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Published in:
Journal of Phycology

Publication date:
2017

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Citation for published version (APA):

Murúa, P., Müller, D. G., Patiño, D. J., & Westermeier, R. (2017). Giant kelp vegetative propagation: Adventitious holdfast elements rejuvenate senescent individuals of the *Macrocystis pyrifera* "integrifolia" ecomorph. *Journal of Phycology*, 53(1), 230-234. <https://doi.org/10.1111/jpy.12493>

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1 Note:

2 **Giant kelp vegetative propagation: Adventitious holdfast elements rejuvenate senescent**
3 **individuals of the *Macrocystis pyrifera* “*integrifolia*” ecomorph.**

4

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15

16

17 **Abstract**

18

19 Recent findings on holdfast development in the giant kelp highlighted its key importance for
20 *Macrocystis* vegetative propagation. We report here for the first time the development of
21 adventitious holdfasts from *Macrocystis* stipes. Swellings emerge spontaneously from
22 different areas of the stipes, especially in senescent or creeping individuals. After being
23 manually fastened to solid substrata, these swellings elongated into haptera, which became
24 strongly attached after one month. Within 4 months new thalli increased in size and vitality,
25 and developed reproductive fronds. Our results suggest the usage of these structures for
26 auxiliary attachment techniques. These could act as a backup, when primary holdfasts are
27 weak, and thus improve the survival rate of the giant kelp in natural beds.

28

29 Key index words: *integrifolia* morph; *Macrocystis pyrifera*; adventitious holdfast; haptera;
30 survival; kelp bed management, vegetative kelp propagation.

31

32 Until a few years ago holdfast morphology was used as a key feature for taxonomic
33 delimitation in *Macrocystis*, which resulted in four different species according to their conic,
34 rhizomatous or mounding holdfast construction (Womersley 1954). Demes et al. (2009)
35 rejected this approach after detecting holdfast polymorphism along a depth gradient in
36 Northeastern Pacific specimens. Beyond these differences by environmentally-mediated
37 plasticity surprisingly little is known about *Macrocystis* holdfast ontogeny. Haptera formation
38 starts with protuberances from the outer edge of the basal stipes (Smith 1939), and eventually
39 they overgrow older haptera and give shape to the adult structure. In the morphotype
40 *integrifolia*, however, the primary dichotomy of the stipe is aggregated, flattened and
41 creeping along the substrate, forming its characteristic holdfast. Sometimes, in older
42 individuals, hapteron-like structures were described below sporophylls by Lobban (1978).
43
44 Westermeier et al. (2014a, 2016) pointed out that these stoloniferous holdfasts may
45 contribute to propagation in “*integrifolia*” populations. Holdfast fragments are able to re-
46 attach to suitable substrata, to grow in two directions when excised, and even to produce
47 more stipes and fronds. They are also suitable for attachment to artificial substrata (e.g. long-
48 lines) and can be used for restoration or aquaculture attempts (Westermeier et al. 2013).
49 Schiel and Foster (2015) concluded that *integrifolia* specimens have longer life spans than
50 any other ecomorph, since leftovers of their rhizome-like holdfast are capable to regenerate
51 the whole thallus after being partially detached for example by storms. As in other kelps,
52 *integrifolia* holdfasts are able to coalesce, and even to divide into two different individuals,
53 increasing the possibility to cover larger surfaces (González et al. 2015, Westermeier et al.
54 2016). These observations raise questions as to what extent this potential occurs in natural
55 populations, and how it is affected by intense harvesting. The *integrifolia* morph is defined
56 by this holdfast trait, and could lead to a differential success in terms of survival and

57 colonization strategies with the ecomorph “*pyrifera*” in response to the environment or
58 disturbance contexts. It also contrasts with the recent findings in molecular, developmental
59 and intercrossing studies (Westermeier et al. 2007, Demes et al. 2009, Macaya and
60 Zuccarello 2010a), which subsume these two entities under the same species *M. pyrifera*.
61

62 The *integrifolia* ecomorph is a conspicuous component of the northern and central Chilean
63 coast to 33°S, Peru and the Northeast Pacific (reviewed in Macaya and Zuccarello 2010b).
64 Within the *integrifolia* population in Bahia Chasco (27° 40’ 41’’ S) we repeatedly noticed
65 structures emerging from the stipes, particularly in senescent individuals, and less frequently
66 in creeping specimens lacking pneumatocysts. Senescent individuals can reach up to 7% of
67 the total population by the end of summer in Bahia Chasco (personal observation). In this
68 time of the year *M. pyrifera* populations decrease in size and productivity not only in
69 Northern Chile but also along the entire Chilean coast (Westermeier et al. 2014b and
70 references therein). These adventitious structures are small and resemble galls (ca. 5 mm
71 diameter), but occasionally larger and resembling haptera (1.0 – 1.5 cm long, same diameter,
72 Fig. 1a). They were especially located at areas where new fronds developed, but sometimes
73 also along the naked stipe. In some senescent specimens the main holdfast started to
74 disintegrate, and highly branched haptera emerged from the basis of their fronds. They
75 became long enough to attach to the substratum and avoid the detachment of the frond (Fig.
76 1b) in a similar way as described by Lobban (1978).

77

78 In an attempt to test if these adventitious haptera structures were able to develop into a
79 holdfast or to support attachment, we collected senescent specimens of *M. pyrifera* during
80 March 2012 (Southern Hemisphere late summer). Zones of stipes with protuberances were
81 excised, taking care to include at least one phylloid to avoid premature mortality due to

82 photosynthesis deficit (Fig. 1c). Some excised fragments had more than one protuberance or
83 even included the original holdfast. Thirty clones were selected and attached to boulders,
84 using elastic bands as described by Westermeier et al. (2016; Fig. 1d), and placed within the
85 *Macrocystis* bed at Bahia Chasco at 6 – 8 m depth. In 4 monthly intervals, all specimens were
86 examined for detachment and the number of reproductive fronds. In addition, the thallus
87 growth and (secondary) holdfast development were quantified in those individuals, and
88 documented by images with a Nikon® D3200 DSLR camera inside an Ikelite® Underwater
89 TTL Housing. In order to know if plants were growing/eroding between months i) total
90 length and ii) secondary holdfast development were compared for every month using a non-
91 parametric Friedman’s ANOVA ($p < 0.05$), since assumptions of homogeneity of variance,
92 normal distributions of the residuals and independence were not fulfilled in our dataset. Post
93 hoc Tests with a Bonferroni adjustment were run to determine different size groups during
94 the experimental time. Graph was plotted using the R package ggplot2 (Wickham 2009).

95

96 As described above for the early development of the primary holdfast, small swellings
97 behaved like early haptera. They showed dichotomous branching until touching a surface,
98 where they flattened and sometimes formed a pad-like structure. This happened within the
99 first month after artificial fastening, and senescent thalli re-attached to hard substrata using
100 their early secondary holdfasts (Fig. 1e). If originally present, primary holdfasts were lost
101 from the second month onward (not shown). By the fourth month the new holdfasts were
102 covering a significant part of the boulders, but typical *integrifolia* holdfast shape was not yet
103 developed (Fig. 1f). Our experimental boulder-transplants decreased the first month in total
104 size. From the second month onwards, however, they showed a steady and significant
105 elongation up to ca. 80 cm length (Friedman’s $\chi^2 = 17.139$, $df = 4$, $p < 0.05$, Fig. 2). These
106 lengths were similar to the highest size values that can be reached from the end of summer to

107 winter in the natural stock of the *integrifolia* population at Bahia Chasco (Westermeier et al.
108 2014b), as well as in repopulation experiments by holdfast fragmentation, recruits and
109 outplants produced in the laboratory (Westermeier et al., 2016). Adventitious holdfasts grew
110 especially well within the first two months (Friedman's $\chi^2 = 24.730$, $df = 4$, $p < 0.05$),
111 reaching 8 cm from 1 cm initial size (Fig. 2). 25% of the total set of plants initially installed
112 did not detach by the fourth month, which agrees with similar values obtained by
113 Westermeier et al. (2016) in the same season, who used holdfast fragments attached to
114 boulders by elastic bands and cyanoacrylate glue during a pilot restoration attempt in the
115 same locality. Over 40% of our experimental specimens reached maturity (in terms of
116 presence of sporophylls with visible sori) after the first month.

117

118 Our observations confirm that the thick protrusions emerging from the stipes of the
119 *integrifolia* morph of *M. pyrifera* are homologous to haptera of early secondary holdfasts,
120 capable to re-attach to new substrate. We suggest the term "adventitious holdfasts" in analogy
121 to adventitious roots formed by land plants. Adventitious root formation is a well-studied
122 process, known in both mono- and dicotyledons (Bellini et al. 2014). Their formation is
123 mediated by either endogenous factors (maturity, phytohormones auxins and cytokinins,
124 reactive oxygen species and calcium reservoir), as well as environmental stimuli (water
125 presence, wounding, oxygen availability and temperature (Guan et al., 2015). In seaweeds,
126 secondary attachment disks have been reported in red algae, sometimes as response to stress
127 conditions, enhancing survival of individuals (Pacheco-Ruíz et al. 2005). In contrast, no cases
128 have been reported for kelps. However, it is known that rhizoid formation and elongation are
129 strongly related to auxin concentration in some Fucales and Ectocarpales (reviewed in
130 Ramakrishna and De Smet 2014). A similar, but still unconfirmed situation may apply to
131 secondary haptera formation in kelps. Likewise, many factors have been discussed to trigger

132 morphogenesis in seaweeds, such as rhizoid formation from totipotent cells (reviewed in
133 Hurd et al. 2014), and holdfast wounding in *Fucus* (McCook and Chapman, 1992). The
134 factors associated with secondary haptera formation in the giant kelp are still unknown, but
135 aging or substrate friction may enhance their development.

136

137 As in many kelps, holdfast development includes haptera formation, growth and attachment.
138 Holdfasts, including the secondary attachment structures described here, are critical for kelp
139 development in a suitable environment and the sporophyte survival in the long term. In the
140 light of our results (and assuming it is not an artefact of its vegetative growth potential),
141 adventitious holdfast formation of the *M. integrifolia* morph may be interpreted as an
142 ultimate effort to re-attach to the substratum in order to survive and/or produce propagules
143 close to a suitable area. After regularly inspecting the giant kelp population at Bahia Chasco
144 in the framework of populations studies (> 40 m² per month), we estimate that successful re-
145 attachment in terms of individuals naturally fastened by adventitious holdfasts (partially or
146 totally as in Fig. 1b) is going on in at least 1 of every 10 m² within this 280 ha kelp bed
147 (unpublished). Perhaps this is contributing a considerable boost to the *integrifolia* population
148 at low densities because of the season or under strong fishery pressure like *Macrocystis* in
149 Chile. The ability to develop secondary holdfasts may be used by local fishermen as a cheap
150 and easy way to restore local kelp stocks by following the techniques described by
151 Westermeier et al. (2016), who recolonized vast areas with *M. pyrifera* by using holdfast
152 fragments. In summary, our experimental clones recovered well. Their re-attachment extents
153 were as good as those of *integrifolia* recruits and seedlings outplanted (> 70%
154 mortality/detachment in boulders and long-lines inoculated in summer after Westermeier et
155 al. 2012), and represent a good alternative for small scale repopulation efforts in a season of

156 lowest kelp productivity.

157

158 Vegetative propagation is an important survival strategy in several species of filamentous
159 algae, some kelps, and green algae from the order Bryopsidales among others (Walters et al.
160 2002, Demes and Graham 2011, Hurd et al. 2014, Westermeier et al. 2014a). It has been used
161 for decades in aquaculture of domesticated seaweeds (Loureiro et al. 2015) and is a basic
162 element for the proliferation of some invasive species (Wright 2005). However, vegetative
163 propagation has not been studied in detail in the giant kelp, since sexual reproduction is
164 considered to be the basic strategy for recruitment. The *integrifolia* morph of *M. pyrifera*
165 constitutes one of the most productive kelp beds in northern Chile. Its vegetative recovery
166 potential by holdfast fragmentation has been studied in detail by Westermeier et al. (2013,
167 2016). In spite of constant harvesting pressure by local fishermen and increasingly severe
168 ENSO events, these populations usually tend to recover rapidly (Vásquez et al. 2006,
169 Westermeier et al. 2014b). Macaya and Zuccarello (2010b) diagnosed a low genetic diversity
170 within northern Chilean kelp beds, possibly caused by consecutive massive mortalities by
171 ENSO events. Our findings of significant natural vegetative propagation in *integrifolia*
172 populations may offer an alternative explanation for low genetic diversity. The proliferation
173 mechanism shown here corresponds to the first tracked evidence of clonal propagation in *M.*
174 *pyrifera* led by adventitious holdfasts, which could imply an important role in the perennial
175 survival of this species either in undisturbed communities or under anthropogenic impacts. It
176 is still pending to know the ecological/biological factors involved in triggering this
177 alternative holdfast developing program and to which extent the population dynamic aspects
178 of *M. pyrifera* are compromised.

179

180 **Acknowledgements**

181 We are grateful to Claudio Soza (Bahia Chasco) for assisting in field sampling, and the three
182 anonymous reviewers that complemented this work after their suggestions. Thanks are due
183 for financial support provided by Gobierno Regional de Atacama, through the grant FIC 2011
184 33-01-212 funded to RW. PM is supported by a scholarship from CONICYT (BECAS
185 CHILE N° 72130422) for PhD studies at the University of Aberdeen.

186

187 **Figure captions**

188 Fig. 1. Development of secondary holdfasts by the *integrifolia* morph of *M. pyrifera*.

189 (a) Swellings sometimes emerge from stipes in senescent plants (arrow).

190 (b) In some cases, an improvised rudimentary holdfast takes the attachment function (arrow),
191 when the primary holdfast is moribund and/or not appropriately fastened (arrowhead).

192 (c) Transplants (clones) of *M. pyrifera* used for cultivation, bearing one protuberance
193 (arrowhead) and at least one frond.

194 (d) Cultivation system in boulders after Westermeier et al. (2016), trying to immobilize the
195 hapteron-like structure using elastic bands (arrow).

196 (e) Development of early secondary holdfasts from a stipe, after the first month in culture.

197 (f) 4-month specimen completely regenerated and attached to a boulder.

198

199 Fig. 2. Recovery of *M. pyrifera* after successful secondary holdfast attachment. Average
200 monthly length in terms of the total size (thallus) and holdfast of specimens attached by their
201 stipe swellings. Error bars indicate the confidence intervals at 95%. Letter on bars were used
202 to designate the statistical differences after the multiple comparisons between groups
203 (Friedman tests), where $a < b$.

204

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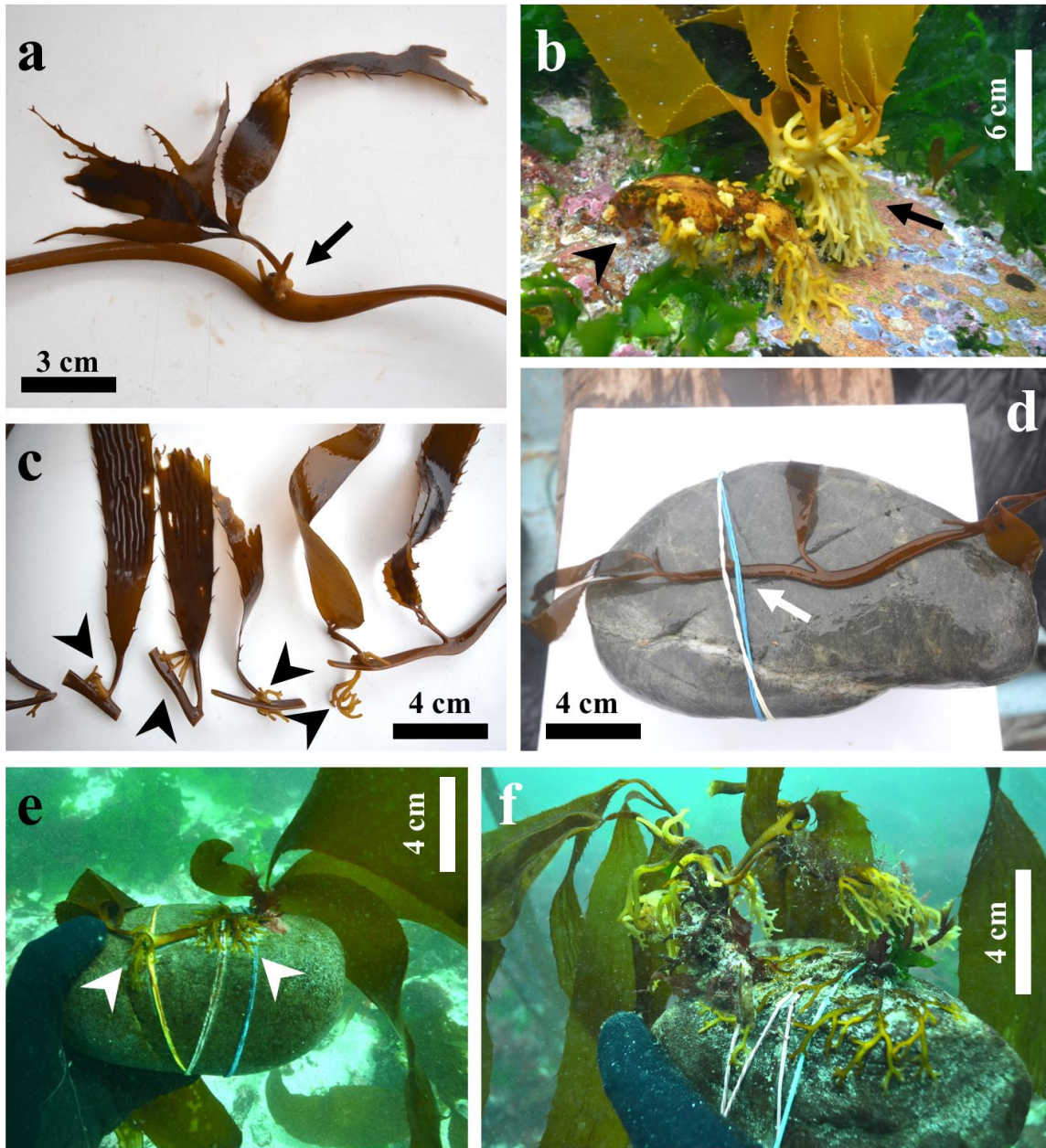
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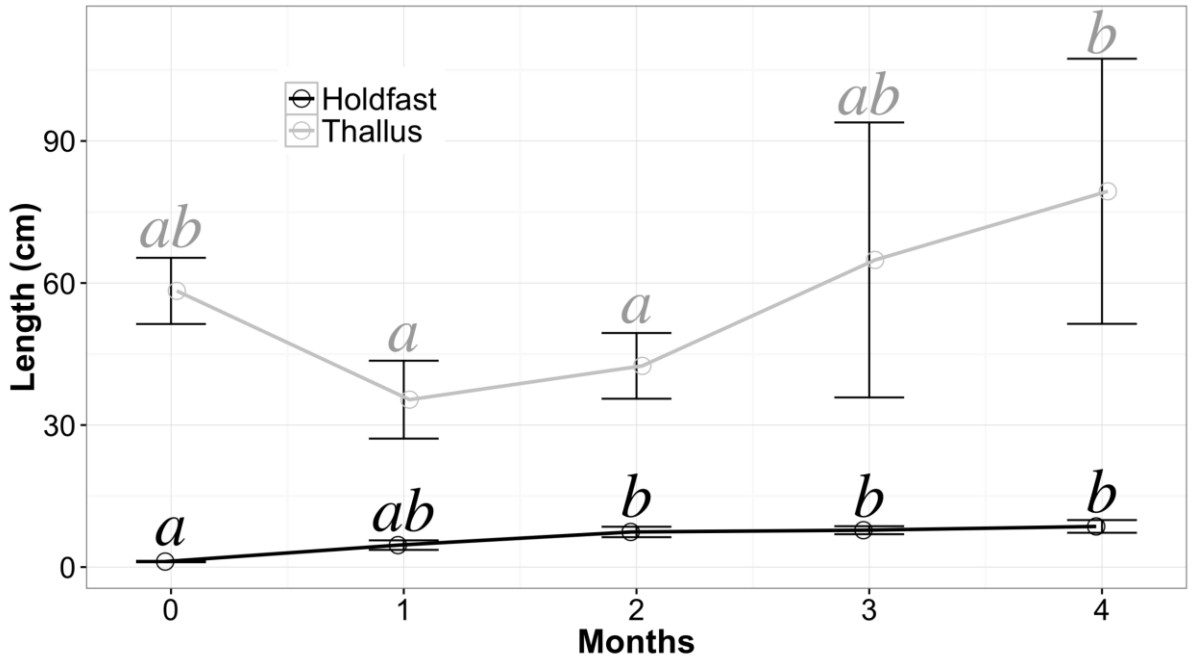


275

276 Figure 1

277

278



279

280 Figure 2.