Cyanobacterial inoculation promotes enhanced clonal growth performance of the aquaticplant Salvinia auriculata.

Larissa Langsdorff Pimenta
Universidade Federal de Lavras
Grécia Andrade Souza
Universidade Federal de Lavras
Luis Carlos Pereira
Universidade Federal de Lavras
Marcelo Gomes Marçal Vieira Vaz
Biodiversita Tecnologia Microbiana
Guilherme Ramos Demetrio
Universidade Federal de Alagoas
Flávia Freitas Coelho
flaviafcoelho@ufla.br
Universidade Federal de Lavras

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Abstract

Clonal plants, like *Salvinia auriculata*, are widespread and perform important ecosystem functions, influencing the structure and composition of the ecosystems in which they occur. Some cyanobacteria perform biological nitrogen fixation (BNF) and can affect plant growth, as nitrogen (N) is a limiting nutrient. Therefore, we carried out a greenhouse experiment with the inoculation of the cyanobacterial strain *Desmonostoc* (UFLA 12) to investigate whether heterocystous cyanobacteria favour individual growth and drives reproductive strategies (sexual reproduction and/or clonal growth) of *S. auriculata*. *Salvinia auriculata* ramets were grown in plastic pots under the following treatments: (D) *Desmonostoc* (UFLA 12) inoculum and (Co) control, in which cyanobacteria were absent. The *Desmonostoc* presence positively influenced the clonal growth of *S. auriculata*, and increased shoots number, plants fresh biomass, and shoot size. We conclude that the inoculation of *Desmonostoc* (UFLA 12) contributes to a more vigorous spread of *S. auriculata* since it enhanced clonal growth. In this sense, the role of *Desmonostoc* (UFLA 12) as a potential biofertilizer may serve as a tool to assist in understanding the excessive growth of *S. auriculata* in aquatic environments.

Introduction

Aquatic macrophytes are widespread plants that inhabits water bodies in several different ecosystems (Alahuhta et al. 2021). Their distribution is linked to many factors that range from the landscape level, as altitude and water body area (Rolon and Maltchik 2006), to local factors as nutrient availability (Dar et al. 2014). Nutrient limitation seems to play a major role in most aquatic macrophyte species growth and development, with carbon, phosphorus and nitrogen being the most important ones (Bornette and Puijalon 2011). Nitrogen (N) is one of the most important nutrients for plant growth, required for the synthesis of essential cellular components such as nucleic acids (Kuypers et al. 2018). However, although it is widely available in the atmosphere in the form of dinitrogen (N_2), most living organisms cannot incorporate it into their metabolism because it is an inert gas (Hoffman et al. 2014). Biological nitrogen fixation (BNF), the conversion of N_2 into molecules that can be used by other organisms, such as ammonium ions and ammonia, is only performed by some prokaryotic microorganisms, including cyanobacteria (Mazhar et al. 2019). Cyanobacteria have the ability to form endophytic associations with various organisms, including plants (mosses, cycads, ferns), fungi (forming lichens), and algae (Adams 2000; Aguiar et al. 2008; Rai et al. 2002). In addition, several strains form epiphytic associations with floating aquatic plants, such as *S. auriculata* (Pimenta et al. 2022), and some even with submerged aquatic plants, such as *Stratiotes aloides*, showing allelopathic activity (Mohamed and Shehri 2010).Endophytic symbiotic associations of cyanobacteria are well studied as they contribute significantly to global BNF (Elbert et al. 2012; Kluge et al. 2002; Meeks 2005). *Trichormus azollae* (*Anabaena azollae*) is a heterocytous cyanobacterium that can reside in the leaf cavities of the aquatic fern *Azolla*, providing nitrogen for use as a biofertiliser in rice crops (Sergeeva et al. 2002; Ahmed et al. 2010; Kollah et al. 2016). In addition to nitrogen fixation, cyanobacteria secrete a variety of secondary metabolites, such as growth-promoting hormones, including auxins, gibberellic acid, and cytokinins

(Haroun and Hussein 2003; Shariatmadari et al. 2013), benefiting the cultivation of various crops such as wheat (Kholssi et al. 2021), tomato (Prasanna et al. 2013), corn (Prasanna et al. 2016a), chickpea (Prasanna et al. 2017), and cotton (Prasanna et al. 2016b).

The relationship between water nutrient content and aquatic macrophytes is also mediated by the plant's life form, and free floating macrophytes depend upon a-high levels of nutrients in the water column to present high physiological performances (Lacoul and Freedman 2006). In addition to different life forms, aquatic macrophytes can also present different reproductive systems. In fact, the ability of clonal reproduction that is present in most of aquatic macrophytes is cited as one of the factors that allow the ecological success of these plants group (Santamaria 2002). In this sense, aquatic clonal plants are widely distributed and play essential ecosystem roles as they are key species in nutrient cycling and present high primary productivity, influencing the structure and composition of aquatic ecosystems (De Kroon and Hutchings 1995; Zuo et al. 2023). They reproduce vegetatively, producing genetically identical individuals named ramets (Zuo et al. 2023), which are physiologically independent of the mother plant (Franklin et al. 2021; Demetrio and Coelho 2023), or by sexual reproduction (Harper 1977; Demetrio and Coelho 2023). In environments with high nutrient availability, clonal plants may produce ramets as an adaptive strategy to utilise all available resources and become dominant over other species (Zheng et al. 2019; Zhang et al. 2020). Therefore, changes in environmental conditions, such as fertilisation (Gonzalez et al. 2016) and nutrient limitation (Dong et al. 1997), can affect plant clonal growth (Geng and He 2021). In this sense *S. auriculata* is a good model to understand the relationship between plants and nitrogen-fixing cyanobatcteria, as it is a common freshwater fern that, under favourable conditions, rapidly colonises large water surfaces through clonal growth (Medeiros et al. 2017). In addition, its submerged leaflets holds a periphyton that encompasses different species cyanobacteria that can influence its growth by providing fixed nitrogen (N) (Pimenta et al. 2022).

Based on the premise that nitrogen-fixing cyanobacteria can influence plant growth (Ahmed et al. 2010; Diez and Ininbergs 2014; Rai et al. 2019), we hypothesized that the inoculation of *Desmonostoc* (UFLA 12) strain will enhance *S auriculata* individual performance and clonal growth, promoting an increase in both size and the number of new ramets.

Materials and Methods

Aquatic plant and their epiphytic cyanobacteria

Salvinia auriculata Aubl. (Salviniaceae) is a free-floating aquatic fern (Coelho et al. 2005b; Miranda and Schwartsburd 2019) that presents clonal reproduction by sprouting, in which each ramet can produce genetically identical ramets. It also presents sexual reproduction, in which spore-producing structures called sori are enclosed by a globular indusium, forming fertile fronds (De La Sota 1962; Miranda and Schwartsburd 2019). The ramets are connected by rhizomes that form colonies and consist of nodes, two aerial folioles responsible for photosynthesis, and submerged, finely divided folioles with a root function responsible for water and nutrient absorption (Room 1983; Sculthorpe 1967). We previously

observed and isolated the *Desmonostoc* strain (UFLA 12) from the submerged folioles of *S. auriculata*, (*see in* Pimenta et al. 2022). *Desmonostoc* (UFLA 12) strains have terminal and intercalary heterocysts, can perform BNF, and form long filaments surrounded by a diffusible mucilaginous sheath. *Desmonostoc* (UFLA 12) filaments are not densely coiled with compact trichomes as in *Nostoc*. In addition, akinetes can be differentiated in long chains as a form of resistance (Hrouzek et al. 2013).

Cyanobacterial inoculum

The cyanobacterial strain used in this study, *Desmonostoc* (UFLA 12) was previously isolated from the epiphyton of *S. auriculata* roots (Pimenta et al. 2022). The culture is deposited in the Collection of Cyanobacteria Cultures (CFC - UFLA), available at the Federal University of Lavras (UFLA). For cyanobacterial biomass production, the strain was inoculated by streaking on 100 Petri dishes (50 for each strain) containing solid BG-11_0 culture medium and placed on a bench to grow under a 12 h light/12 h dark photoperiod. After 60 days, the biomass was scraped from the plates and transferred to 2 litres of liquid BG-11_0 (1 litre for each strain) and homogenised using 20 mL syringes. To obtain an inoculum free of agar particles and dead cells, the pre-inoculum was centrifuged at 5000 rpm for 5 minutes (Thermo Scientific MULTIFUGE X1R centrifuge). After centrifugation, the supernatant was removed, and the pellet was resuspended in 5 L of liquid medium (2.5 L for each strain) to obtain the desired optical density, measured by light spectrophotometry at a wavelength of 680 nm. The cyanobacterial density of the inoculum was estimated using a spectrophotometer (SHIMADZU UV-1800), obtaining an optical density of 0.283. This inoculum was divided into 50 mL Falcon tubes containing 15 mL of inoculum (1% of the total volume of the trays) for each replicate of the greenhouse experiment. In total, there were 10 tubes containing 15 mL of BG-11_0 medium with *Desmonostoc* (UFLA 12), 10 tubes containing 7.5 mL of BG-11_0 medium with *Desmonostoc* (UFLA 12), and 10 tubes containing only 15 mL of BG-11_0 medium for the control treatment.

Greenhouse experiment

Salvinia auriculata ramets were collected in summer from a permanent lagoon in the south-eastern region of Brazil (21°08'56"S, 44°52'53"W). After collection, the plants were carefully washed with distilled water to remove solid particles, dead parts and any cyanobacteria and eukaryotic algae attached to the roots. Healthy and morphologically similar ramets were selected and weighed to ensure that each tray contained a colony of three ramets weighing between three and four grams. After this process, colonies were transferred to a greenhouse with a 30% shade condition, and were acclimatized during seven days, prior to the start of the experiment..

After the acclimatization phase, colonies containing three initial ramets, called mother plants, were placed in plastic trays containing 1.5 L of filtered tap water and subjected to two treatments: (D) – 15 mL of *Desmonostoc* (UFLA 12) inoculum, and (Co) - No cyanobacterial inoculum, only 15 mL of BG-11_0.

Each treatment had ten replicates, totalling 20 trays (N = 20). Water was added to the trays every seven days to maintain the 1.5 L level. At the end of the 28-day experiment, the number of new ramets (shoots) and sori was counted, and the length and width of the aerial and submerged folioles of both the mother plant and the shoots were measured using calipers. After the measurements, the plants were dried in an oven at 60°C for 48 hours and weighed. Individual growth (dry biomass and size of mother plant), clonal growth (number of shoots, dry biomass and size of shoots) and investment in sexual reproduction (number of sori) were evaluated. Clonal growth (mother plant plus + shoots) was calculated by subtracting the initial fresh weight from the final fresh weight and dividing by the total duration of the experiment (28 days), as shown in the equation below (Gufu et al. 2019).

$$Tx = \frac{PFF - PFI}{t}$$

Data analysis

All response variables (mother plant dry weight, mother plant aerial folioles length and width, mother plant submerged folioles length and width, number of shoots, shoots dry weight, shoots aerial folioles length and width, shoots submerged folioles length and width, sori number per colony, and growth rate) were checked for normality with Shapiro-Wilk tests. We built generalized linear models (GLM's) to test the effect of treatments (predictor variables) on *S. auriculata* mother plants and shoot traits. We built an individual model for each response variable that was performed with a gaussian distribution when data were normal and with quasipoisson distribution when data was not normal, to account for overdispersion (Crawley, 2012). All GLM's were performed in the R environment, using the *glm* function of the base package (R Core Team, 2023). After this, we used the function *rsquared* of the "piecewiseSEM" package (Lefcheck, 2016) to obtain the R^2 for the predictor variables (Treatment) in each model.

Results

Cyanobacteria did not promote the general individual growth of initial ramets (mother plants) of *S. auriculata* (Fig. 1A-E), but enhanced the growth rate and increased the foliole widthtable (Table 1). The treatments did not affect dry biomass of the mother plant (F = 1.791 p = 0.1975, Fig. 1B), aerial leaf length (F = 0.5066, p = 0.4857; Fig. 1D),, submerged leaf width (F = 1.2639, p = 0.2757, Fig. 1E), and submerged leaf lenght (F = 2.3186, p = 0.1452, Fig. 1E). In contrast to the individual growth of the mother plant of *S. auriculata*, there were differences in the clonal growth of *S. auriculata* (Fig. 1G). The number of shoots varied among treatments (Table 1), with the highest number of shoots observed in the presence of *Desmonostoc* (D treatment) compared to control treatment (Co treatment). Sexual reproduction, however, was not affected by the presence of *Desmonostoc* (F = 2.1687, p = 0.1581, Fig. 1G). On the other hand, the presence of *Desmonostoc* was highly important for the growth of *Salvinia auriculata* clonal offspring. The shoots that grew in the presence of *Desmonostoc* presented larger aerial and submerged leaves length and width (Table 1, Fig. 2A-E).

Discussion

The presence of *Desmonostoc* presented different effects on *Salvinia auriculata* that depended upon the level of plant organization that was sampled. *Salvinia auriculata* mother ramets were not affected *Desmonostoc* presence, while the shoots generated by these ramets were greatly affected in a positive way by the Cianobatceria presence. According to Gadgil and Bossert (1970), the life history of an organism can be seen as the result of three biological processes: survival, growth, and reproduction, which compete for resources. *Salvinia auriculata* can differentially allocate resources between these biological processes (Medeiros et al. 2016), and since we set up the experiment with already developed and grown ramets capable of clonal and sexual reproduction (De La Sota 1962; Miranda and Schwartsburd 2019), these ramets were likely not growing because they were allocating more resources in clonal growth. In addition, investment in sori production tends to occur in dry conditions and at high population densities, as observed by Coelho and colleagues (2005b). In habitats with high environmental variability, this strategy is crucial to the plant's persistence (Coelho et al., 2021). In our experiment, we did not induce drought and we did not observe excessive ramet growth, which explains the lack of differences in sori production between treatments.

Clonal growth, resulting in the rapid spread of ramets, is an important feature of the life history of aquatic plants, allowing them to efficiently occupy space (Seastedt 2009). The inoculation of the *Desmonostoc* (UFLA 12) strain contributed to the clonal growth of *S. auriculata* in all aspects, including numerical increase, biomass, and shoot size, as well as growth rate. Under favourable conditions, *S. auriculata* exhibits vigorous clonal growth (Julien et al. 2002). In terms of nutrients, nitrogen (N) and phosphorus (P) are the most limiting factors affecting the growth of aquatic plants (Duan et al. 2007; Smith 2014). In addition to their ability to fix nitrogen and release it into the environment, cyanobacteria are also partially capable of converting insoluble mineral phosphorus into soluble forms that can be used by other organisms. (Cameron and Julian 1988; Rai et al. 2019; Yandigeri et al. 2011). Therefore, we suggest that aquatic plants may benefit from the FBN that occurs in the periphytic community formed by cyanobacteria (Srivastava et al. 2017). Hempel and colleagues (2008) reported positive effects of periphyton on aquatic plants, such as the provision of organic compounds and carbon dioxide, making nutrient cycling more efficient. Therefore, we suggest that the FBN performed by *Desmonostoc* (UFLA 12) improves the nutritional conditions of the environment and favours the clonal growth of *S. auriculata*.

The strength of the plant-cyanobacteria interaction and the resulting effects depend on both the plant and the cyanobacteria and are often highly specific (Kollmen and Strieth 2020; Stewart et al. 1983). Initially, the most studied and used genera for fertilisation belonged only to the genera *Nostoc* and *Anabaena*. (Bergman and Rai 1993). However, other genera such as *Cylindrospermum*, *Synechococcus*, *Aulosira*, *Scytonema*, *Tolypothrix*, *Westiellopsis*, *Calothrix*, *Phormidium*, *Oscillatoria*, *Plectonema*, and *Gloeotrichia* have been used (Manjunath et al. 2001; Osman et al. 2010; Prasanna et al. 2016b; Singha 2009; Verma et al. 2016).

In this experiment, we used *Desmonostoc* (UFLA12), which is phylogenetically close to *Nostoc*. *Nostoc* is a genus with a wide geographical distribution and more than 250 described taxa; however, molecular analyses have shown that the genus is not homogeneous, and some new taxa belonging to *Desmonostoc* (Hrouzek et al. 2013) and *Mojavia* (Řeháková et al. 2007) are based on *Nostoc* species (Sant'Anna et al. 2020).

We suggest that the increased clonal growth of *S. auriculata* was greater in the treatment with *Desmonostoc* inoculation due to the specificity of the plant-cyanobacteria relationship (Kollmen and Strieth 2022; Stewart et al. 1983). The *Desmonostoc* (UFLA 12) strain used in the experiment was isolated from the roots of this aquatic plant. In addition, *Desmonostoc* produces a mucilaginous sheath that can facilitate the attachment of cyanobacteria to surfaces and nutrient deprivation, and store them (Droop 1968; Kollmen and Strieth 2022; Schmitt and Flemming 1999; Schooling and Beveridge 2006).

The *Desmonostoc* (UFLA 12) strain is nitrogen-fixing and therefore capable of performing FBN. It can produces both terminal and intercalary heterocytes (Hrouzek et al. 2013) what may explain the enhanced growth observed on the *S. auriculata* individuals submitted to the strain addition. Studies have shown that periphyton and aquatic plants compete for nutrients (O'Hare et al. 2018; Périllon and Hilt 2019; Romo et al. 2007; Xie et al. 2013). Thus, *S. auriculata* shoots may have invested available resources in root growth for nutrient uptake rather than in aerial folioles, as no different light conditions were imposed between treatments that could shape resource allocation for increased leaf area and photosynthetic rates (Medeiros et al. 2017). Biomass allocation to different parts of aquatic plants, such as roots and leaves, may occur due to limitation of available nutrients, as observed by Zhou and colleagues (2011).

Several authors have reported plant growth in the presence of cyanobacteria. Soil fertilisation by different cyanobacterial strains increased root length, height, and fresh and dry biomass of pumpkin, cucumber, and tomato plants (Shariatmadari et al. 2013; Hashtroudi et al. 2013). *Cylindrospermum muscicola* and *Anabaena oryzae* increased the growth of *Lupinus termis* (Haroun and Hussein, 2003), and *Nostoc entophytum* and *Oscillatoria angustissima* contributed to the growth of peas. It was also observed that the consortium of *Calotrix* sp., *Anabaena cylindrica*, and rhizobacteria promoted the growth of wheat in a hydroponic system (Kholssi et al. 2021). Due to the social and environmental problems caused by the overuse of agricultural inputs, combined with the high cost of fertilisers, the search for more sustainable alternatives, such as biofertilisers, has increased, and the potential for fertilisation by cyanobacteria has been explored (Sadvakasova et al. 2023).

We observed the fertilisation capacity of *Desmonostoc* (UFLA 12) in the growth of *S. auriculata*, contributing to its more vigorous clonal spread. The fact that *Desmonostoc* (UFLA 12) favoured the clonal growth of *S. auriculata* can be used as a tool to understand the excessive growth of these plants in aquatic environments, with the aim of biofertilising plants or managing them.

In conclusion, the *Desmonostoc* (UFLA 12) strain proved to be an excellent growth promoter for *S. auriculata*, a wild plant. It is therefore a promising strain for use in fertilisation.

Table 1

Table 1
Values of the statistical tests, for the mother plant and shoots

Ramet type	Model	Variation Source	Estimate	Std Error	t-value	p-value	R^2
Mother plant	Growth rate ~ Treatment	Intercept	0.083	0.013	6.037	**< 0.01**	0.44
		Desmonostoc presence	0.074	0.019	3.806	**< 0.01**	
	Mother plant aerial leaf width ~ Treatment	Intercept	2.766	0.072	37.966	**< 0.01**	0.25
		Desmonostoc presence	-0.258	0.103	-2.504	**< 0.05**	
Shoot	Shoot dry weight ~Treatment	Intercept	-3.649	0.245	-14.857	**< 0.01**	0.23
		Desmonostoc presence	0.785	0.296	2.648	**< 0.01**	
	Shoot aerial leaf width ~Treatment	Intercept	0.306	0.294	1.039	0.312	0.57
		Desmonostoc presence	1.072	0.341	3.145	**< 0.01**	
	Shoot aerial leaf length ~Treatment	Intercept	0.124	0.248	0.498	0.624	0.45
		Desmonostoc presence	0.911	0.294	3.09	**< 0.01**	
	Shoot submerse leaf width ~Treatment	Intercept	-1.435	0.298	-4.816	**< 0.01**	0.34
		Desmonostoc presence	1.029	0.347	2.963	**< 0.01**	
	Shoot submerse leaf length ~Treatment	Intercept	-0.1803	0.306	-0.589	0.562	0.45
		Desmonostoc presence	1.050	0.355	2.955	**< 0.01**	

Declarations

Competing Interests

The authors declare no competing interests.

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Author Contribution

LLPConceptualization, Methodology and Investigation (execution of the experiment) , Formalanalysis, Writing – original draft, ValidationGASMethodology and Investigation (Inoculum preparation and experiment maintenance)LCPMethodology and Investigation (Inoculum preparation and experiment maintenance)MGMVVMethodology (inoculum preparation) and Investigation (identification of Cronbergia sp.(UFLA 35) and Desmonostoc sp. (UFLA 12) strains.GRDConceptualization, Formal analysis, Writing – original draft, ValidationFFCConceptualization, Formal analysis, Writing – original draft, Validation

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Availability of data and material

The data will be made available for consultation, if required

Code availability

Not applicable

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Figures

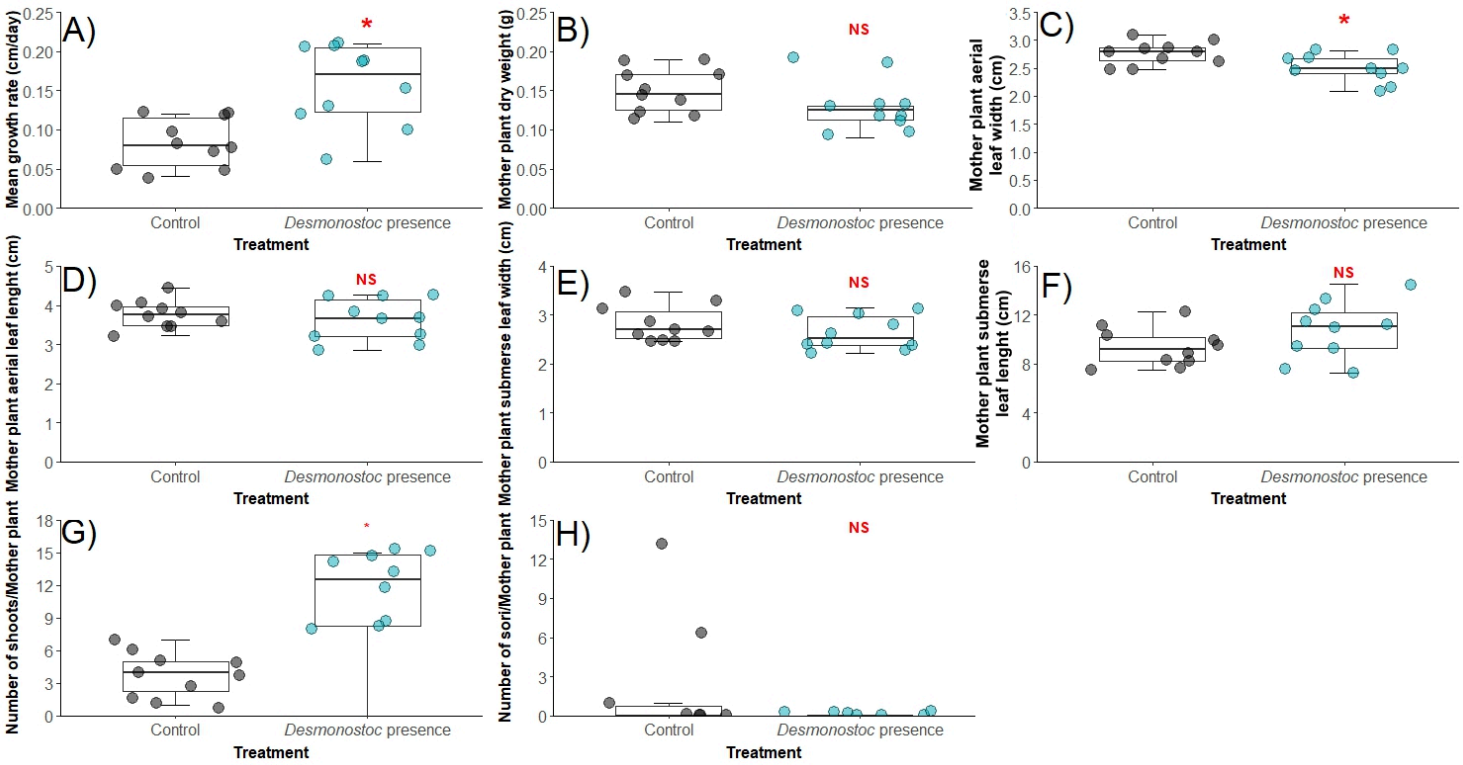

Figure 1

Individual growth of the mother plant of *S. auriculata* in each treatment. (A)Mean growth rate (cm/day); (B) Mother plant dry weight (g); (C) Mother plant aerial leaf width (cm); (D) Mother plant aerial leaf length (cm); (E) Mother plant submerse leaf width (cm); (F) Mother plant submerse leaf length (cm); (G) Number of shoots/Mother plant; (H) Number of sori/Mother plant

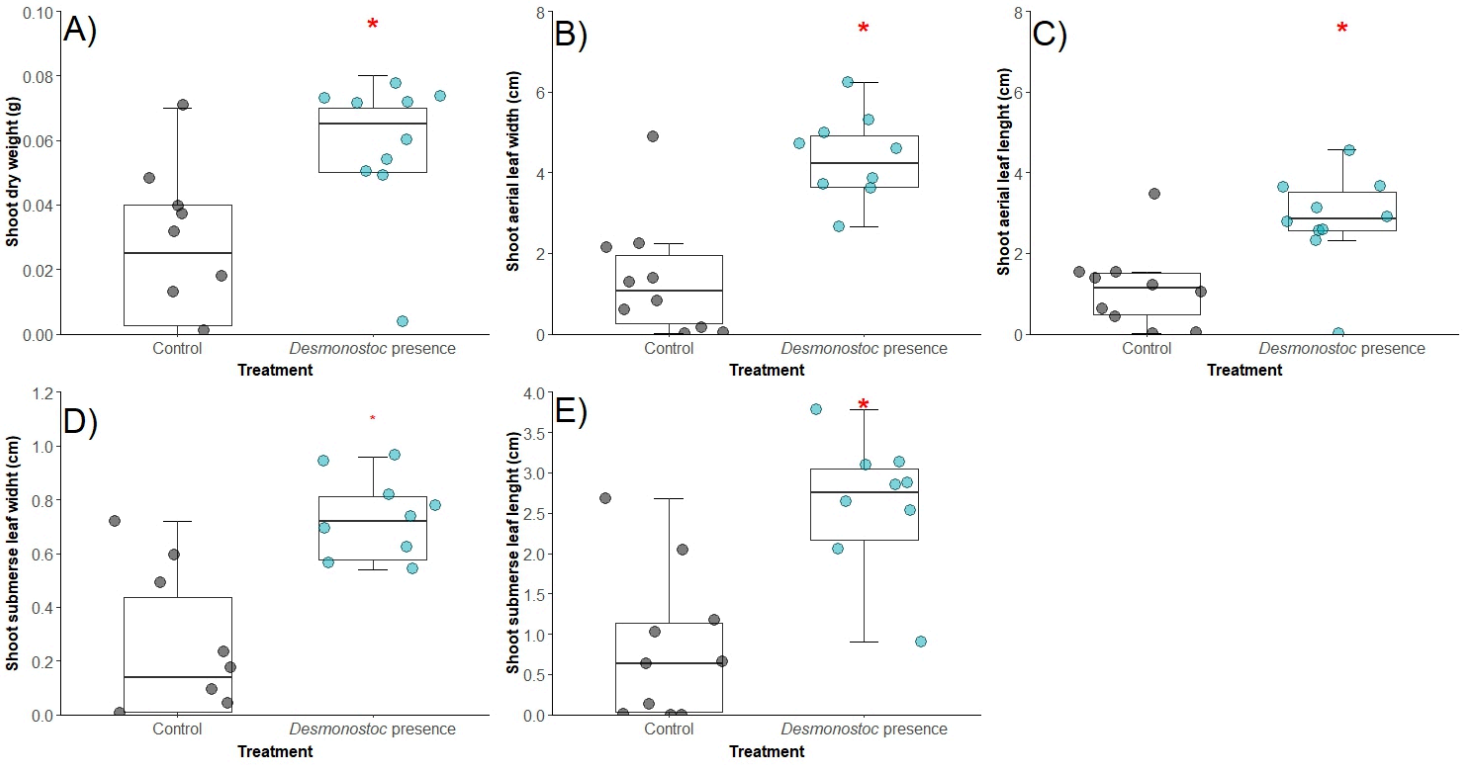

Figure 2

Clonal growth of *S. auriculata* in each treatment. (A) Shoot dry weight (g); (B) Shoot aerial leaf width (cm); (C) Shoot aerial leaf length (cm); (D) Shoot submerse leaf width (cm); (E) Shoot submerse leaf length (cm)