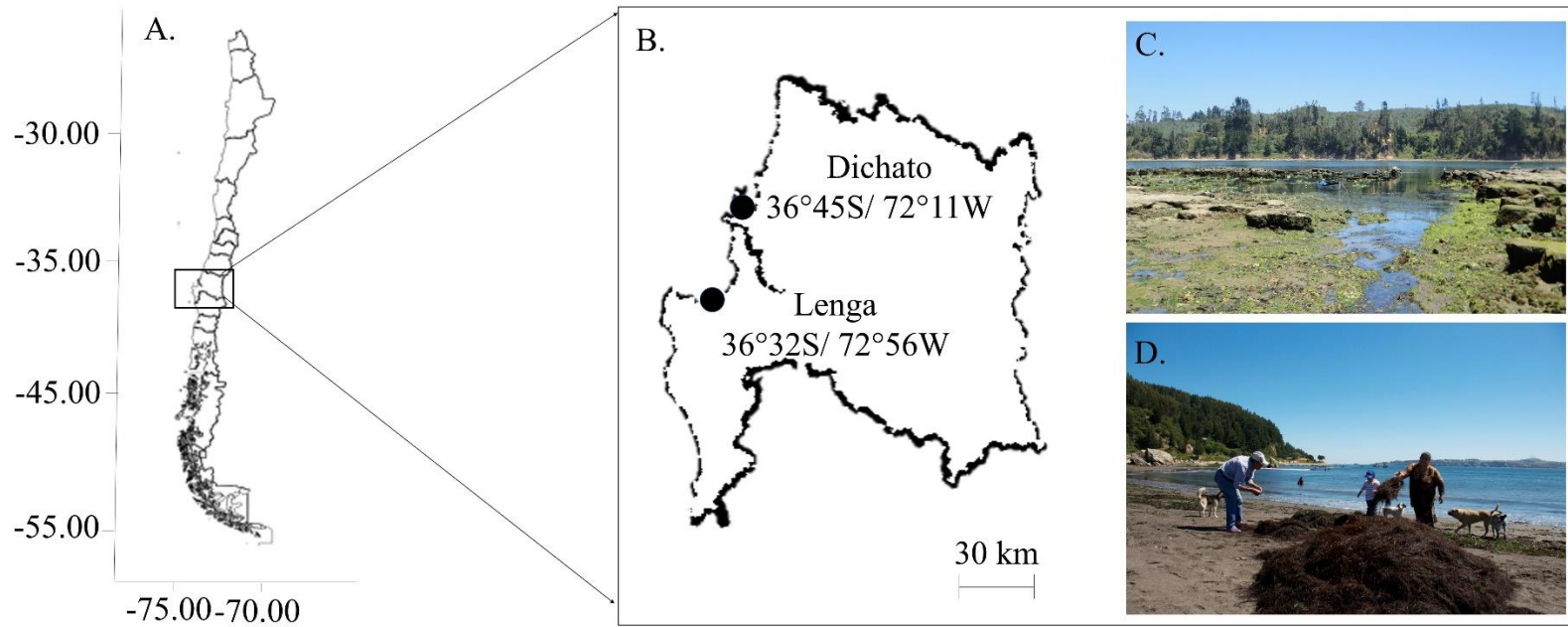


Fig. 1

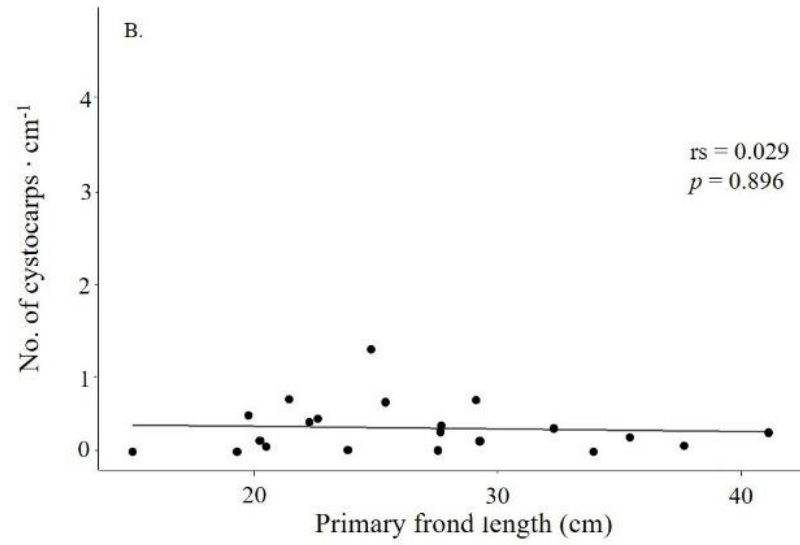
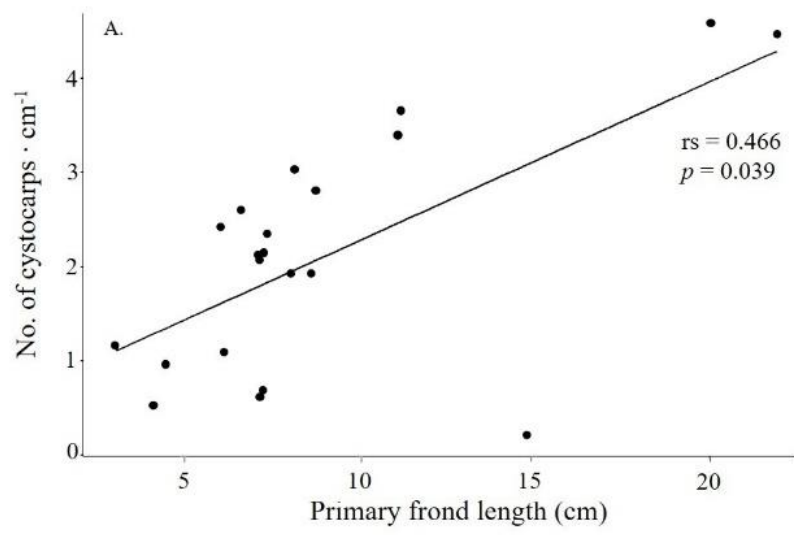


Fig. 2

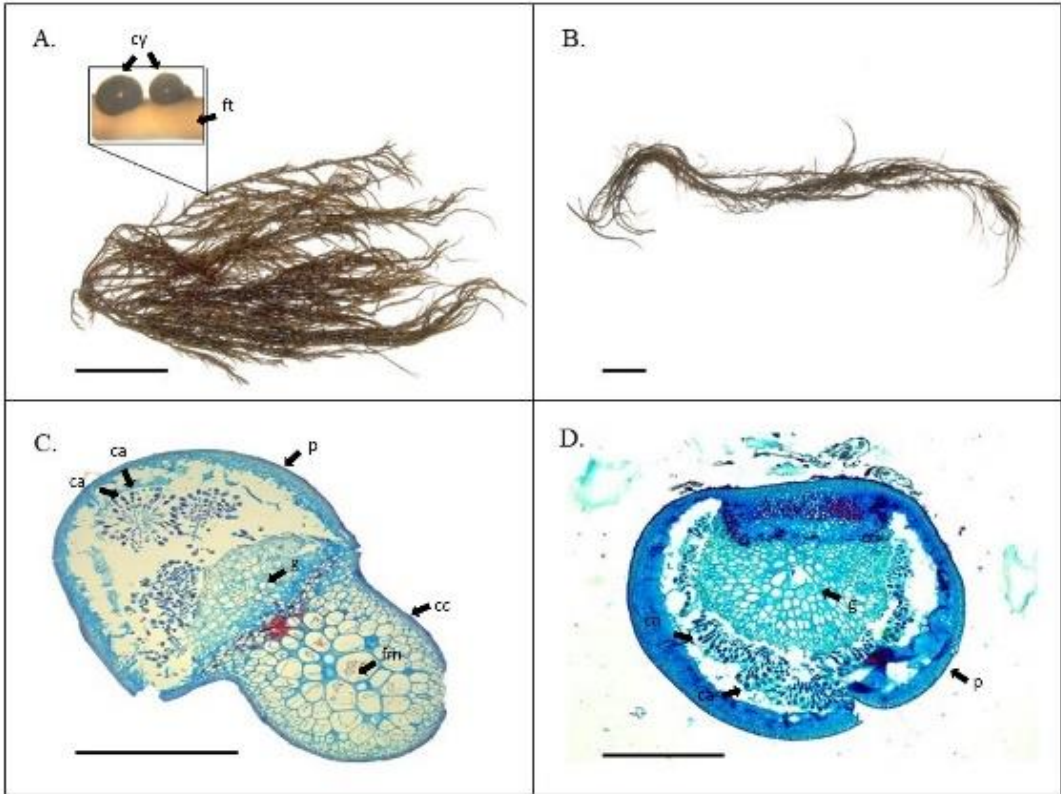


Fig. 3