Phenology of farmed seaweed Kappaphycus alvarezii infestation by the parasitic epiphyte Polysiphonia sp. in Madagascar

Gaëtan Tsiresy, Jérémy Preux, Thierry Lavitra, Philippe Dubois, Gilles Lepoint & Igor Eeckhaut

Springer

Springer

CrossMark

Phenology of farmed seaweed *Kappaphycus alvarezii* infestation by the parasitic epiphyte *Polysiphonia* sp. in Madagascar
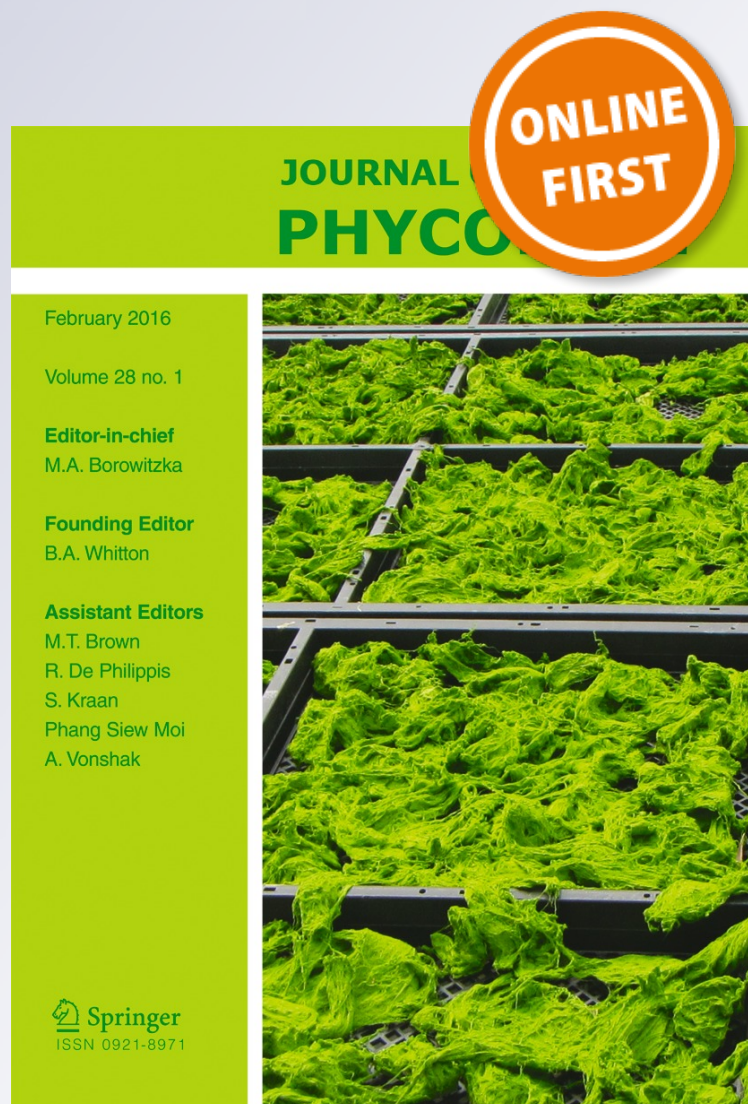
Gaëtan Tsiresy [1,2] · Jérémy Preux [1] · Thierry Lavitra [2] · Philippe Dubois [3] · Gilles Lepoint [4] · Igor Eeckhaut [1,2]

Abstract With the increase of seaweed farming activities, epiphytic filamentous algae (EFA) disease has appeared in many regions of Madagascar. This infestation has dramatic consequences for local farmers as it alters drastically farmed algal growth and has caused farming activity to collapse in many places. The present study characterizes the structure and ultrastructure of the stages observed in the life cycle of *Polysiphonia* sp. and gives the results of a monitoring of 18 months made in three *Kappaphycus alvarezii* farming sites in the southwest of Madagascar. Transmission electron microscopy (TEM) was used to analyze the ultrastructure of the cortex in infested *K. alvarezii*. Five stages have been observed in the life cycle of *Polysiphonia* sp.: the infesting stage that is a small dark spot observed at the surface of *K. alvarezii*, the male gametophyte, the female gametophyte, the tetrasporocysts, and the undifferentiated stage where individuals show normal thalli without sexual differentiation. EFA infestation was never recorded in Sarodrano, but often in the two other monitored villages (Lambohara, Tampolove). Prevalence of infestation varied from 40 to 100 % and the rates of infestation from 42 to 78 epiphytes cm^{-2}. Prevalence of infestation showed significant seasonal variation and a between-sites variation; the rates of infestation were not significantly different between sites and did not vary with the period. The ways of infestation between *K. alvarezii* individuals in an infested field and from infested to healthy fields are discussed at the light of the present results.

Introduction

The 'cottonii,' *Kappaphycus alvarezii* (Doty) Doty ex P.C.Silva, is an important source of raw material for the carrageenan industry (Bindu and Levine 2010; Hayashi et al. 2010). *Kappaphycus alvarezii* farming does not escape parasitic infestations, and it is mainly affected by two diseases: the "ice-ice" and the epiphytic filamentous algal (EFA) diseases. The "ice-ice" was observed for the first time in 1974 in some Philippine farms (Trono 1974) where the initial stress allowed pathogenic microorganisms (mainly bacteria or fungi) to infect the algal thalli (Largo et al. 1995a, b; Mendoza et al. 2002; Loureiro et al. 2009; Solis et al. 2010; Guiry, 2011). The second was reported by Doty and Alvarez (1975) on *K. alvarezii* in some Philippines farms, but since then, the epiphyte outbreak has been occurring regularly in major carrageenophyte farms in the Philippines, Indonesia, Malaysia, and Tanzania. EFA infestations are caused by diverse algae, generally of the genera *Polysiphonia* and *Neosiphonia* (Rhodomelaceae, Rhodophyta: Kim and Lee 1999) (Hurtado et al. 2006). The species *Neosiphonia savatieri* represents 80–85 % of the infesting epiphytic algal species checked in Malaysia (Vairappan 2006; Vairappan et al. 2008) and is prevalent in China (Tong et al. 2011).

Upon maturation, infected seaweed takes on a "hairy" appearance with "goose-bumps"-like cortical swellings

✉ Gaëtan Tsiresy
tsiresygaetan@gmail.com

1 Biology of Marine Organisms and Biomimetics, Biosciences Institute, University of Mons, 6, Avenue Champ de Mars, Pentagone 2B 7000 Mons, Belgium

2 Polyaquaculture Research Unit, Institut Halieutique et des Sciences Marines, University of Toliara, Route du Port Mahavatse, 601 Toliara, Madagascar

3 Marine Biology, Free University of Brussels, Brussels, Belgium

4 Laboratory of Oceanology—MARE center, University of Liège, B6c, 15 allée du 6 août, 4000 Liège, Belgium

Springer

(Vairappan et al. 2007). At the last stage of infestation, the epiphytes leave dark pits on the cortical swelling. *N. savatieri* causes damage on the thallus surface of its host, induces the global weakening of the cultured algae, and increases its exposure to herbivore attacks and to opportunistic bacteria like *Alteromonas* sp., *Flavobacterium* sp., and *Vibrio* sp. (Vairappan 2006; Vairappan et al. 2008). Furthermore, *N. savatieri* infestations have been shown to cause damage of the photosynthetic oxygen-evolving complex, decrease of active reaction centers and the plastoquinone pool as well as significant reduction in the performance indexes (PI) of PSII (Pang et al. 2011). Epiphyte outbreaks in Malaysia and the Philippines lead to remarkable reductions of the *K. alvarezii* biomass production and a decline of the carrageenan quality (Critchley et al. 2004; Hurtado et al. 2006; Vairappan et al. 2007, 2008). Because of the weak level of production caused by the parasitic algae, some countries such as China are no longer continuing to cultivate *K. alvarezii* on a large scale (Pang et al. 2011). Nevertheless, to our best knowledge, not enough ultra-structural studies have been done on the EFA infection on cottonii, proving definitively the parasitic aspect of EFA.

Emergence of an epiphytic outbreak is a complex problem, and the extent of the outbreak often depends on the quality of the cultivated strain, abiotic parameters of the culture site, and seasonal weather fluctuations (Borlongan et al. 2011). In Malaysia, the occurrence of epiphytes was correlated with the abrupt changes (either increase or decrease) in temperature and salinity, the EFA appearing twice a year, from late February to June and from September to November (Vairappan 2006).

Seaweed farming in Madagascar started more recently, in 1989, at Songeritelo under the aegis of the "Institut Halieutique et des Sciences Marines" (IH.SM, Toliara). Today, Madagascar still develops this sector that has become a permanent source of income for many coastal communities. The production of private companies depends largely of the appearance or not of the EFA in culture fields. Nosy Ankao (northeast region) production, for example, reached more than 1000 t year^{-1} but declined to less than 100 t year^{-1} in the last years due to EFA disease (Eeckhaut *pers. com.*). With the increase of seaweed farming activities these last years, EFA disease has appeared in many regions of Madagascar. The disease occurs not only in the southwest of Madagascar but also in the north and the east (Antalaha) that face a serious problem as EFA infestations appear several times a year: EFA reduces drastically the farming production, and the farmers, which are primarily fishermen, are quickly demotivated when they earn less than fishing.

The recent study of Ateweberhan et al. (2015) on *K. alvarezii* gives some insights on the ecology of EFA in the southwest of Madagascar although nothing is still known about the seasonal variations of the different stages that are present in the life cycle of the algae. The duration of EFA life cycle is not known but supposed to be around 10 weeks (Vairappan et al. 2007), but previous works have suggested that all stages can be observed during a given period of EFA infestation. Here, we monitored the appearance of these stages during the survey and characterized the structure and ultra-structure of all stages observed during the life cycle of *Polysiphonia* sp. over an 18-month (December 2012 to May 2014) period in three farming sites in the southwest of Madagascar.

Materials and methods

Study area and farming technique

Monitoring was conducted monthly from December 2012 to May 2014 on three sites around Toliara, SW Madagascar: Sarodrano, Tampolove, and Lambohara. In the three sites, the methods conducted for *K. alvarezii* farming were the "off-bottom" and "long line": off-bottom is the system where seaweed lines (rope of 10 m length populated with 50 seedlings of 100 g wet weight, each placed at equal intervals of 20 cm) are attached to wooden stakes at each end and suspended about 60–80 cm from the bottom. It is practiced in Sarodrano and Lambohara. The same seaweed lines were installed in the second system called "long lines" but the lines were attached to a rock at both ends (in Tampolove). This second method uses plastic bottles as floats to maintain the seaweed line level. About 5000 and 30,000 seaweed lines are installed in the three sites, respectively, for long lines and off-bottom systems. In the three sites, the farmed seaweeds are not exposed to waves nor to violent tide currents as they are grown out of channels. The substrata below the seaweeds are silty with a large proportion of fine sand grains at the three sites. The proportion of particles <250 μm varied from 40 to 70 % (at Sarodrano and Tampolove, respectively) (Plotieau et al. 2014).

Seawater temperatures were recorded at the three sites with data loggers (HOBOware pro waterproof, 8 K) recording the temperature automatically every hour. The data loggers were placed in the seaweed farming sites, at the same level as the farmed seaweeds. Monthly average temperatures were obtained in integrating all temperatures recorded in 1 month divided by the number of records during this month.

The infestations were recorded twice a month by technicians living in villages. During the infestation record, scientists collected infested thalli and fixed them in a solution of 3 % glutaraldehyde in cacodylate buffer. The proportions of the infested field surface were evaluated, and infested thalli fixed in glutaraldehyde served to record the rate of infestation or to characterize the fine structure of the various stages observed in the life cycle of the epiphytic filamentous algae. The epiphytic filamentous alga infesting *K. alvarezii* around

Toliara is a single species (Bertiaux 2013) that falls into the *Polysiphonia* group defined by Choi et al. (2001) in having four pericentral cells with absence of cortication and a straight arrangement of tetrasporangia.

We classified the development cycle into five stages to facilitate our evaluation during the observation. To record the rate of infestation under binocular microscope, the numbers of EFA individuals infesting *K. alvarezii* on a 1-cm^2 area were counted for five samples coming from five different *K. alvarezii* (individual seedlings) from five different lines at each period of infestation. The sum of the epiphytes observed on 1 cm^2 for the five samples was divided by 5 to get the average (infestation rate). Infested areas were evaluated during our monitoring to obtain the percentage (prevalence of infestation) relative to the total cultivated area.

A comparison of the infestations between the periods was performed with the Kruskal-Wallis test, at the 95 % confidence level ($p < 0.05$) (Villares et al. 2002). Spearman's correlation coefficient was used to study a potential correlation between temperatures and EFA occurrences. Non-parametric statistical, Mann-Whitney, and chi-squared tests were used to compare the development stage rate between EFA occurrence times and between male and female gametophytes. Statistical analyses were conducted using Prism 6 (GraphPad software, USA).

Epiphyte ultrastructure

Scanning electron microscopy Glutaraldehyde-fixed infested *K. alvarezii* were sampled to observe the external morphology of the various *Polysiphonia* sp. stages under a scanning electron microscope. The samples were rinsed three times in ethanol 70 % and dehydrated in graded ethanol solutions of 90 and 100 %. The samples were then dried using the critical point method with a Polaron critical point device, placed on aluminum stubs, and treated for 4 min in a JEOL JFC-1100^E ion coater (Bozzola and Russell 1992). Samples were observed with a JEOL JSM-6100 scanning electron microscope.

Histological observations and transmission electron microscopy Specimens fixed in glutaraldehyde were also used for analyzing the pathologies induced by *Polysiphonia* sp. on *K. alvarezii* at the attachment sites. The samples were rinsed three times for 10 min in a cacodylate solution (1 volume of 0.4 M sodium cacodylate and 1 volume of 3.7 % saline). Then, they were post-fixed for 1 h in 1 % osmium tetroxide (2 volumes of 2 % osmium tetroxide 2 %; 1 volume of 0.4 M sodium cacodylate; 1 volume of 9.2 % NaCl). After three rinses, the samples were dehydrated in a series of baths of increasing concentrations of ethanol (25, 50, 70, 90, and 100 %) and embedded in Spurr's resin. Semi-thin sections of 1 μm thick were cut, and the sections were stained for 15 s

with an equal volume mixture of 1 % methylene blue and 1 % Azur II before being observed under an optical microscope (Bozzola and Russell 1992).

A dozen samples were also observed by transmission electron microscopy after first reaching the area of interest using semi-thin sections as explained above. Ultrathin sections with a thickness of 70 nm were cut using a Leica ultra-section ultramicrotome with a diamond knife and placed on copper grids. Finally, they were contrasted with 7.5 % uranyl acetate and 6.18 % lead citrate. Samples were observed on a Zeiss LEO 906^E transmission electron microscope.

Results

The surface of healthy *K. alvarezii* was smooth (Fig. 1a), and the normal colors of individuals varied from a yellow-green, in most cases, to red. The earliest sign of infestation by *Polysiphonia* sp. was the appearance of dark spots on the surface of the hosts that can be observed by eye in the field (Fig. 1b). These spots, when detected early, were in small numbers, less than 10 on a whole *K. alvarezii* individual. They became numerous a few days after, giving a rough appearance to the infested thalli as the epiphytes emerged from the host cortex at each dark spot (Fig. 1b, c, e). Thereafter, algae became feathery due to the presence of many adult epiphytes that appear quickly after thalli emergence and formed a dense cover on the surface of the infested *K. alvarezii* (Fig. 1d). At the end of the infestation, seaweeds were whitened and developed symptoms similar to ice-ice disease: the coloration is lost and *K. alvarezii* becomes very fragile with parts of individuals breaking easily (Fig. 1f).

Morphology and ultrastructure of the stages observed in the life cycle of *Polysiphonia* sp.

With careful microscopic observation, five stages were recorded, which are (i) the infesting stage (i.e., a small dark spot observed at the surface of *K. alvarezii*), (ii) the male gametophyte stage (i.e., individuals with sporangia), (iii) the female gametophyte stage (i.e., individuals with cystocarps), (iv) the tetrasporophyte stage (i.e., individuals with spore tetrads), and (v) the undifferentiated stage (i.e., sexually undifferentiated individuals that will evolve into gametophytes or tetrasporophytes). The two last stages were very similar morphologically as the spore tetrads were internal, and it has been impossible to separate them during the monthly counts that were realized with a binocular microscope, these two stages being consequently mixed together under the label "mixed stage" in the figures illustrating the monthly infestations.

The infesting stage This stage appears in the shape of a dark spot at the surface of the infested algae. It always appeared as a

Fig. 1 Evolution of EFAD symptoms on *K. alvarezii* during the infestation. **a** Healthy seaweed, **b** *K. alvarezii* spotted, the first stage of infestation, **c** the beginning of the feathery stage after emergence of epiphytes, **d** an advanced feathery stage, **e** the creepy stage of the disease with goose bumps, **f** ultimate stage where *K. alvarezii* shows symptoms of bleaching. *H.T* healthy thalli, *Epi* epiphyte, *Hos* host, *D.S* dark spot, *G.B* goose bump, *W.T* whitened thallus
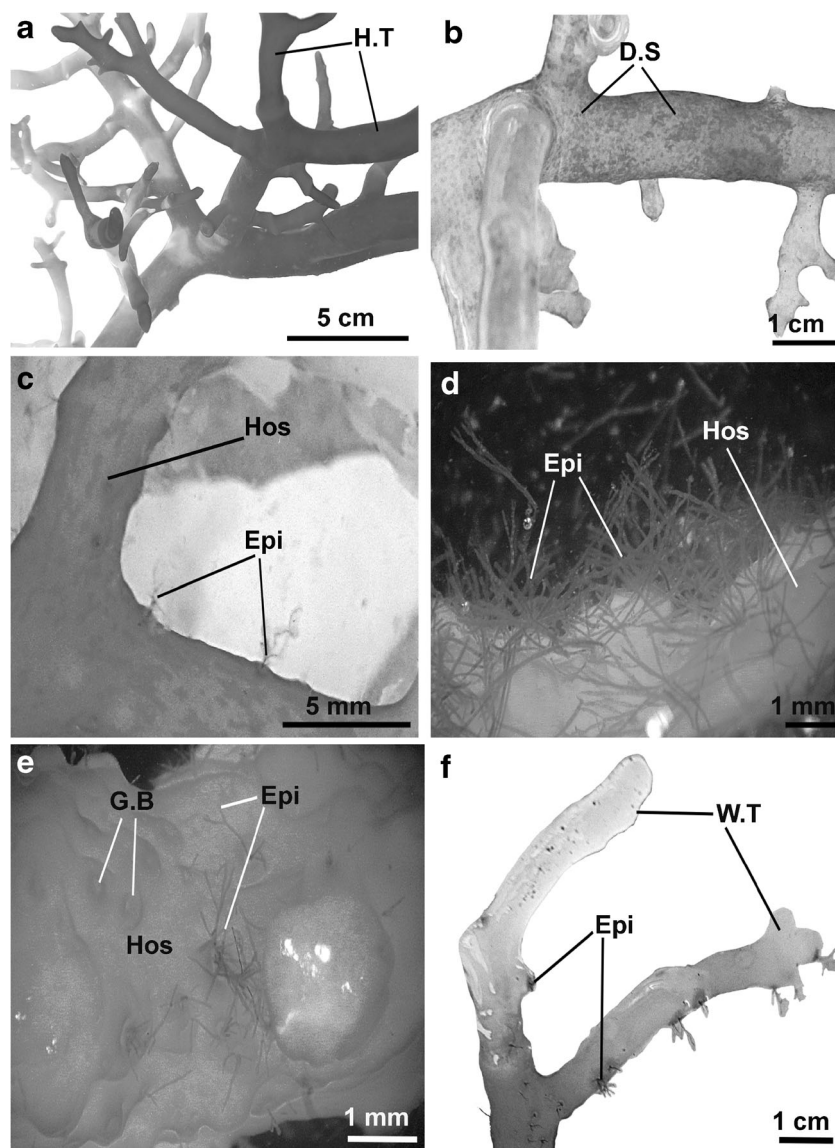
small, unbranched epiphyte of 50 to 100 μm long emerging from a flat pit or a small wart. SEM views of the small, emerged epiphytes showed that they were small, unsegmented primary rhizoids of ca. 40 μm in diameter arising from flat pits of ca. 60 μm (Fig. 2a). When the rhizoids were a bit longer, the pit top warts were about 200 μm high. Semi-thin cross sections through the infesting stage showed that the emerging rhizoid of the epiphyte was made of a large central cell (50–70 μm in diameter) and four flat pericentral cells (Figs. 2g, 3b, and 4b).

The infesting stage was implanted inside the first 200 μm from the surface of *K. alvarezii* (Fig. 4a). Semi-thin sections though healthy thalli of *K. alvarezii* show that the first 200 μm are composed of three layers (Fig. 3a). The inner layer is made of many cells that compose the core of the thallus. These cells cells are 40 to 80 μm in diameter, and they have a well-developed cell wall 5 μm thick. The middle layer is ca.

60 μm thick. It is composed of two to three superimposed cell layers. The cells of the middle layer are of 10 to 20 μm in diameter, and their cell wall is thin (less than 1 μm thick). Their cytoplasmic content appears darker than the one observed in the cells of the inner layer. The outer layer is 10 to 20 μm thick. It is made only of a dark extracellular material that also fills the space between the cells of the middle and the inner layers.

Sections made through infested thalli of *K. alvarezii* showed that the epiphyte was inserted into the extracellular material that coats the cells of the inner layer (Figs. 3b and 5a). Cells of the inner layer were not killed by the presence of the epiphyte, although in some sections, we observed a breakdown of their cell wall. Yet, in longitudinal sections through the infesting stage, the outer layer of the infested cortex, made in the healthy stage of a dark extracellular matrix, is absent where the epiphyte emerges and in its close vicinity. Instead of

Fig. 2 The major EFA stages observed by scanning electron microscopy. **a** The infesting stage appearing as a button emerging growing out of *K. alvarezii*, **b** the undifferentiated stage sorting out from a pit on a *K. alvarezii* thallus, **c** cystocarp observed laterally and implanted on a female gametophyte, **d** two spermatangia of the male gametophyte, **e** two tetraspores inside a tetrasporocyst, **f** spermatangia with filament attached on the epiphytic peduncle, **g** a terminal branch showing the four pericentral cells and one central cell. *E.B* epiphytic branch, *W.E* wart emersion, *F.G* female gametophyte, *Hos* host, *Epi* epiphyte, *Tri* trichocyst, *Seg* segment, *Spe* spermatangia, *Cys* cystocarp, *Ped* peduncle, *C.C* central cell, *P.C* pericentral cell, *C.P.T* cell protecting tetraspore
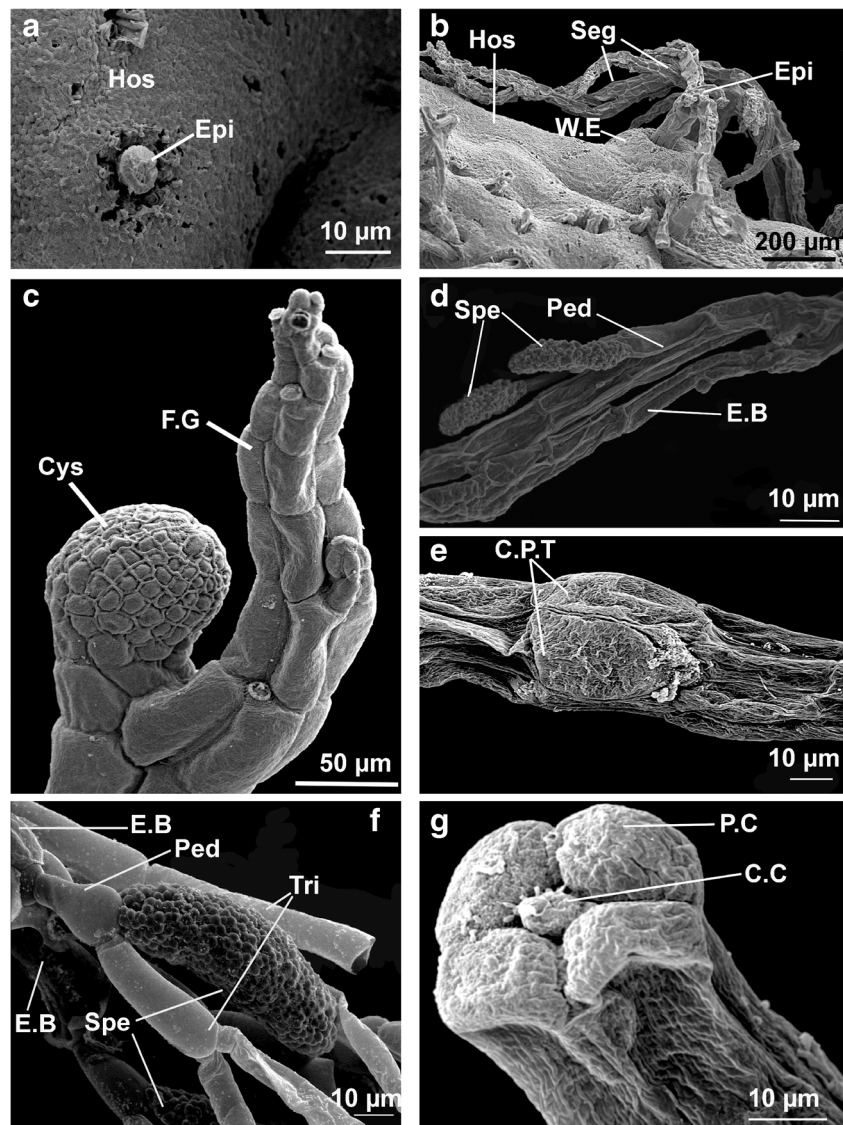
this outer layer, remnants of cells from the middle layer formed the surface of *K. alvarezii* (Fig. 3c, d).

The undifferentiated stage and the tetrasporophyte The undifferentiated stage measures from 0.2 to 2.5 mm long (Fig. 2a). It started from the wart from which it emerges by a 200-µm-long, 100-µm-wide, unsegmented central branch that splits on top into 2 to 15 branches. Each branch divided two to four times forming branches made of 10 segments (Fig. 2b). From the base to the top, a branch of an epiphyte is formed of maximum 40–50 segments, one segment being of 50 µm long. Each segment is made of one central cell with its four pericentral cells. TEM revealed that the central cell included the core of the cell with peripheral chloroplasts and a ca. 15-µm-thick extracellular cell wall (Fig. 5a–c). The cell wall was made of superposed layers of about 500 nm thick each (Fig. 5b). These layers showed various electron densities with

the ones closer to the cytoplasmic membrane being more pleated than those at the periphery (Fig. 5c). The pericentral cells are flat cells ca. 5 µm thick, 15 µm wide, and 50 µm long (Fig. 5a). The cell wall of these cells is not well developed, forming a small layer of 1 µm thick.

The undifferentiated stage did not show any male or female reproductive structures and no tetraspores. Buds of 20 to 50 µm long were often observed at the start of new segments, along the whole length of the branches, and at their tips. Traces of the loss of buds and of trichocysts were also frequently present along the branches and appeared as circular scars of 20 µm in diameter bordered by a lip of a few micrometers high, most of them being at the tips of the branches (Fig. 6e).

The tetrasporophyte stage was characterized by the presence of tetrasporocysts (Figs. 2e and 4c). Tetrasporocysts often appear from the fourth branching to the tips. The tetrads

Fig. 3 Semi-thin sections. **a** Cross section of a healthy thallus of *Kappahycus alvarezii* showing the three layers composing its surface. **b** Cross section through an infesting stage implanted into the third layer of *K. alvarezii*. **c** Longitudinal section through an epiphyte at the point of emergence. **d** Cross section of an infested thallus of *Kappahycus alvarezii* showing the three layers composing its surface. *C.A* cortical area, *S.A* subcortical area, *E.C* epiphytic cell, *E.C.W* epiphytic cell wall, *Epi* epiphyte
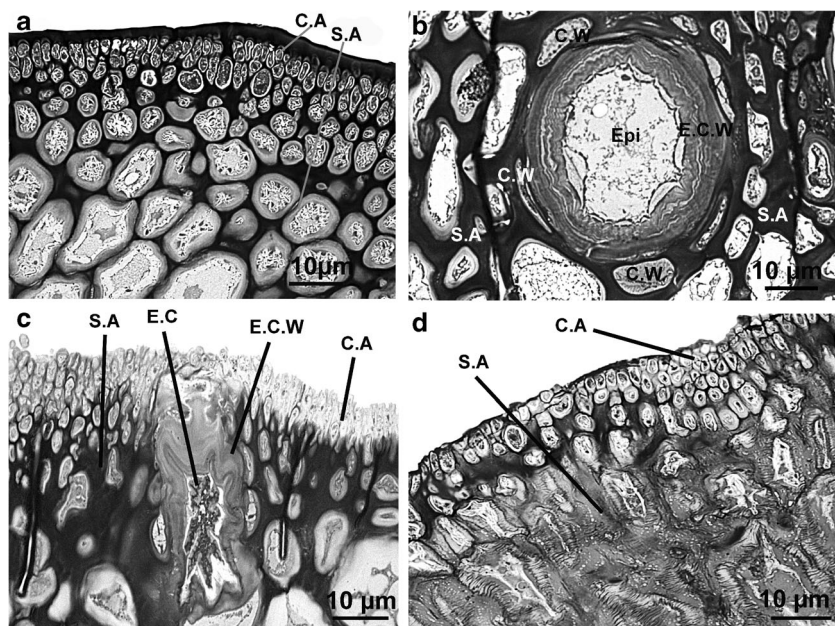
are 40 μm long for 30 μm wide. The maximum tetrasporocysts observed on a tetrasporophyte was of 28.

The male and female gametophyte stages The male gametophytes are recognizable when they bear spermatangia with conical structures 130 to 200 μm long and 45 to 60 μm wide. They were supported by a peduncle of 30 μm long that links them to the main branches (Fig. 2d, f). Most of the spermatangia were supported by the third and fourth branching of the epiphytes.

The female gametophytes are recognizable when they bear cystocarps with a spherical to oval structure 60 to 200 μm in

length (Figs. 2c and 6a) on top of which is a ca. 10-μm-diameter pore. Some carpospores of 10 μm were sometimes observed attached to the cystocarp close to the pore or within it (Fig. 6c). The cystocarp was implanted laterally on a branch of the epiphyte, supported by a short 40-μm-long peduncle (Fig. 6a, c). Its surface consists of 20 to 40-μm-long polyhedral cells forming the pericarp that surrounds the internal carposporophyte (Figs. 4d and 6b, d). In young cystocarps, polyhedral cells are separated from each other by a lip that is not present in older cystocarps which showed many 5- to 8-μm-long comma-shaped bacteria typical of *Vibrio* bacteria (Fig. 6b, f).

Fig. 4 EFA semi-thin sections (classic histological). **a** Epiphyte implantation on its host. **b** Cross section of epiphyte thallus. **c** Cross section of tetrasporocyst. **d** Cross section of carposporophyte. *C.A* cortical area, *C.C* central cell, *E.C.W* epiphyte cell wall, *SA* subcortical area, *C.P* pericentral cell, *Epi* epiphyte, *Chl* chloroplast
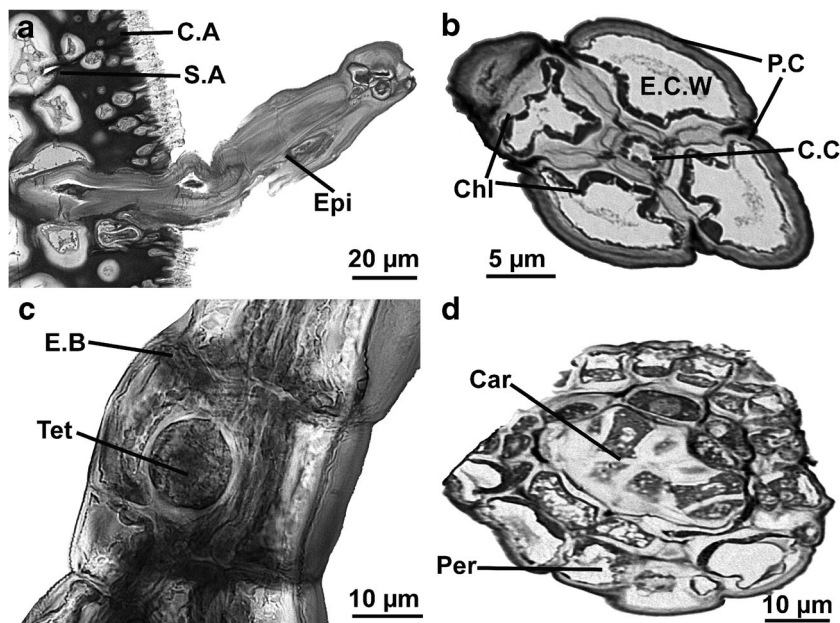
Fig. 5 Transmission electron micrographs of *Polysiphonia* sp. implantation on its host. **a** Transverse section of an epiphyte in its host. **b** Detail of the epiphyte cell wall surface in contact with its host. **c** Longitudinal section in an epiphyte before emersion, cross sections on infested *K. alvarezii*. *P.C.* pericentral cell, *S.A* subcortical area, *C.C* central cell, *E.C.W* epiphyte cell wall, *E.C.M* epiphyte cell membrane, *H.C.W* host cell wall, *E.M* extracellular matrix, *Hos* host, *Chl* chloroplast
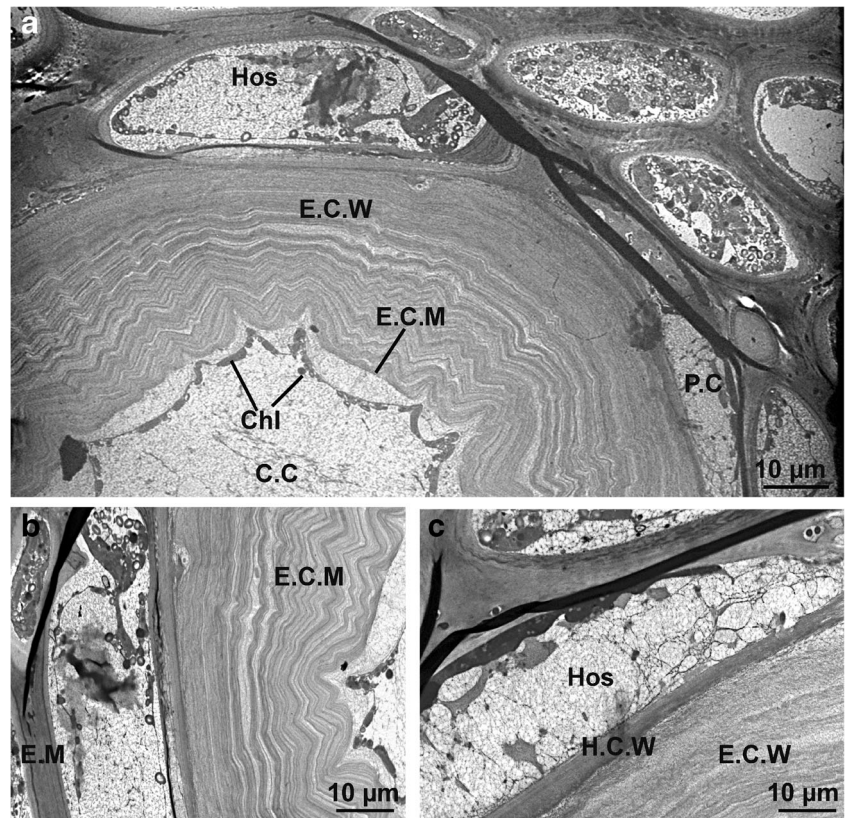

Description and seasonal occurrence of epiphyte infestations

The minima and maxima of temperatures observed each month in the three sites were analyzed with respect to the epiphyte occurrence times. There was no significant difference (ANOVA; $p > 0.05$) between the average temperatures recorded monthly when we compared the three villages, although the pattern of temperature variation is slightly different in Sarodrano (Fig. 7a, b, c). In the southwest of Madagascar, the cold season occurs from the beginning of March to the end of August and the warm season begins in September to the end of February. The rainy season generally occurs from November to February, but the seawater is influenced by terrestrial runoff and thus by the inland rains that occur mainly over the whole warm season. The patterns of temperature variation in Tampolove and Lambohara show that maxima decrease sharply from April, below the average of 32 °C, increasing to maxima greater than 32 °C in September. In Sarodrano, the maxima decreased in April below 32 °C, increasing above 32 °C in November. The average monthly temperatures recorded in Sarodrano varied between 21.86 and 31.15 °C, the minimum recorded was 19.47 °C and the maximum 33.43 °C. In the two other sites, Tampolove and Lambohara, the average monthly temperatures varied, respectively, from 22.24 to 29.97 °C and from 23.11 to 30.66 °C.

During the 18 months of monitoring, we never recorded EFA infestation in Sarodrano, the southern farm. EFA infestations were recorded at six periods in Lambohara and at four periods in Tampolove. EFA infestations were observed in December 2012, February 2013, September 2013, December 2013, March 2014, and May 2014 at Lambohara. Infestations occurred in February 2013, April 2013, December 2013, and March 2014 at Tampolove. The prevalence of infestation when arriving on site varied from 30 to 100 % in Lambohara and from 40 to 100 % in Tampolove. The rate of infestation varied from 42 to 57 epiphytes cm^{-1} in Lambohara and 46 to 78 epiphytes cm^{-2} in Tampolove (Fig. 8). There is no significant difference between the average number of epiphytes cm^{-2} of Lambohara and Tampolove when all the recorded infestation rates are polled together ($p > 0.05$). Also, in the three periods where infestation was observed in the two villages (February 2013, December 2013 and March 2014; Fig. 7), there was no significant difference in the rates of infestation ($p > 0.05$) between the two sites. No correlation was observed between temperature-prevalence and between temperature-infestation rates ($p > 0.05$).

In the two villages where infestations were recorded, EFA never occurred from June to August, a period where the maxima were always below 30 °C. Nevertheless, EFA was observed during the cold season in the two villages: in April 2013 in Tampolove and in May 2014 in

Fig. 6 Development of female gametophyte. **a** Young female gametophyte, **b** smooth membrane of a cystocarp, **c** old cystocarp with carpospore, **d** surface of a cystocarp with bands, **e** Female gametophyte with bud and scar, **f** bacteria on the of a cystocarp with bacteria. *E.B* epiphytic branch, *Y.G* young cystocarp, *Bac* bacteria, *F.G* female gametophyte, *Bac* bacteria, *Car* carpospore, *Cys* cystocarp, *Sca* scar, *Bud* buds
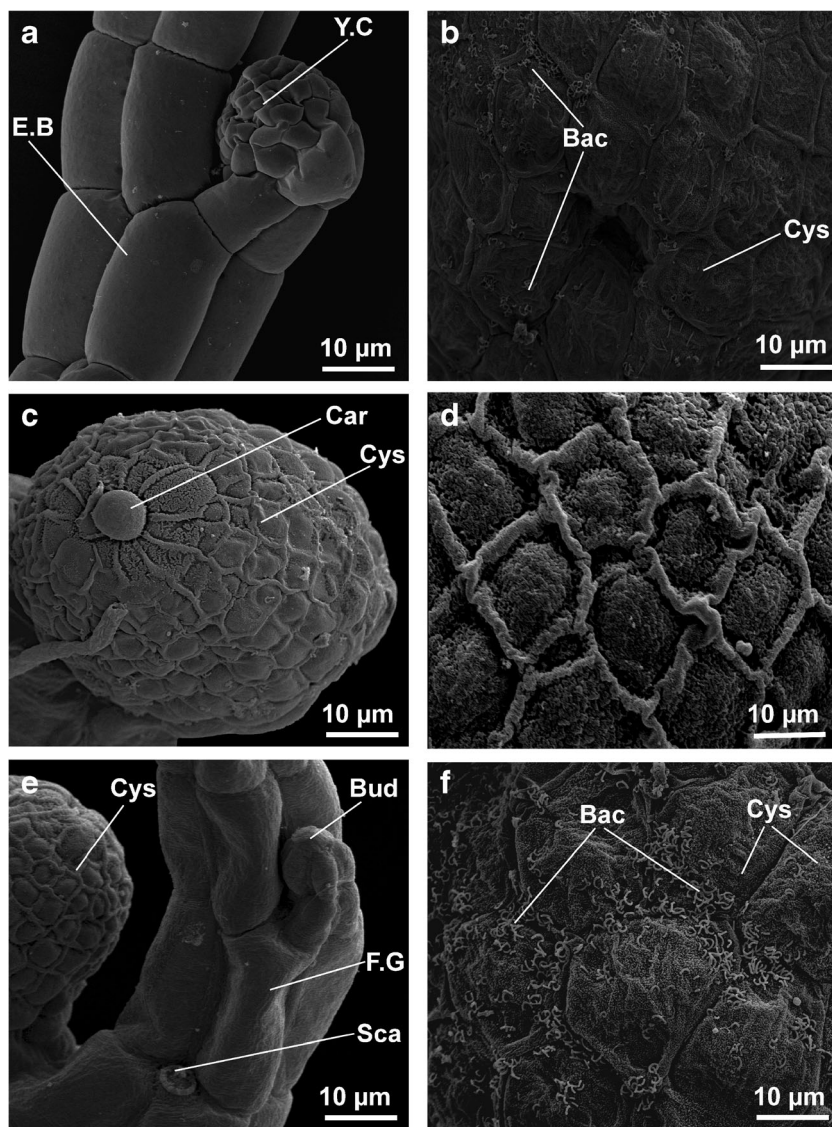
Lambohara. However, the two villages were not touched by the disease in 3 months of the warm season: October, November, and January. There is no correlation between the rate of infestation and temperature, nor between the prevalence of infestation and temperature. Concerning the prevalence of infestation, minimal values of 40 % or less were observed both in April and in February when average temperatures of 27.96 and 30.33 °C, respectively, were recorded. The maximum values of 100 % were observed in September, December, and March.

The proportions of the various stages in the two sites are shown in Fig. 9a, b. A total of 1606 epiphytes were recorded on the 30 cm^2 of *K. alvarezii* observed in Lambohara (all periods combined). In this village, individuals of the mixed stage and the female gametophytes were observed during the six periods, individuals at the infesting stage were observed at four periods, and male gametophytes only twice. A total of 1225 epiphytes were observed in Tampolove with the individuals of the infesting stage and of the mixed stage having been observed at each period, the female gametophytes during three periods and the male gametophytes at only one period. No periodic cycle of the EFA stages was highlighted by the graphs suggesting that the life cycle was certainly accomplished in a short time and all stages were often simultaneously present. The least observed stage was the male gametophyte. Significant differences were observed between male and female gametophytes in the two sites. Comparisons between male and female gametophytes at each occurrence times show that only in September 2013 were these two gametophytes not significantly different. Tetrasporophytes, even if it was not possible to account them accurately, were observed at each period of infestation.
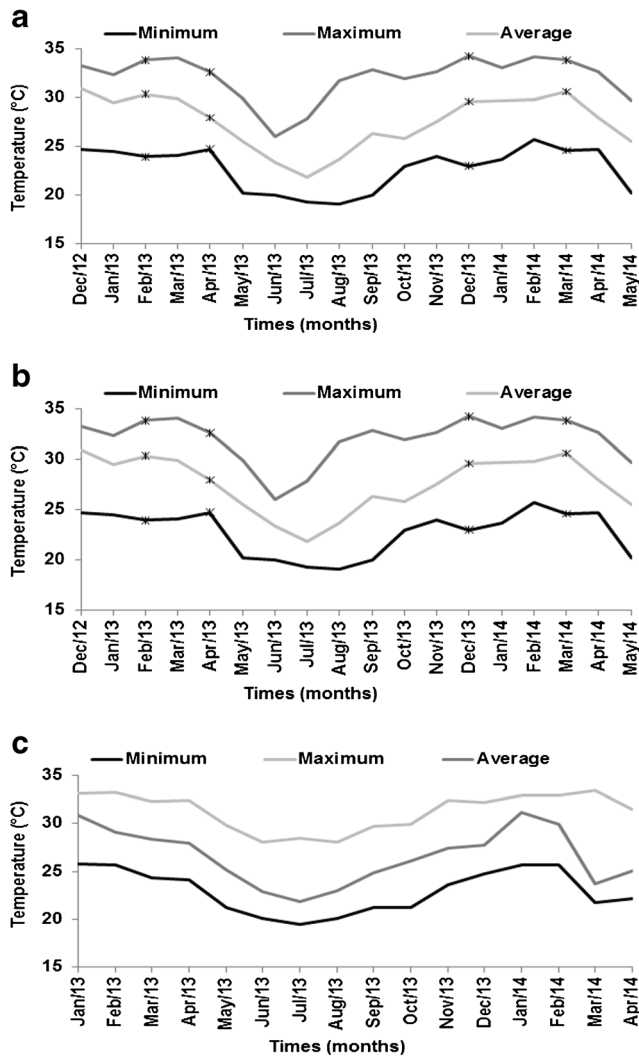
Fig. 7 The minima, maxima, and average temperatures observed each month in the three sites with the epiphytes occurrence times (*x* on the curves): **a** in Tampolove, **b** in Lambohara, and **c** in Sarodrano

Fig. 8 Epiphyte density (epiphytes $cm^{-2} \pm SD$, $n = 5$) on *K. alvarezii*. *Bars in black* are epiphyte density in Lamohara, and *bars in grey* are epiphyte density in Tampolove

Discussion

Our work first considered the life cycle of the EFA observed in three villages of the southwest of Madagascar. The epiphytic algae infesting *K. alvarezii* in the southwest and also in the north of the island (Nosy Ankao) from where we obtained some samples were all identified by a species falling into the *Polysiphonia* group as defined by Choi et al. (2001) in having four pericentral cells with absence of cortication and a straight arrangement of tetrasporangia. Also, a phylogenetic analysis carried by our team (Bertiaux 2013) on a 253-bp portion of the rbcL gene of EFA samples from Madagascar (including some samples from Nosy Ankao, northeast from Madagascar) indicated that the Malagasy EFA forms a monophyletic sister group of a clade including eight species of the genera *Polysiphonia* (3 species) and
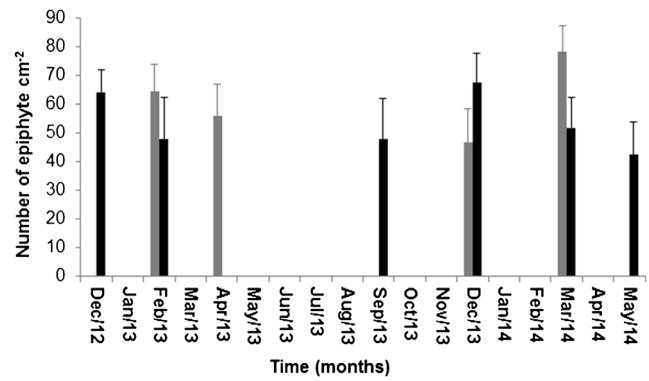
Neosiphonia (5 species) (Bertiaux 2013). We named the Malagasy EFA "*Polysiphonia* sp." mainly because of their morphological characters.

Gametophytes (haploid plants), carposporophytes (diploid plants), and tetrasporophytes (diploid plants), observed in the Malagasy *Polysiphonia* species, are very characteristic of the common triphasic life cycle observed in red seaweeds (Fritsch 1959; Roland and Vian 1985). Tetrasporic and gametophytic plants (male and female) are morphologically similar (isomorphic), whereas the carposporophyte is developed on female plant after fertilization (Jadiye et al. 2006). There was no difference in the cycle of our *Polysiphonia* sp. as observed by Jadiye et al. (2006) on another species of *Polysiphonia*.

Vairappan (2006) identified tetraspores with optical microscope as being the infesting stage of *N. savatieri*. We did not identify the nature of the infesting stage, but we suppose that it can be tetraspores, carpospores, and buds produced by emerged epiphytes. Indeed, at many periods during the cold and warm seasons, cystocarps, tetrasporophytes, and buddings were observed simultaneously. Male gametophytes with spermatangia were the less-recorded stage and their lifetime is certainly very short. Ateweberhan et al. (2015) found a significant seasonal and between-sites variations in infestation, the prevalence of infestation varying between 30 and 100 %. We also observed a significant seasonal variation and a between-sites variation of the prevalence. The rates of infestation were not significantly different between sites and were similar to rates of infestation observed in the Philippines (88.5 epi cm^{-2}), Tanzania (69.0 epi cm^{-2}), Indonesia (56.5 epi cm^{-2}), and Malaysia (42.0 epi cm^{-2}) (Vairappan 2006). Interestingly, in the Ateweberhan et al. (2015) study, some farming sites were healthy as was Sarodrano in our study. These authors observed that the lowest temperature variations and the lowest maximum temperatures occurred in healthy sites as we also observed

Fig. 9 Proportions of the various stages in the two sites: **a** in Tampolove and **b** in Lambohara
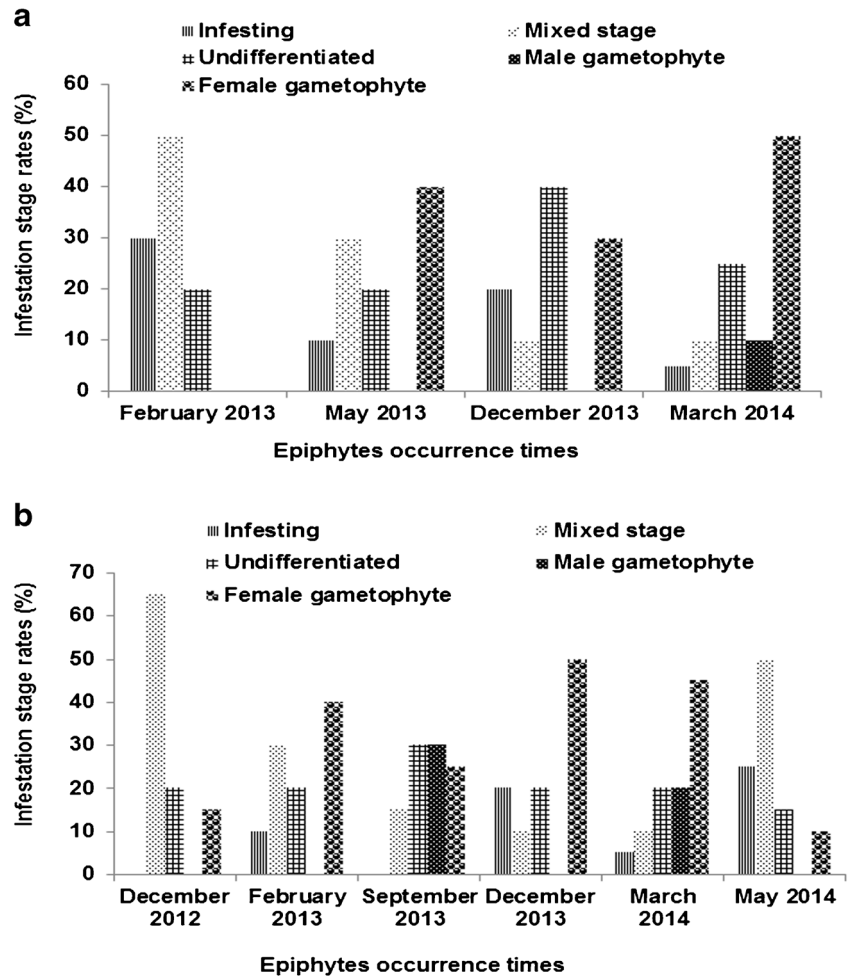
in Sarodrano. This suggests that the least the minimum temperature in farmed field is, the best the farming conditions to avoid EFA infestation are. The companies managing the farmers in the southwest of Madagascar should carefully study the temperature regime of their future farming sites for their selection criteria. A suggestion would be that they could place data loggers in potential sites during the cold and hot seasons to oversee the

Fig. 10 The supposed ways of infestation between *K. alvarezii* individuals in an infested field and from infested to healthy fields
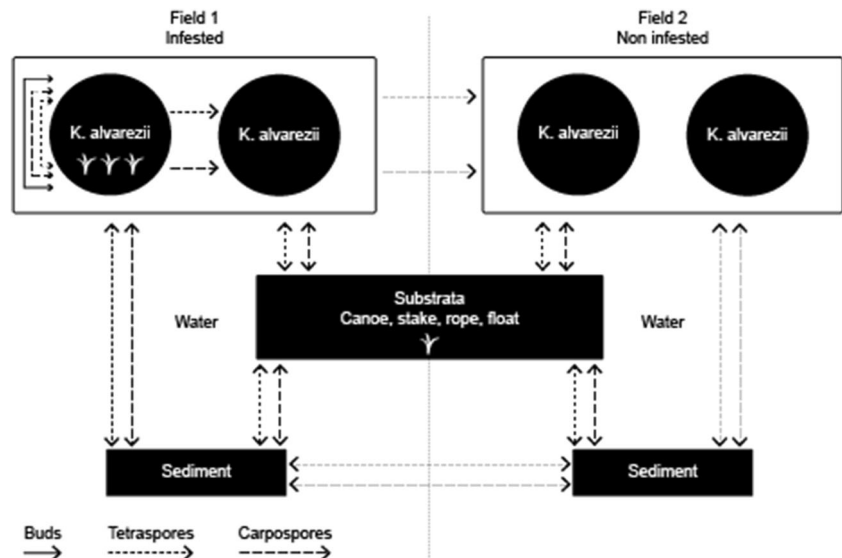
temperature extreme variations (the maximum temperature stays below 32 °C).

The ultrastructure analysis revealed the presence of buds on *Polysiphonia* individuals that were single stems or finger-like processes of a few hundred micrometers. Buds were located between two segments or at the tips of the branches, where many scars were also observed suggesting that buds are often released and certainly participate to the global infestation of *K. alvarezii* individuals. The smallest *Polysiphonia* individuals observed on *K. alvarezii* emerged from warts. They were unbranched thalli implanted into the cortex of *K. alvarezii*. Such warts were first described by Critchley et al. (2004) and Hurtado et al. (2006) who reported goose bumps as primary symptom of the infestation of an unidentified species of *Polysiphonia*. The symptoms induced by *Neosiphonia savatieri* (Vairappan 2006) and *N. apiculata* (Vairappan et al. 2007) on *K. alvarezii* farmed in the Philippines, Indonesia, Malaysia, and Tanzania were described afterward. Warts and the dark spots, similar to the ones described in the present work, were only observed during *N. apiculata* infestations (Vairappan et al. 2007), suggesting that *Polysiphonia-Neosiphonia* species exhibit different symptoms upon infecting *K. alvarezii* (Vairappan et al. 2007; present work). Ultrastructure of the infesting stage and of the cortex of infested *K. alvarezii* was also analyzed in the present work. This showed that infestation affects algae internally contrary to most epiphytes which generally adhere on the plant surface without internal effect. This internal infestation allows us to qualify EFA as parasitic epiphytes, implying that the benefit for EFA to infect cottonii is not only to find a surface to develop on (like other epiphytes) but also to have the possibility to derive a part of its host resources. Therefore, effects of EFA on its host are not restricted to a decrease in light available for photosynthesis (like many epiphytes), but could be also to derive nutrients and/or photosynthetates from its host like other algal parasites (e.g., *Harveyella mirabilis*; Kremer 1983). Infestation has also a physical effect. Indeed, as in our study, Vairappan et al. (2007) highlighted that infestation by *N. apiculata* and *N. savatieri* leads to tissue disintegration and secondary bacterial infection culminating in thallus rupture and breaking-off of seaweeds from culture lines. The disappearance of the extracellular matrix bordering the cortex we observed certainly opens the way to bacterial infection and explains the *K. alvarezii* breaking-off observed macroscopically. We also observed high concentrations of *Vibrio*-shaped bacteria on cystocarps and the surface of infested *K. alvarezii*.

The supposed ways of infestation between *K alvarezii* individuals in an infested field and from infested to healthy fields are summarized in Fig. 10. In an infested field, the number of epiphytes increases on infested *K. alvarezii* by the implantation of tetraspores, carpospores, and buddings coming from their own EFA flora. Transmission from infested to healthy individuals certainly occurs through water transportation of both spores, increasing the prevalence of infestation in a field. Spores can deposit into sediments and stay latent until favorable conditions enable them to infect healthy seaweeds at another period. Spores could also adhere to and grow on artificial substrata like dugout canoes, stakes, ropes, and floats that are used by farmers. Sediments of infested fields can act as reservoirs where spores may be disseminated towards healthy fields. Furthermore, artificial substrata can also be reservoirs where infested individuals are transported into healthy fields. *Polysiphonia-Neosiphonia* occurrence on artificial substrata has been shown (Bertiaux 2013), and canoes with other farming equipment have to be cleaned when passing from one farmed site to another. Also, recently, wild-caught juvenile sea cucumbers *Holothuria scabra* and red seaweed *Kappaphycus striatum* were co-cultured in Zanzibar (Beltran-Gutierrez et al. 2014). Growth performance for seaweed and sea cucumbers did not differ between monoculture and co-culture treatments, the results indicating that *H. scabra* is a highly viable candidate species for lagoon co-culture with seaweed (Beltran-Gutierrez et al. 2014). As sediments might also be a reservoir of spores, the effects on EFA prevalence of polyculture using deposit feeders like sea cucumbers in farmed fields should be analyzed. Investigations should be made on the three compartments: the infested field, the sediments, and the artificial substrata—that potentially play a role in the dissemination of the disease.

References

Ateweberhan M, Rougier A, Rakotomahazo C (2015) Influence of environmental factors and farming technique on growth and health of farmed *Kappaphycus alvarezii* (cottonii) in south-west Madagascar. J Appl Phycol 27:923–934

Beltran-Gutierrez M, Sebastian CAF, Kunzmann1 A. Stead SM, Msuya FE, Hoffmeister TS, Slater MJ (2014) Co-culture of sea cucumber *Holothuria scabra* and red seaweed *Kappaphycus striatum*. Aquac Res 1–11 DOI:10.1111/are.12615

Bertiaux F (2013) «DNA tracking» du réservoir de «Neosiphonia sp.» (Rhodophyta), agent de l'«Epiphytic filamentous algal disease» sévissant dans les champs de l'algue *Kappaphycus alvarezii*, aquacultivée à Madagascar. Master II Thesis, Umons: 120p

Bindu MS, Levine IA (2010) The commercial red seaweed *Kappaphycus alvarezii*. An overview on farming and environment. J Appl Phycol 23:789–796

Borlongan IAG, Tibudos KR, Yunque DAT, Hurtado AQ, Critchley AT (2011) Impact of AMPEP on the growth and occurrence of epiphytic *Neosiphonia* infestation on two varieties of commercially cultivated *Kappaphycus alvarezii* grown at different depths in the Philippines. J Appl Phycol 23:615–621

Bozzola JB, Russell LD (1992) Electron microscopy: principles and techniques for biologists. Jones and Bartlett Publishers, Boston, pp 14–62

Critchley AT, Largo DB, Wee W, Bleicher LG, Hurtado AQ (2004) A preliminary summary on *Kappaphycus* farming and the impact of epiphytes. Jap J Phycol 52:231–232

Choi HG, Kim MS, Guiry MD, Saunders GW (2001) Phylogenetic relationships of polysiphonia (Rhodomelaceae, Rhodophyta) and its relatives based on anatomical and nuclear small-subunit rdna sequence data. Can J Bot 79(12):1465–1476

Doty MS, Alvarez VB (1975) Status, problems, advances and economics of *Eucheuma* farms. Mar Technol Soc J 9:30–35

Fritsch FE (1959) The structure and reproduction of the algae, vol. II, Cambridge University Press, Cambridge pp. 722–724

Guiry MD (2011) *Kappaphycus alvarezii* (Doty) Doty ex PC Silva. AlgaeBase. World-wide electronic publication. National University of Ireland, Galway, **http://www.algaebase.org**

Hayashi L, Hurtado AQ, Msuya FE, Bleicher-Lhonneur G, Critchley AT (2010) A review of *Kappaphycus* farming: prospects and constraints. In: Seckbach J, Einav R, Israel A (eds) Seaweeds and their role in globally changing environments. Springer, Dordrecht, pp 251–283

Hurtado AQ, Critchley AT, Trespoey A, Bleicher-Lhonneur G (2006) Occurrence of *Polysiphonia* epiphytes in *Kappaphycus* farms at Calaguas Is., Camarines Norte, Phillippines. J Appl Phycol 18: 301–306

Jadiye S, Anilkumar C, Rao PSN (2006) Sexual anomaly in a marine red alga *Polysiphonia unguiformis* Boergesen. Curr Sci 90:289–290

Kim MS, Lee IK (1999) *Neosiphonia flavimarina* gen. et sp. nov. with a taxonomic reassessment of the genus *Polysiphonia* (Rhodomelaceae, Rhodophyta). Phycol Res 47:271–28

Kremer B (1983) Carbon economy and nutrition of the alloparasitic red alga *Harveyella mirabilis*. Mar Biol 76:231–239

Largo DB, Fukami F, Nishijima T, Olino M (1995a) Laboratory-induced development of the ice-ice disease of the farmed red algae *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Solieriaceae, Gigartinales, Rhodophyta). J Appl Phycol 7:539–543

Largo DB, Fukami F, Nishijima T (1995b) Occasional bacteria promoting ice-ice disease in the carrageenan-producing red algae *Kappaphycus*

alvarezii and *Eucheuma denticulatum* (Solieriaceae, Gigartinales, Rhodophyta). J Appl Phycol 7:545–554

Loureiro RR, Reis RP, Critchley A (2009) In vitro cultivation of three *Kappaphycus alvarezii* (Rhodophyta, Areschougiaceae) variants (green, red and brown) exposed to a commercial extract of the brown alga *Ascophyllum nodosum* (Fucaceae, Ochrophyta). J Appl Phycol 22:101–104

Mendoza WG, Montaño NE, Ganzon-Fortes ET, Villanueva RD (2002) Chemical and gelling profile of ice-ice infected carrageenan from *Kappaphycus striatum* (Schmitz) Doty "sacol" strain (Solieriaceae, Gigartinales, Rhodophyta). J Appl Phycol 14:409–418

Pang T, Liu J, Liu Q, Lin W (2011) Changes of photosynthetic behaviors' in *Kappaphycus alvarezii* infected by epiphyte. eCAM. doi:10. 1155/2011/658906

Plotieau T, Lepoint G, Baele JM, Tsiresy G, Rasolofonirina R, Lavitra T, Eeckhaut I (2014) Caractéristiques minérales et organiques des sédiments des enclos d'élevage d'*Holothuria scabra* (Holothuroidea, Echinodermata). Bêche-de-mer Bulletin de la CSP 34:29–33

Roland JC, Vian B (1985) Atlas de Biologie Végétale. Ed Masson, Paris, 110 p

Solis MJL, Draeger S, Thomas Edison E, de la Cruz TEE (2010) Marine-derived fungi from *Kappaphycus alvarezii* and *K. striatum* as potential causative agents of ice-ice disease in farmed seaweeds. Bot Mar 53:587–594

Tong P, Jianguo L, Qian L, Wei L (2011) Changes of photosynthetic behaviors in *Kappaphycus alvarezii* Infected by Epiphyte. Evid Based Complement Alternat Med 2011:658906

Trono GC Jr (1974) *Eucheuma* farming in the Philippines. U. P. Natural Science Research Center, Quezon City

Vairappan CS (2006) Seasonal occurrences of epiphytic algae on the commercially cultivated red alga *Kappaphycus alvarezii* (Solieriaceae, Gigartinales, Rhodophyta). J Appl Phycol 18:611–617

Vairappan CS, Chong SC, Lhonneur GB, Critchley AT (2007) Epiphyte outbreak in *Kappaphycus alvarezii* (Gigartinales, Rhodophyta) farms in South East Asia: impact on the yield and quality of carrageenan. XIXth International Seaweed Symposium, 26–31, Kobe, Japan, p 80

Vairappan CS, Chung CS, Hurtado AQ, Soya FE, Lhonneur GB, Critchley A (2008) Distribution and symptoms of epiphyte infection in major carrageenophyte-producing farms. J Appl Phycol 20:27–33

Villares R, Puente X, Carballeira A (2002) Seasonal variation and background levels of heavy metals in two green seaweeds. Environ Pollut 119:79–90