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Short-term salt spray reveals high salt tolerance in a neotropical orchid species

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Abstract Salt is a limiting factor of plant growth in natural environments. Although not commonly exposed to salt, epiphytic orchids have some drought-related phenotypical traits that could grant some tolerance to the ionic component of salinity. *Epidendrum fulgens* Brongn. is a terrestrial orchid with recent epiphytic ancestry and it shares many phenotypic traits with epiphytic orchids. Here we investigated the salinity response of the orchid species *E. fulgens* by exposing plant stems to varying concentrations of artificial seawater spray (0, 200, 400 and 800 mM Na^+). Each plant had four stems, each stem corresponding to a salt treatment. We measured the maximum quantum efficiency of photosystem II (PSII) over 10 days and at the end of this period, we measured the relative water content (RWC), osmotic potential, chlorophyll index, and leaf Na^+ concentration. Negative effects of salt were only observed above 400 mM Na^+. While stems treated with 800 mM Na^+ reduced the osmotic potential, the RWC remained similar to control stems. Unexpectedly, leaf Na^+ concentration was similar for salt-treaded and control stems, suggesting that *E. fulgens* might be able to avoid the leaf absorption of this ion. Therefore, *E. fulgens* can tolerate salt spray in similar concentrations as the seawater and some of its physiological responses, such as maintaining a high RWC when facing osmotic stress, resemble the response of epiphytic orchids when facing drought stress.

Keywords Stress physiology · Dose-response · Salt spray · Seawater

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1 Introduction

Salt spray is a ubiquitous and important factor in coastal zones and is one of the many ways salt affects plants in the natural environment (Du and Hesp 2020). The spray is an aerosol of minuscule seawater droplets formed by the breaking waves, which are then carried out by the winds and precipitate over the coastal vegetation (Du and Hesp 2020). Once the salt reaches the plant, it causes an osmotic effect, reducing the leaf expansion rate, and the plant growth (Munns 2002). This initial phase of salinity closely resembles the effects of drought on plants (Munns 2002, 2011). The concentration of the salt inside the plant cell increases gradually over time, eventually reaching toxic concentrations after some days or weeks (Munns 2002). This toxicity causes cell death and generates a further reduction of plant growth (Munns 2002). The osmotic effect might be circumvented by osmotic adjustment, either by means of the production of compatible osmolytes or the absorption of the salt ions themselves (Ogburn and Edwards 2010). On the other hand, ionic stress is managed by the capacity of tolerating high concentration of ions inside the cell, which is usually called tissue tolerance (Munns and Tester 2008).

The Orchidaceae is one of the largest plant families (Christenhusz and Byng 2016) and most (~70%) orchid species are epiphytes (Gravendeel et al. 2004). Studies investigating drought on orchids (e.g., Zotz and Tyree 1996; Stancato et al. 2001; Cui et al. 2004; De La Rosa-Manzano et al. 2014; Gantiva et al. 2020), indicate that water scarcity is the critical factor for their development in plant canopies (Zotz and Hietz 2001). Most orchids are drought-resistant and feature adaptations such as root velamen, pseudobulbs and highly succulent leaves (Zhang et al. 2018). Of all these traits, succulency is also commonly found on halophytes; a more succulent leaf provides tolerance to the ionic component of salinity by allowing the dissolution of a larger amount of Na^+ ions without increasing leaf Na^+ concentration (Ogburn and Edwards 2010). Therefore, one could expect succulent epiphytic plants to be pre-adapted to salinity, specially to the ionic component of salinity. The hypothesis that epiphytic-related traits could confer salt tolerance to orchids has never been tested, and studies about salinity in orchids are rare. One of the few exceptions is the study by Abdullakasim et al. (2018), who tested the physiological response of a cultivar of *Dendrobium* spp. to different salt concentrations in the substrate. Therefore, the question of whether orchids' tolerance to drought might also allow them to tolerate salinity at least partially remains to be tested.

Epidendrum L. is a Neotropical orchid genus, the largest within the Orchidaceae family in this biogeographical realm, with more than 1,800 species described (Karremans 2021). Most species of the genus are epiphytes, but some are terrestrial and/or lithophytes (Benzing and Atwood Jr 1984; Karremans 2021). Of special relevance to this work is the subgenus *Amphiglottium*, whose species inhabit disturbed sites like rock outcrops, lava flows, roadsides, and sand dunes (Pinheiro et al. 2009). One of the species of this subgenus is *Epidendrum fulgens* Brongn., a sympodial and terrestrial orchid that inhabits the south and southeastern regions of Brazil, growing in contrasting habitats such as coastal sandy plains and rocky outcrops (Pinheiro et al. 2011). Despite its terrestrial habit, *E. fulgens* phylogenetic position suggests that its recent ancestral was an epiphyte (Cardoso-Gustavson et al. 2018). Indeed, several traits related to drought resistance and commonly observed in epiphyte orchids, such as fast water uptake via root velamen and thick leaves (Hietz et al. 2022) are also present in *E. fulgens*. Therefore, *E. fulgens* is the ideal study system to test whether plants with epiphytic traits and of epiphytic ancestry are preadapted to salinity. Our hypothesis is that *E. fulgens* exhibits high tolerance to salinity, attributed to its highly succulent leaves, which is expected to function as a buffer against the ionic effect of salinity. If our hypothesis is validated, it would help explain how this genus, majorly composed of epiphytes, could successfully colonize the restingas. Here, individuals of this species were sprayed with salt solutions during a short-term experiment and some physiological parameters were measured.

2 Material and methods

To test the tolerance of *E. fulgens* to salt stress, we designed a salt spray stress experiment, selecting 10 potted specimens of *E. fulgens* from the living collection maintained by our research group. All of them were growing in the living collection common garden for at least 2 years. Each plant was composed of several sympodial stems, which are produced annually (Fig. 1). We assigned four stems from each plant to one of the four groups: the control group, which was sprayed with distilled water, or one of the three treatment groups, sprayed with varying concentrations of seawater solution. Therefore, we exposed different modules of the same individual to four conditions simultaneously. The four solutions were:

Fig. 1 Photograph of an *Epidendrum fulgens* individual used in the experiment, highlighting the different sympodial stems of a single plant

distilled water (control), 200 mM Na^+ (50% seawater concentration), 400 mM Na^+ (100% seawater concentration) and 800 mM Na^+ (200% seawater concentration) (Dickson and Goyet 1994). The osmotic potential and electrical conductivity of each solution was −0.24 MPa and 0.02 dS m^{-1}, −1.14 MPa and 14.1 dS m^{-1}, −2.53 MPa and 32.4 dS m^{-1}, and −4.43 MPa and 49.9 dS m^{-1} for distilled water (control), 200, 400 and 800 mM Na^+, respectively. The stems were sprayed every day for ten consecutive days with 10 mL of solution, which was enough to wet all its aerial surface. When one stem was being sprayed, the others were protected by plastic bags. The experiment was terminated after 10 days as the stems treated with 800 mM Na^+ started to show pronounced signals of senescence. The experiment was carried out in a greenhouse, so plants were sheltered from rainfall, and no watering was applied during the 10 days to avoid washing the salt from the leaf surface. Air temperature and relative humidity were monitored throughout the experiment with a THC-4 logger (INKBIRD, Shenzhen, China). The average air temperature was 18.5 °C ranging from 29.4 to 1.1 °C, while air relative humidity ranged from 22.3 to 89.5%.

To test to which degree the osmotic stress could disturb the photochemistry of *E. fulgens*, we selected for each stem the 4th or 3rd basipetal leaf—the same throughout the experiment—and measured the maximal (F_m) and basal (F_o) fluorescence signals in dark-adapted (30 min) leaves using a saturating light pulse (1 s, 8000 μmol photons m^{-2} s^{-1}, 655 nm) (Baker 2008). Using these signals, we further calculated the maximum quantum efficiency of PSII (F_v/F_m) as [F_m – F_o]/F_m. These measurements were taken on days 1, 2, 4, 7 and 10, always before the daily salt application, between 10:00 h and 14:00 h, using a PAM-2000 chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany).

At the end of the experiment, we measured the leaf chlorophyll index of the selected leaves using a chlorophyll meter (ClorofiLOG CFL 1030, Falker, Porto Alegre, RS, Brasil) and then harvested those leaves to make further measurements. Leaf osmotic potential was estimated using a Wescor HR-33T dew-point microvoltmeter and the thermocouple psychrometer mode of operation connected to C-52 sample chambers (Wescor Inc., Logan, UT, USA). The leaf discs (1 cm^2) placed inside the chambers were previously frozen in liquid nitrogen (Brown and Oosterhuis 1992). Also using 1 cm^2 leaf discs we estimated the leaf relative water content (RWC) as RWC = [(fresh mass – dry mass)/(turgid mass – dry mass)] × 100 (Barrs and Weatherley 1962). Finally, leaf Na^+ concentration was measured through the nitric-perchloric acid digestion method (Zasoski and Burau 1977).

To test the effect of salinity on photochemical activity, leaf water status, and leaf Na^+ concentration, we ran a one-way REML mixed-effect ANOVA—following the model below—using the R package 'lme4' (Bates et al. 2015):

$$Y_{nsg} = \mu + T_s + G_g + \epsilon_{nsg} \quad (1)$$

where Y_{nsg} is the parameter's value measured in the n stem of the g genet exposed to treatment s. μ is the grand mean. T_s is the fixed factor that estimates the effect of the salt treatment. The random factor G_g accounts for the effect of each genet. Finally, ϵ_{nsg} is the residual error term for each observation. We also ran a Tukey post hoc test to evaluate differences among salt spray treatments. All statistical tests were performed in the R software v. 3.6.2 (R Core Team 2022).

3 Results

At the end of the experiment, on day 10, the cumulative effect of salt spray on the photochemical activity was clearly negative and proportional to the salt concentration (Fig. 2a). The mixed-effects ANOVA corroborates the negative effect of salt in F_v/F_m ($\chi^2(3) = 14.1$, $p < 0.01$). Moreover, the mean F_v/F_m of control stems, sprayed with distilled water, did not differ from the F_v/F_m of stems sprayed with either 200 mM Na^+ (t-ratio = 0.5, p = 0.9) or 400 mM Na^+ (t-ratio = 2.0, p = 0.2). Only the 800 mM Na^+ treatment was significant on F_v/F_m as compared to the control (t-ratio = 4.1, $p < 0.01$).

The chlorophyll index decreased with increasing salt spray concentration ($\chi^2(3) = 20.6$, $p < 0.001$) (Fig. 2b). Compared with control stems, the salt-induced reduction of leaf chlorophyll index only appeared in the stems treated with 400 mM Na^+ (t-ratio = 2.8, $p < 0.05$) and 800 mM Na^+ (t-ratio = 4.3,

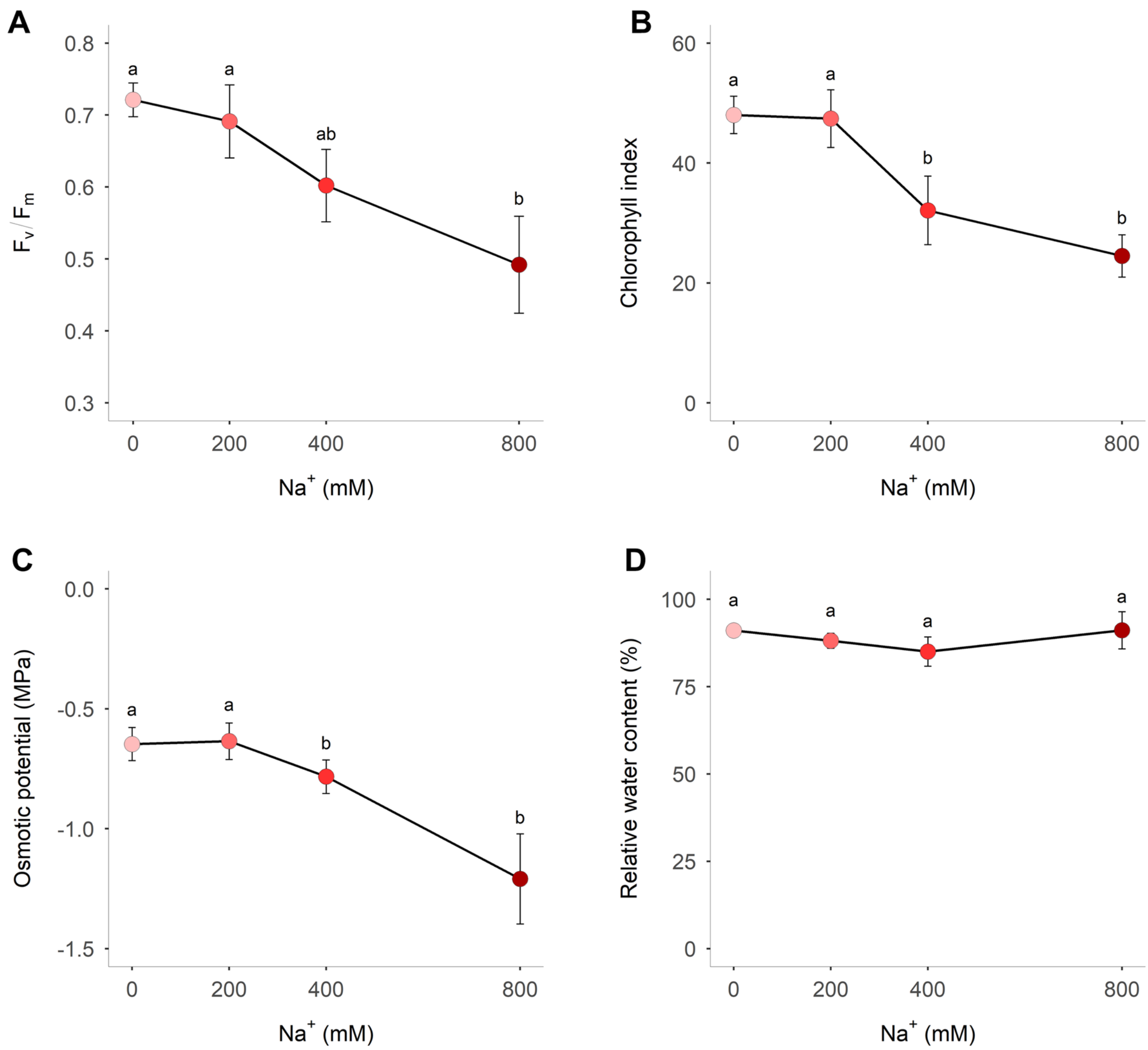

Fig. 2 The maximum quantum efficiency of PSII (F_v/F_m) (**a**), chlorophyll index (**b**), osmotic potential (**c**) and leaf relative water content (**d**) of *Epidendrum fulgens* after 10 days of salt spray. Each individual had one of four stems sprayed with distilled water (0 mM Na^+), 200 mM Na^+, 400 mM Na^+ or 800 mM Na^+ solutions. Darker red points are solutions with higher Na^+ concentrations. Each symbol is the mean value (n = 10) ± SE. Different letters indicate statistical differences among salt concentrations ($p < 0.05$)

$p < 0.01$) solutions. The salt treatment also caused a reduction in osmotic potential ($\chi^2(3) = 15.5$, $p < 0.01$), but the osmotic potential of treated stems differed from control ones only in the group sprayed with the 800 mM Na^+ solution (t-ratio = 3.5, $p < 0.01$) (Fig. 2c). The leaves of both control and treatment stems reached the end of the experiment without displaying signals of desiccation, as the RWC of control and salt-treated stems did not differ ($\chi^2(3) = 2.2$, $p = 0.5$) (Fig. 2d). The leaf Na^+ concentration was not affected by salt spray ($\chi^2(3) = 2.5$, $p = 0.4$), hence control and treatment plants exhibited similar Na^+ concentrations (Fig. 3).

4 Discussion

E. fulgens appears to employ an ability to avoid salt absorption by leaves, as leaf Na^+ concentration did not differ between salt treatments and control stems (Fig. 3). The ability of *E. fulgens* to maintain a stable Na^+ concentration throughout a wide range of salt conditions resembles that of *Sporobolus airoides* (Torr.) Torr. and *Muhlenbergia rigens* (Benth.) Hitchc., two grass species that are considered salt excluders and are adapted to live in saline areas (Hunter and Wu 2005). Such species of grass were exposed to approximately 60 applications of salt spray for 5 months in a salinity tolerance experiment.

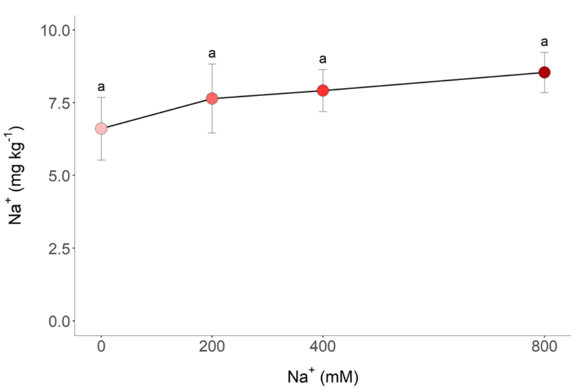

Fig. 3 The Na^+ concentration in the leaves of *Epidendrum fulgens* after 10 days of salt spray. Each individual had one of four stems sprayed with distilled water (0 mM Na^+), 200 mM Na^+, 400 mM Na^+ or 800 mM Na^+ solutions. Darker red points are solutions with higher Na^+ concentrations. Each symbol is the mean value ($n = 10$) ± SE. Different letters indicate statistical differences among salt concentrations ($p < 0.05$)

This is six-times more applications than we did here, but the concentration of the solution was much smaller—26 mM Na at most—than the ones used here. The final leaf Na^+ concentrations for those two species were between 4 and 9 g kg^{-1} (Hunter and Wu 2005), which is similar to the ones found here.

Stems of *E. fulgens* sprayed with the 800 mM Na^+ solution exhibited a significant decrease of leaf osmotic potential from −0.64 to −1.2 MPa, on average, when compared to control ones (Fig. 2c). This appears to have been successfully translated into maintenance of leaf water status since leaf RWC remained unchanged for 800 mM Na^+ (Fig. 2d), as well for all other treatments, when compared to control stems. Such osmotic adjustment allows the plant to cope with osmotic stress caused by salinity (Munns 2002) and can be mediated by the production of organic osmolytes such as proline and glycine betaine (Munns 2002) and accumulation of salt ions within their vacuoles. This reduces the cell osmotic potential whilst avoiding ion toxicity caused by high ion concentrations in the cytoplasm (Munns 2002; Munns and Tester 2008). However, this strategy is a double-edged one: while reducing osmotic potential with ions that come from the environment is cheaper energetically than producing organic osmolytes, they can only reduce the potential up to a certain point because their excessive accumulation will be toxic to the cell (Munns 2002). In our study, given that the leaf Na^+ concentration did not differ among treatments, accumulation of Na^+ ions does not seem to be an important strategy to adjust the leaf osmotic potential.

One of the first symptoms of salt-induced stress in *E. fulgens* appears to be a decrease in chlorophyll index as this was the only physiological parameter measured that responded to 400 mM Na^+ treatment, being reduced by 33% when compared to the control (Fig. 2b). Decreasing chlorophyll concentration is a natural step of leaf ontogeny, occurring during leaf senescence (Hörtensteiner 2006). However, it can also be induced by environmental stresses, and it is generally interpreted as a symptom of oxidative damage (Xie et al. 2019). It is not simple to determine which component of salt stress—ionic or osmotic—generated the oxidative damage that caused chlorophyll degradation since virtually all environmental stresses can eventually lead to oxidative stress (Xie et al. 2019). Since the main ion related to salt stress, Na^+ did not accumulate differently among treatments, ionic stress does not seem to be the cause of decreased chlorophyll index (Figs. 2b and 3). On the other hand, there was a decrease of osmotic potential in salt-treated stems (Fig. 2c). Therefore, it is more reasonable to conclude that osmotic stress rather than ionic stress has led to chlorophyll degradation.

Here we found no reduction of F_v/F_m in the milder treatment of 400 mM Na^+ despite the reduction of chlorophyll index. Similarly, Santos (2004) reported a decrease of leaf chlorophyll concentration and F_v/F_m of *Helianthus annuus* L. caused by root salinity, but with chlorophyll concentration reduction manifesting first, in the mildest treatment of 25 mM Na^+. One hypothesis that could explain this sequence of symptom manifestation is that chlorophyll degradation, despite its negative effects on photosynthesis, could be a strategy to reduce energetic pressure on PSII, avoiding decreases in F_v/F_m during mild stress. In fact, less chlorophyll means reduced light harvesting by leaves, which prevents excessive light capture and formation of reactive oxygen species (Munné-Bosch and Alegre 2000).

Epidendrum fulgens has a considerable tolerance to salt stress since most of its physiological parameters was significant even when sprayed with a 400 mM Na^+ solution, which is similar in concentration to the seawater (Dickson and Goyet 1994). One unexpected result was that all treatment groups had the same leaf Na^+ concentration as the control group (Fig. 3). The ability to avoid the uptake of salt ions and other solutes by leaves is considered to be negatively related to the leaf wettability (Ahmad and Wainwright 1976; Fernández et al. 2017). Therefore, this result could indicate a possible low wettability of *E. fulgens* leaves. However, considering *E. fulgens* epiphytic ancestry, this result becomes interesting because high wettability is considered to be adaptative in epiphytic environments (Gotsch et al. 2015), since it would allow a more efficient leaf water uptake (Schreel and Steppe 2020). These two perspectives are obviously conflicting and a fruitful avenue for future research would be to test if *E. fulgens* does have low leaf wettability and how much it differs from its epiphytic ancestors in this trait. Importantly, the maintenance of high RWC and high F_v/F_m under osmotic stress is an ability that resembles that of epiphytic orchids (De La Rosa-Manzano et al. 2014).

Na^+ recirculation via phloem (Wu 2018) could have impacted our results since the control and all three salt concentration treatments were represented in four stems of the same individual. This could have allowed a reduction in salt stress intensity on the stems treated with higher concentrations of salt by transporting Na^+ to other stems under lower salt stress intensity. This would explain, for example, why all treatment and control groups had similar Na^+ concentration and then mean that we overestimated the tolerance of *E. fulgens* to salt stress. However, electrical conductivity of 800 mM Na^+ solution was about 2500 times higher than one for control solution (49.9 vs. 0.02 dS m^{-1}) and it is unlike that such huge difference could be offset by internal redistribution. Even if phloem Na^+ recirculation was a major process controlling Na^+ tissue concentration in our experiment, *E. fulgens* would still be regarded as a highly salt-tolerant plant since most plants have their growth inhibited by Na^+ concentrations as low as 50 mM (Wu 2018).

There are very few studies addressing salt stress tolerance in orchids, which limits our ability to compare *E. fulgens* tolerance to other species of the same family. That said, one study shows that *Dendrobium* L. specimens, which are mostly epiphytic, are sensitive to irrigation with salt solution of electrical conductivity of 2 dS m^{-1} or higher (Abdullakasim et al. 2018). This is a much higher sensitivity than the one we found here for *E. fulgens*, which was able to withstand, with virtually no harmful effects, salt solutions with electrical conductivity of 14.1 dS m^{-1}. In conclusion, our study shows that *E. fulgens*, as terrestrial species with epiphytic traits, has a high tolerance to salt. We believe this is mostly due to its succulent leaves, which would confer high tissue tolerance. This suggests that epiphytic traits could promote salt tolerance. Although there are still few studies allowing a general conclusion, we suggest the Orchidaceae family is a good study system to answer whether plants with epiphytic-like traits are pre-adapted to salinity since it is a diverse family with many epiphytic species (Gravendeel et al. 2004).

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Declarations

Conflict of interest The authors declare there is no known conflict of interest regarding the publication of this article.

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