Temperature is not a limiting factor for the expansion of *Halophila stipulacea* throughout the Mediterranean Sea

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ABSTRACT: The seagrass *Halophila stipulacea* is a Lessepsian migrant that has spread from the Red Sea into the Mediterranean Sea where some authors consider it invasive. It has been suggested that the range of expansion of the species in the Mediterranean will be limited by the 15°C sea surface isotherm. Here we tested the effects of temperature on survival, photosynthesis, leaf growth and clonal growth of *H. stipulacea*. We analysed the temporal and spatial variation of sea surface temperature (SST) in the Mediterranean Sea and the species’ spread rate since its introduction to forecast its future expansion and potential changes in the spread rate due to increased SST. We estimated that the species has been spreading throughout the Mediterranean with a variable rate averaging 12 km yr⁻¹. Despite being a species native to the tropics, it was able to survive, photosynthesise and grow within a broad range of temperatures (10 to 30°C). At 10°C, a temperature colder than the winter’s lowest isotherm for most of the Mediterranean Sea, the clonal growth ceased but plants did not die and continued to photosynthesise and produce new leaf biomass. The maximum photosynthetic rate peaked at 30°C but the optimal leaf growth rate was within the range of temperatures for temperate seagrass species (11.5 to 26°C). Based on the present spread rate and on the non-limiting effect of temperature, we estimate that in the next 100 yr *H. stipulacea* will be present throughout the whole Mediterranean Sea (but perhaps not the north Adriatic), potentially spreading into the Atlantic.

KEY WORDS: Biological distribution limits · Geographical distribution · *Halophila stipulacea* · Mediterranean Sea · Spread rate · Temperature

INTRODUCTION

Since the opening of the Suez Canal in 1869, the eastern Mediterranean Sea has become prone to Lessepsian migrants, i.e. tropical species that migrate from the Red Sea into the Mediterranean Sea through the canal. *Halophila stipulacea* (Forsskål) Ascherson, the dominant seagrass in the northern Red Sea (Short et al. 2007), is one of those species. The occurrence of growing, living plants of *H. stipulacea* in the Mediterranean was first recorded in 1926 off Rhodes (Forti 1927). The species has spread into other areas, colonising several locations around Cyprus and Rhodes (Lipkin 1975). Its progressive colonisation westwards is well documented, with records of its presence in Malta (Lanfranco 1970), Sicily (Biliotti & Abdelahad 1990, Alongi 1993, Di Martino et al. 2006), Vulcano Island (Acunto et al. 1997, Procaccini et al. 1999) and the harbour of Palinuro, in the Tyrrenhian Sea (Gambi et al. 2009). Its westernmost records are in the Gulf of Gabes (Missaoui et al. 2003) and more recently at Cap Monastir, Tunisia (Sghaier et al. 2011).

*H. stipulacea* is sometimes classified as an invasive species (sensu Strefatis & Zenetos 2006) but no reports have been published on the displacement of native species by *H. stipulacea* in the Mediterranean.
(Williams 2007, Tsiamis et al. 2010). In contrast, such displacement has been reported in the Caribbean, where the species has been introduced, probably by boats, and is expanding in areas formerly covered by the local seagrass *Syringodium filiforme* (Willette & Ambrose 2009, 2012, Willette et al. 2014).

Temperature is a major driver for the geographical distribution of marine plants and in particular for the colonisation of tropical species in the western Mediterranean (Bianchi 2007, Samperio-Ramos et al. 2015). For many years, the 15°C sea surface isotherm was considered the geographical distribution limit for *H. stipulacea* in the Mediterranean, because the species was confined to the eastern, warmer areas of the basin (Por 1990, Bianchi 2007). More recently, settlements of *H. stipulacea* were found in western Italy (Gambi et al. 2009), but this range expansion was considered to be associated with the higher seawater temperature caused by the presence of hydrothermal vents.

Even though it is generally accepted that the expansion of the distribution range of the tropical seagrass *H. stipulacea* in the Mediterranean is limited by temperature, this has never been investigated. Temperature may limit the geographical distribution of a species by imposing 3 types of biological limit: lethal, growth and reproductive (Lüning 1990). The growth limits are particularly important for species that may persist and disperse without sexual reproduction, as is often the case with seagrasses.

Here we tested experimentally the effects of temperature on survival, photosynthesis and growth of *H. stipulacea*. Potential areas in the Mediterranean where temperatures may be suitable for *H. stipulacea* expansion were identified after computing the spatial and seasonal variation of sea surface temperature (SST) using satellite data. The historical spread rate of the species was also estimated, to analyse if it has changed recently due to increased SSTs and to predict a time frame for the future expansion of the species in the Mediterranean.

**MATERIALS AND METHODS**

**Image analysis of SST**

Satellite grids of monthly averages of SST in the Mediterranean Sea, with a spatial resolution of 4.4 × 4.4 km, were obtained from the National Oceanic and Atmospheric Administration (NOAA) Satellite and Information Service (http://data.nodc.noaa.gov/pathfinder/Version5.2/). The Pathfinder v.5.2 monthly mean SST data covered a period of 28 yr ranging from 1982 to 2009, with a total of 336 images. Only nocturnal SST grids were considered, to avoid potential bias related to solar radiation that originates diurnal fluctuations in SST (Raitos et al. 2006). SST images were processed and analysed using Mirone software (Luis 2007). The average sea surface isotherms of February and August in the Mediterranean were computed and represented using a greyscale palette of 7 shades, each representing 1°C, with a precision of 0.001°C. In order to compute the yearly variation of SST in the Mediterranean basin, following the procedure described in Relvas et al. (2009), all 336 monthly grids were organised in a 3-dimensional stack, with the third dimension corresponding to time. During this process the data were checked against a quality flag file that assigns a quality factor of between 0 (worst) and 7 (best) to each value. To ensure that only the best quality data were used, only data values with a quality factor of 6 and 7 were retained. The outcome of 28 yearly averages was ‘piled up’ once again and used to obtain the mean annual variation rate of the SST, fitting a linear regression model to data of each grid cell. The mean seasonal variation of SST was obtained by performing 4 repetitions of the process described above, each time using the monthly mean grids of 3 mo (spring: March–May; summer: June–August; autumn: September–November; winter: December–February) (Luis 2007).

**Experimental procedure**

Plants of *Halophila stipulacea* were collected in January 2012 from a shallow, turbid meadow (average water depth of 3 m) located close to the old port in Akrotiri Bay in Limassol, Cyprus (34°42′22″N, 33°07′26″E; Fig. 1). The plants were immediately sent in moist and dark conditions to the Centre of Marine Sciences, Portugal, where they arrived 2 d after collection. Plants were kept in a growth chamber where light and temperature were controlled (Arablab), for 2 wk to acclimate to the experimental conditions (aerated natural seawater at 18°C, salinity of 35, light level of 60 to 70 µmol quanta m⁻² s⁻¹ and 12:12 h photoperiod, with the addition of 5 µM NH₄⁺). Plants were acclimated at 18°C because it is an intermediate temperature within the experimental range (10 to 30°C). Although in Akrotiri Bay, where *H. stipulacea* was collected, the average salinity is 39 (Forchino 2010), an acclimation and experimental salinity of 35 was selected. Seawater was renewed every 2 d to
keep oxygen at saturating levels. During acclimation, the physiological condition of the plants was monitored by measuring the maximum electron quantum yield of Photosystem II \(Y = F_v/F_m\). Yield was measured on a daily basis using a pulse amplitude modulated (PAM) fluorometer (Diving-PAM, Heinz Waltz). Yield steadily increased from 0.4 to values around 0.76, similar to those measured \textit{in situ} by Sharon et al. (2009) in native populations of \textit{H. stipulacea} in the Gulf of Aqaba (23°C, 8 m depth). When yields attained this value, we considered plants to be fully recovered and started the experiment.

The effect of temperature on photosynthesis and growth of \textit{H. stipulacea} was experimentally assessed at a range of values (10, 13, 16, 20, 25 and 30°C) that should encompass those experienced by the species in most of the Mediterranean Sea (see Fig. 1). A total of 6 aquaria containing distilled water were placed inside the growth chamber, which was set to the lowest experimental temperature (10°C). Temperatures from 13 to 30°C were achieved using heaters that were placed inside the aquaria, and checked regularly with a thermometer. A total of 5 replicate flasks (600 ml seawater) of each temperature, each containing 5 plants of \textit{H. stipulacea}, were placed inside each aquarium. Each plant included a piece of rhizome and respective roots, and at least 2 attached shoots. The flasks were sufficiently high to prevent the entry of distilled water from the aquarium. Plants were transferred from the acclimation temperature (18°C) to experimental temperatures by increments of ±2 to 3°C d\(^{-1}\) to avoid stressful thermal shock. The plants were exposed to the experimental temperatures for 7 d. During the experiment, the seawater (salinity of 35, 5 \(\mu\)M NH\(_4^+\)) in the flasks was renewed every 2 d. Water renewals were pre-warmed to avoid thermal stress. At the end of the experiment, the photosynthetic and growth rates of \textit{H. stipulacea} exposed to the 6 different temperatures were measured.

Photosynthetic oxygen production was measured in randomly chosen leaves of each temperature treatment, in a square section incubation chamber coupled to a Clark-type oxygen electrode (DW3/CB1, Hansatech) calibrated with N\(_2\) and air-saturated water. For each temperature treatment, 6 replicate measurements were performed. In each measurement, 3 to 4 leaf sections were inserted into the chamber and incubated in 15 ml GF/F filtered seawater. During the measurements, the temperature of the filtered seawater inside the chamber was similar to that of the respective temperature treatment and was kept constant by a circulator thermostat bath. Oxygen evolution was measured at 8 gradually increasing light intensities of 5, 12, 39, 85, 195, 390, 501 and 875 µmol quanta m\(^{-2}\) s\(^{-1}\). These were set using a series of neutral slide filters. Actinic light was provided by a slide projector (Pradovit 150, Leica) equipped with a halogen lamp. Each experimental light step lasted approximately 10 min. Respiration was also measured as oxygen depletion inside the chamber in the dark after setting the leaves in oxygen-saturated water. After measurements, leaf tissues were dried at 60°C for 48 h. Gross photosynthesis (mg O\(_2\) g\(^{-1}\) DW h\(^{-1}\)) was calculated as the sum of respiration and net photosynthesis. The Smith and Talling model (Smith 1936, Talling 1957) was fitted to gross photosynthesis versus irradiance data and the photosynthetic parameters \(P_{max}\) (maximum photosynthetic rate, mg O\(_2\) g\(^{-1}\) DW h\(^{-1}\)), \(\alpha\) (initial slope, mg O\(_2\) g\(^{-1}\) DW h\(^{-1}\)/µmol quanta m\(^{-2}\) s\(^{-1}\)), \(I_c\) (compensation irradiance, µmol quanta m\(^{-2}\) s\(^{-1}\)) and \(I_s\) (saturation irradiance, µmol quanta m\(^{-2}\) s\(^{-1}\)) were calculated. \(I_c\) was estimated from the photosynthesis–irradiance curve obtained for each temperature,
therefore resulting in a single \( I_c \) value per temperature treatment.

Leaf growth (mm\(^2\) leaf\(^{-1}\) d\(^{-1}\)) was measured by the increment in the leaf surface area of shoots \( (n = 5 \) shoots per treatment\() \), whereas clonal growth (no. of new shoots plant\(^{-1}\) d\(^{-1}\)) was measured by counting the number of new \textit{H. stipulacea} shoots developed from the apical meristematic activity during the experiment \( (n = 5 \) per treatment\() \). Shoots were tagged using numbered fine plastic fibres, in order to identify them when measuring initial and final leaf surface areas. Measurements were performed in the apical shoot and the second and third shoot of the rhizome. All shoots in each temperature level were photographed before and after the experiment and their leaf surface areas were calculated using the software Image J \( (\text{Abràmoff et al. 2004}) \).

**Data analysis**

The geographical spread rate of \textit{H. stipulacea} throughout the Mediterranean Sea since the opening of the Suez Canal in 1869 was estimated based on the time series of all reports of new sites in the basin and their distances to the origin (the Suez Canal). Only new sites lying outside the previously known geographical distribution were considered to estimate the spread rate (see Fig. 3, points marked with circles), as new sites found within the known species distribution at the time of the report do not contribute information on increasing geographical expansion. A linear regression was fitted to the distance/year data set and the slope of the regression was used as an estimate of the spread rate (km yr\(^{-1}\)).

The effect of temperature on the leaf growth rate of each leaf size class (0–2 and 2–4 cm length) was tested using 2 separate Kruskal-Wallis non-parametric tests, one for the size class of 0–2 cm and another for the size class of 2–4 cm length, because data were not normally distributed, even after transformation. Post hoc analysis was performed using the Tukey multiple comparison test. One-way analysis of variance was used to test the effects of temperature on the clonal growth and on photosynthetic parameters (except for \( I_k \), where the Kruskal-Wallis non-parametric test and the multiple comparison Dunn’s method were used). Multiple comparisons were performed using the Holm-Sidak method. Effects were considered significant at \( p \leq 0.05 \).

**RESULTS**

**Spatial and seasonal variation of STT**

Fig. 1 shows the present distribution of \textit{Halophila stipulacea} throughout the Mediterranean based on the available literature, superimposed on the average STTs in February and August obtained from the image analysis of the SSTs. The geographical distribution of the species is presently confined between minimum winter temperatures of 14°C in the western Mediterranean and maximum summer temperatures of 29°C in the eastern Mediterranean. The computation of the yearly variation (1982 to 2009) of the SST in the Mediterranean basin showed that the rate of temperature increase in winter has been higher in the southeast Mediterranean (Fig. 2a), coinciding with the geographical expansion of \textit{H. stipulacea}, except in the Aegean Sea, where the increase rate has been close to zero. In summer, the rate of temperature increase has been much higher than in winter throughout the whole Mediterranean, but more marked in the eastern basin (Fig. 2b). The increase of the distribution range of the species through time (Fig. 3, points marked with circles) was significant \( (R^2 = 0.80, p = 0.007) \). The estimated spread rate of \textit{H. stipulacea} throughout the Mediterranean since its first report in the basin averaged 12 km yr\(^{-1}\) (Fig. 3).
The effects of temperature on the gross photosynthesis response to irradiance of *H. stipulacea* showed no signs of photoinhibition at high irradiances at any of the temperatures tested (curves not shown). Significant effects of temperature on the photosynthetic parameters of *H. stipulacea* were found (Fig. 4). The maximum photosynthetic rate, \(P_{\text{max}}\), increased about 2-fold at 30°C (\(F = 12.23\), dfn = 5, dfd = 24, \(p < 0.001\)) (Fig. 4a), whereas the irradiance at the onset of saturation, \(I_k\), increased about 4-fold at 25 and 30°C (\(H = 14.82\), df = 5, \(p = 0.011\)) (Fig. 4c). The photosynthetic efficiency at low irradiances, \(\alpha\), decreased about 5-fold between 10 and 30°C (\(F = 4.20\), dfn = 4, dfd = 20, \(p = 0.012\)) (Fig. 4b). The compensation irradiance, \(I_c\), when respiration and photosynthesis are balanced, was much higher (about 10-fold) in plants exposed to the highest temperatures (25 and 30°C) (Fig. 4d), following the exponential response of respiration to temperature (\(F = 40.66\), dfn = 5, dfd = 27, \(p < 0.001\)) (Fig. 5).

**Fig. 3.** Spread rate of *Halophila stipulacea* throughout the Mediterranean. The plot shows the distance to the Suez Canal (distribution range) of the sites where the species was observed and the year of the report. Maximum distance values observed through time (circled points). A linear regression was fitted to these values to estimate the spread rate (the slope of the regression, in km yr\(^{-1}\)).

**Fig. 4.** Effects of temperature on *Halophila stipulacea* photosynthetic parameters: (a) \(P_{\text{max}}\), maximum photosynthetic rate (mg O\(_2\) g\(^{-1}\) DW h\(^{-1}\)); (b) \(\alpha\), photosynthetic efficiency (mg O\(_2\) g\(^{-1}\) DW h\(^{-1}\)/µmol quanta m\(^{-2}\) s\(^{-1}\)); (c) \(I_k\), irradiance at the onset of saturation (µmol quanta m\(^{-2}\) s\(^{-1}\)); and (d) \(I_c\), compensation irradiance (µmol quanta m\(^{-2}\) s\(^{-1}\)). Values are mean ± SE. Different letters indicate significant differences (\(p \leq 0.05\)).
The growth rate of *H. stipulacea* leaves decreased with leaf size. Younger leaves (0–2 cm) showed higher leaf area growth compared to older ones (2–4 cm) (Fig. 6). However, the temperature effect on leaf area growth rates was similar for younger and older leaves. Growth rate was maximal at 20°C but differences among temperatures were not generally significant (leaf size class 0–2 cm: $H = 18.50, df = 5, p = 0.002$; leaf size class 2–4 cm: $H = 9.36, df = 5, p = 0.096$). The clonal growth increased significantly with temperature to a maximum production of 1 shoot every 2 d at 30°C (Fig. 7) ($F = 6.76, df_n = 5, df_d = 24, p < 0.001$). The lowest temperature of 10°C inhibited the clonal growth of *H. stipulacea*.

**DISCUSSION**

*Halophila stipulacea* has been expanding throughout the Mediterranean since its putative introduction in 1869 when the Suez Canal was opened (Por 1971), with no indication of jump-type introductions in very distant areas, characteristic of new introductions. The spread rate at which it has been dispersing since its first reported observation in 1926 (Forti 1927) is not constant through time, as seen for other invasive macrophytes (Montefalcone et al. 2015), but nevertheless continuous, averaging 12 km yr$^{-1}$. This rate is low when compared with the spread rates estimated for seaweeds, which can be higher than 300 km yr$^{-1}$ (Lyons & Scheibling 2009).

A lower temperature limit of 15°C has been hypothesised as the physiological barrier for the expansion of the tropical *H. stipulacea* throughout the Mediterranean Sea (Bianchi 2007). However, our experimental results on the effects of temperature on the photosynthetic and growth rates provide clear evidence that the expansion of *H. stipulacea* throughout the Mediterranean is not likely to be limited by temperature because the species revealed a large thermal tolerance. Furthermore, the overlaid map of the surface isotherms with the current distribution of *H. stipulacea* in the Mediterranean Sea (Fig. 1) indicates that the species has already expanded below the 15°C isotherm of February (the coldest month of the year) into the Adriatic and the Aegean Seas, and along the western Mediterranean.

Despite being a species native to the tropics, *H. stipulacea* was able to survive, photosynthesise and
grow within a broad range of temperatures (10 to 30°C). The maximum photosynthetic rate was highest at 30°C, whereas the leaf growth rate peaked at 20°C in both leaf size classes. The optimal temperature for photosynthesis of *H. stipulacea* was similar to values reported for other *Halophila* species (27.5°C for *H. ovalis* in Western Australia, Hillman et al. 1995; 30°C for *H. decipiens* in Virginia, Dennison et al. 1993; and 32.5°C for *H. johnsonii* in Florida, Torquemada et al. 2005). On the other hand, the optimal temperature for leaf growth of *H. stipulacea* (20°C) was lower compared to *H. ovalis* from Western Australia (25°C) (Hillman et al. 1995). The optimal growth temperature of *H. stipulacea* is within the range of optimal growth temperatures of warm temperate seagrass species (11.5 to 26°C), lying outside the range of tropical/subtropical species (23 to 32°C) (Lee et al. 2007). This may well justify the presence and spread of the tropical *H. stipulacea* into colder and western regions of the Mediterranean Sea. Clonal growth occurred within a broad range of temperatures (13 to 30°C). To our best knowledge, there are no reports of clonal growth rates of *H. stipulacea*. However, values of new leaf production of the tropical species *H. ovalis* average 0.5 leaves per apex shoot per day (Nakaoka & Aioi 1999), which is within the range of the average rate of clonal growth determined in *H. stipulacea* in this study. While most of the photosynthetic parameters increased with temperature, the photosynthetic efficiency, α, of *H. stipulacea* showed a reverse pattern, with higher values at lower temperatures. This pattern contrasts with that of *H. johnsonii*, which exhibited higher α values at higher temperatures in Florida (Torquemada et al. 2005). The high photosynthetic efficiency of *H. stipulacea* at lower temperatures suggests the existence of a mechanism of photo-acclimation to maximise carbon fixation under low water temperatures and low irradiiances during winter months. At 10°C, colder than the winter’s lowest temperature for most of the Mediterranean Sea, the clonal growth ceased but the plants did not die and continued to photosynthesise and produce new leaf biomass. This contrasts with other *Halophila* species such as *H. ovalis* in Western Australia, in which biomass production stopped at 10°C (Hillman et al. 1995).

Given the tropical origin of *H. stipulacea*, it is surprising that the species is functional at such a wide range of temperatures and that the optimal temperature for its leaf growth is 20°C. We do not know if this trait is specific to the population sampled in Cyprus or if it is consistent throughout the Mediterranean Sea. If general, one possible explanation is that the environmental conditions of the Mediterranean Sea may have selected for particular genotypes with such traits. Genetic analyses of Mediterranean meadows and parent population in the Red Sea are necessary to test this hypothesis.

If the temperature traits of *H. stipulacea* reported here are not highly variable along the Mediterranean Sea, the current warming trend of the SST may eventually reduce the presence of *H. stipulacea* in the easternmost and warmest part, the Levant Sea, as growth and photosynthetic efficiency of the species are reduced at temperatures higher than 20°C. On the other hand, the increase in the SST might favour the growth of the species during winter months, allowing it to expand into western and colder areas. The satellite data analysis showed that the SST of the Mediterranean Sea increased at a relatively fast rate from 1982 to 2009, an average increase of 1 and 1.5°C for the western and eastern Mediterranean, respectively. These values compare well with those of recent studies, which estimated an average increase of approximately 1°C based on satellite image data from 1985 to 2007 (Vera et al. 2006, Raitos et al. 2010). Longer-term studies using high-resolution models predict that the SST of the Mediterranean will increase by 3°C by 2100 (Somot et al. 2006). This may contribute to the success of *H. stipulacea* expansion throughout the Mediterranean Sea, particularly in the western Mediterranean, where the species is not yet present.

It should be noted, however, that temperature will not act in isolation on the spread of *H. stipulacea*, and therefore other interacting variables such as water circulation, light, salinity, nutrient availability, herbivory and interspecific competition may also affect the species expansion. Concerning nutrient availability, Alexandre et al. (2014) showed that the N₂ acquisition strategy of *H. stipulacea* is characterized by a similar uptake capacity and uptake efficiency of leaves and roots. This favours the species dispersal potential because *H. stipulacea* is not limited to sites where nutrients are only available in either water or sediment. Considering the actual spread rate of the species and the temperature tolerance values reported here as representative of Mediterranean stands, we estimate that in the next 100 yr *H. stipulacea* will be present throughout the whole Mediterranean Sea and will potentially spread into the Atlantic. An exception may be the north Adriatic, where winter temperatures in coastal waters may fall below 10°C. To confirm this spread potential of *H. stipulacea*, longer-term studies with genetically different populations from the Mediterranean Sea.
should be carried out, combined with other biological limits for geographical distribution such as sexual reproduction and seed dispersal.

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