Cyanobacterial inoculation promotes growth of the aquatic plant Salvinia auriculata

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Abstract

Clonal plants, like cyanobacteria, are widespread and perform important ecosystem functions, influencing the structure and composition of the habitats in which they occur. Some cyanobacteria perform biological nitrogen fixation (BNF) and can affect plant growth as nitrogen (N) is a limiting nutrient. Therefore, to investigate whether heterocystous cyanobacteria favour individual growth and reproductive strategies (sexual reproduction and clonal growth) of *Salvinia auriculata*, we carried out a greenhouse experiment with the inoculation of two strains of cyanobacteria, *Desmonostoc* (UFLA 12) and *Cronbergia* (UFLA 35). *S. auriculata* ramets were grown in plastic pots with the following treatments: (D) *Desmonostoc* (UFLA 12) inoculum; (C) *Cronbergia* (UFLA 35) inoculum; (D + C) *Cronbergia* (UFLA 35) + *Desmonostoc* (UFLA 12) inoculum, and (Co) control, absence of cyanobacteria. Treatments (D) and (D + C) positively influenced the clonal growth of *S. auriculata*. *Desmonostoc* inoculation contributed to numerical increase in shoots, biomass gain, and shoot size. *Cronbergia* (UFLA 35) alone was not able to promote the growth of *S. auriculata*, only in consortium with *Desmonostoc* (UFLA 12). We conclude that the inoculation of *Desmonostoc* (UFLA 12) and *Cronbergia* (UFLA 35) favours the clonal growth of *S. auriculata* contributing to its more vigorous spread. The fact that *Desmonostoc* (UFLA 12) and *Cronbergia* (UFLA 35) favoured the clonal growth of *S. auriculata* may serve as a tool to assist in understanding the excessive growth of these plants in aquatic environments, for acting as a potential biofertiliser.

Introduction

Clonal plants are widely distributed and play essential ecosystem roles such as nutrient cycling and high primary productivity, influencing the structure and composition of aquatic and terrestrial ecosystems (De Kroon and Hutchings 1995; Zuo et al. 2023). They reproduce vegetatively, producing genetically identical individuals called 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). The aquatic plant *Salvinia auriculata* is a common freshwater fern that, under favourable conditions, rapidly colonises large water surfaces through clonal growth (Medeiros et al. 2017). In its submerged leaflets there is a periphyton of cyanobacteria that can influence its growth by providing fixed nitrogen (N).

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 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). 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).

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 hypothesised that the inoculation of *Desmonostoc* (UFLA 12) and *Cronbergia* (UFLA 35) strains, individually and in consortium, will enhance both individual and clonal growth, promoting an increase in both size and the number of new ramets of *S. auriculata*.

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 reproduces asexually by producing genetically identical ramets or sexually, where 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). In the submerged folioles of *S. auriculata*, the *Desmonostoc* strain (UFLA 12) was observed and subsequently isolated (*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).

The *Cronbergia* strain (UFLA 35) was observed and subsequently isolated from the aquatic macrophyte *Pistia stratiotes* (M. Biondi, UFLA, Brazil, unpublished research). *Cronbergia* is characterised by

unbranched, metameric trichomes, short with a single row of cells, with both ends of the filament equal, capable of differentiating akinetes (Genuário et al. 2018). Like *Desmonostoc* (UFLA 12), *Cronbergia* can also perform nitrogen fixation BNF, but can only produce terminal heterocysts (Genuário et al. 2018).

Cyanobacterial inoculum

The cyanobacterial strains used in this study, *Desmonostoc* (UFLA 12) and *Cronbergia* (UFLA 35), were previously isolated from the epiphyton of *S. auriculata* roots (Pimenta et al. 2022)d *stratiotes* (M. Biondi, UFLA, Brazil, unpublished research), respectively. Both cultures are deposited in the Collection of Cyanobacteria Cultures (CFC - UFLA), available at the Federal University of Lavras (UFLA). For cyanobacterial biomass production, the strains were 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 0.2 for *Cronbergia* (UFLA 35) and 0.283 for *Desmonostoc* (UFLA 12). 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 15 mL of BG-11_0 medium with *Cronbergia* (UFLA 35), 10 tubes containing 7.5 mL of BG-11_0 medium with *Desmonostoc* (UFLA 12) and 7.5 mL of BG-11_0 medium with *Cronbergia* (UFLA 35), 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 transferred to a greenhouse with 30% shade and washed to remove soil particles and dead parts. Prior to the start of the experiment, the plants were acclimatised for seven days with tap water and 30% shade. 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.

Colonies containing three initial ramets, called mother plants, were placed in plastic trays containing 1.5 L of filtered tap water and subjected to four treatments: T1- *Desmonostoc* (UFLA 12) inoculum (D); T2- *Cronbergia* (UFLA 35) inoculum (C); T3- *Desmonostoc* (UFLA 12) + *Cronbergia* (UFLA 35) inoculum (D + C); and T4- No cyanobacterial inoculum, only 15 mL of BG-11_0 (Co). Each treatment had ten replicates, giving a total of 40 trays (N = 40). Each tray received 15 mL of inoculum, previously separated in Falcon tubes. 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

A Kolmogorov-Smirnov test was performed to check the normality of the data. To assess the effect of treatments (predictor variables) on individual growth, clonal growth and investment in sexual reproduction, an ANOVA was performed on the following response variables: aerial folioles width of aerial folioles of the mother plant and shoots, submerged folioles width of submerged folioles of the mother plant and shoots, aerial folioles length of aerial folioles of the mother plant and shoots, and submerged folioles length of the mother plant. The Kruskal-Wallis test was performed for the following response variables: growth rate, dry biomass of the mother plant and shoots, dry biomass of the shoots, number of shoots and sori number, and length of submerged folioles of shoots, length of submerged folioles of shoots. In addition, pairwise differences were evaluated using Tukey's test for parametric data and Dunn's test for non-parametric data. Analyses were performed using IBM SPSS Statistics version 26.

Results

Cyanobacteria did not promote the individual growth of initial ramets (mother plants) of *S. auriculata* (Fig. 1). The treatments did not affect dry biomass of the mother plant (KW = 5.68; p = 0.128; Fig. 1a), aerial leaf length (F = 0.50; p = 0.684; Fig. 1b), aerial leaf width (F = 2.52; p = 0.074; Fig. 1c), submerged leaf length (F = 1.78; p = 0.168; Fig. 1d) and submerged leaf width (F = 0.524; p = 0.669; 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. 2). The number of shoots varied among treatments (KW = 18.191; p < 0.001), with the highest number of shoots observed in the (D) treatment compared to (Co) (Dunn = 20.3; p < 0.001) and (C) (Dunn = 15.3; p = 0.003). The (D + C) treatment produced more shoots than (Co) (Dunn = 13.9; p = 0.008; Fig. 2a) and (C) produced a lower number of shoots than (D) (Dunn = 15.3, p = 0.003). For the shoot dry biomass, there was a difference among treatments (KW = 8.743; p = 0.033), with the highest shoot biomass obtained in treatment (D) compared to (Co) (Dunn = 15.0; p = 0.004; Fig. 2b).

The treatments also affected the size of shoots produced by *S. auriculata*. The (D) treatment increased the length (F = 5.304; p = 0.002) and width (F = 5.274; p = 0.002) of aerial folioles and shoots compared to (Co). The (D + C) treatment increased width of aerial folioles compared to (Co) (Figs. 2c and 2d). The

length of submerged folioles and shoots also differed among treatments (KW = 10.856; p = 0.013; Fig. 2e), with the longest submerged folioles observed in the (D) treatment (Dunn = 14.85; p = 0.004), followed by (D + C) (11.4; p = 0.029) compared to (Co). The (D) treatment had longer submerged folioles than the (C) treatment (Dunn = 12.1; p = 0.020). Regarding the submerged folioles width, there was a difference between (D) and (Co) (F = 4.228; p = 0.012) and between (Co) and (D + C), with the (D + C) treatment being wider (F = 4.228; p = 0.039). The widest submerged folioles were observed in the (D) treatment, followed by (D + C) (Fig. 2f). There was a difference among treatments, when comparing the growth rate of all ramets of *S. auriculata* (mother plant + shoots) (KW = 8.229; p = 0.041; Fig. 2g), with the (D) treatment having a higher growth rate than (Co) (Dunn = 14.45; p = 0.006).

Sori production was not affected by cyanobacteria inoculation. Cyanobacterial inoculation did not affect sori production (KW = 6.82; p = 0.07; Fig. 3).

Discussion

Individual growth and sexual reproduction of *S. auriculata* were not affected by inoculation of (D), (C) and (D + C). According to Gadgil and Bossert (1970), the life history of an organism can be seen as the result of three biological processes: maintenance, growth, and reproduction, which compete for resources. Plants 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 or sexual reproduction (De La Sota 1962; Miranda and Schwartsburd 2019), these ramets were likely not growing because they were invested 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. In our experiment, we did not induce drought and we did not observe excessive ramet growth, which explains the lack of differences between treatments in sorus production.

Clonal growth, resulting in the rapid spread of ramets, is an important feature of the life history of aquatic plants, allowing them to occupy space efficiently (Seastedt 2009). The inoculation of *Desmonostoc* (UFLA 12) 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 genera phylogenetically close to *Nostoc* and *Cylindrospermum*. *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). The genus *Cronbergia* was derived and separated from *Cylindrospermum* and, as presented by Komárek et al. (2010), is a genus with few previously described species, such as *C. amazonensis*, isolated from the Solimões River in the Brazilian Amazon (Genuário et al, 2018), and the species *C. siamensis*, *C. paucicellularis* and *C. planctonica*, isolated from wet soils and rice fields in Thailand, periphyton in small lagoons in Slovakia and plankton in brackish water in Sweden, respectively (Komárek et al. 2010).

We suggest that the increased clonal growth of *S. auriculata* was greater in (D) 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, unlike *Cronbergia* (UFLA 35), which was isolated from *P. stratiotes*. 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).

Both strains are nitrogen-fixing and therefore capable of performing FBN. However, *Cronbergia* is limited in the number of heterocytes per filament, producing a maximum of two terminal heterocytes at the end of the filament (Genuário et al. 2018), while *Desmonostoc* (UFLA 12) can produce both terminal and intercalary heterocytes (Hrouzek et al. 2013). *Cronbergia* (UFLA 35) alone did not increase any of the measured morphological characteristics, including number of shoots, dry biomass and growth rate of *S. auriculata*. There was an increase with the addition of *Cronbergia* (UFLA 35) only in the consortium with *Desmonostoc* (UFLA 12). In the (D + C) consortium there was half the initial amount of each cyanobacterial strain, which may have resulted in lower nutrient availability. 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) and *Cronbergia* (UFLA 35) in the growth of *S. auriculata*, contributing to its more vigorous clonal spread. The fact that *Desmonostoc* (UFLA 12) and *Cronbergia* (UFLA 35) 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. Furthermore, *Cronbergia* (UFLA 35) alone is not able to promote the growth of *S. auriculata*, but only in consortium with *Desmonostoc* (UFLA 12).

Declarations

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Competing Interests

The authors declare no competing interests.

Availability of data and material

The data will be made available for consultation, if required

Code availability

Not applicable

Author Contributions

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Methodology (inoculum preparation) and Investigation (identification of *Cronbergia* sp. (UFLA 35) and *Desmonostoc* sp. (UFLA 12) strains.

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Figures

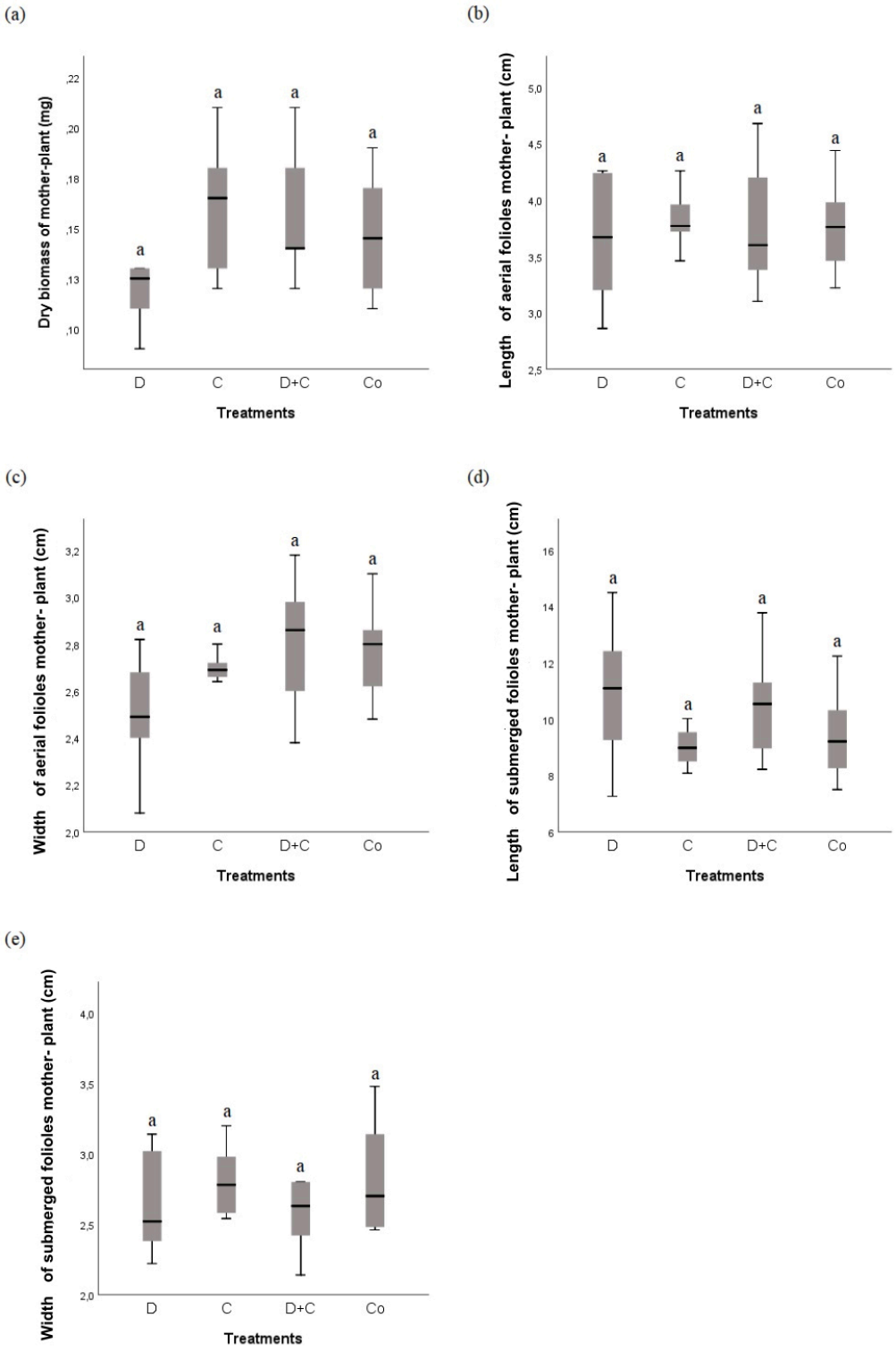

Figure 1

Individual growth of the mother plant of *S. auriculata* in each treatment. (a) Dry biomass (milligrams) of the mother plant of *S. auriculata* in each treatment (Min=0.09; Max=0.21; Median=0.14; Standard error=0.031). (b) Mean values of aerial folioles lengths (centimetres) (Min=2.0; Max=3.18; Median=2.68; Standard error=0.44). (c) Mean widths (centimetres) of aerial folioles (Min=2.86; Max=4.70; Mean=3.75; Standard error=0.25). (d) Means of lengths (centimetres) of submerged folioles (Min=2.14; Max=3.64;

Mean=2.74; Standard error=1.169). (e) Mean widths (centimetres) of submerged folioles (Min=7.26; Max=14.50; Mean=10.02; Standard error=0.36).

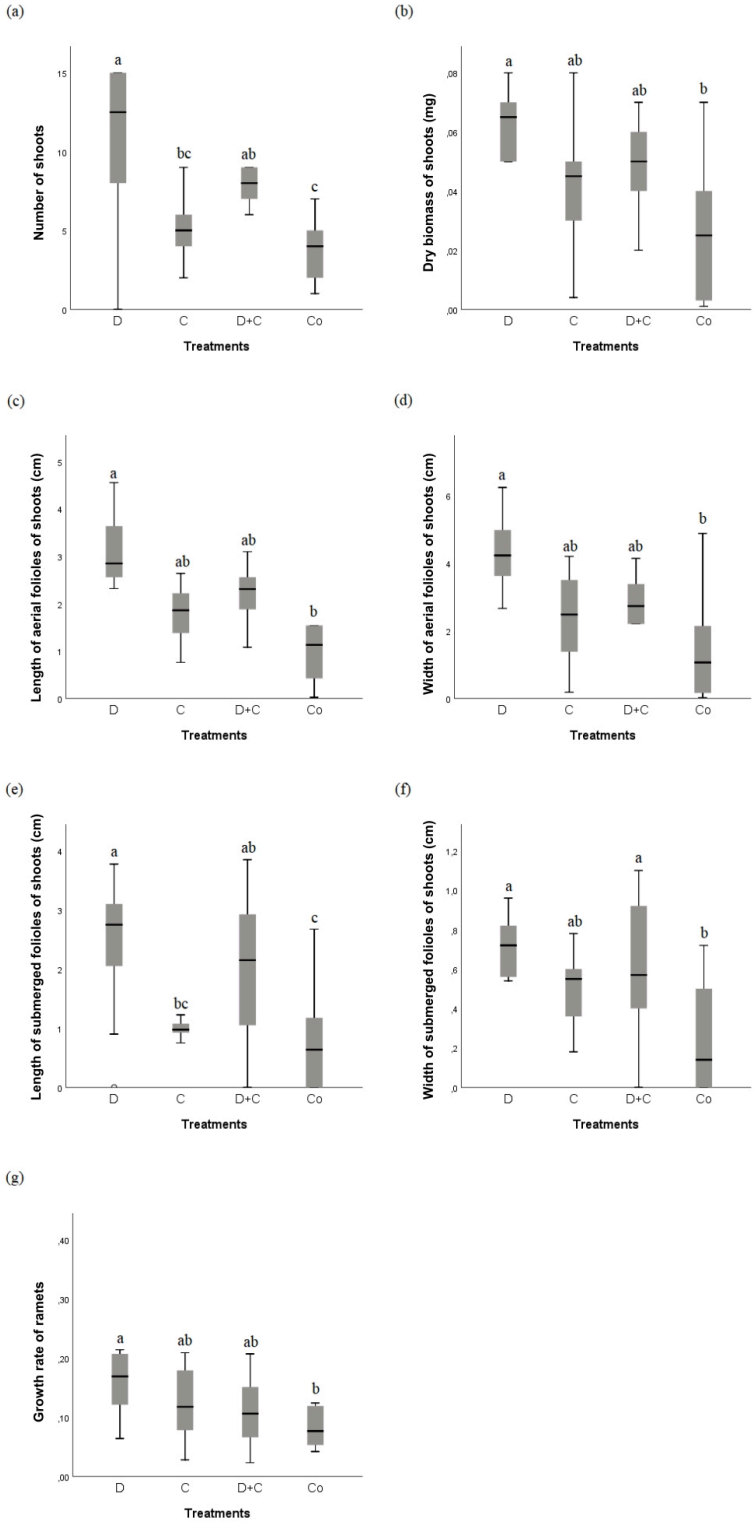

Figure 2

Clonal growth of *S. auriculata* in each treatment. (a) Number of shoots per treatment (Min=0; Max=15; Median=6.5; Standard error=4.00); (b) Dry biomass (milligrams) of shoots per treatment (Min=0.00; Max=0.08; Median=0.05; Standard error=0.02); (c) Mean values of lengths of aerial folioles of shoots

(Min=0; Max=4.56; Mean=1.93; Standard error=1.10). (d) Mean values of widths of aerial folioles of shoots (Min=0; Max=6.24; Mean=2.52; Standard error=1.71). (e) Mean values of lengths of submerged folioles of shoots (Min=0; Max=3.85; Median=1.12; Standard error=1.16). (f) Mean values of widths of submerged folioles of shoots (Min=0; Max=1.10; Mean=0.51; Standard error=0.32). (g) Growth rate of ramets (mother plant + shoots) of *S. auriculata*(Min=0.023; Max=0.335; Median=0.11; Standard error=0.06).

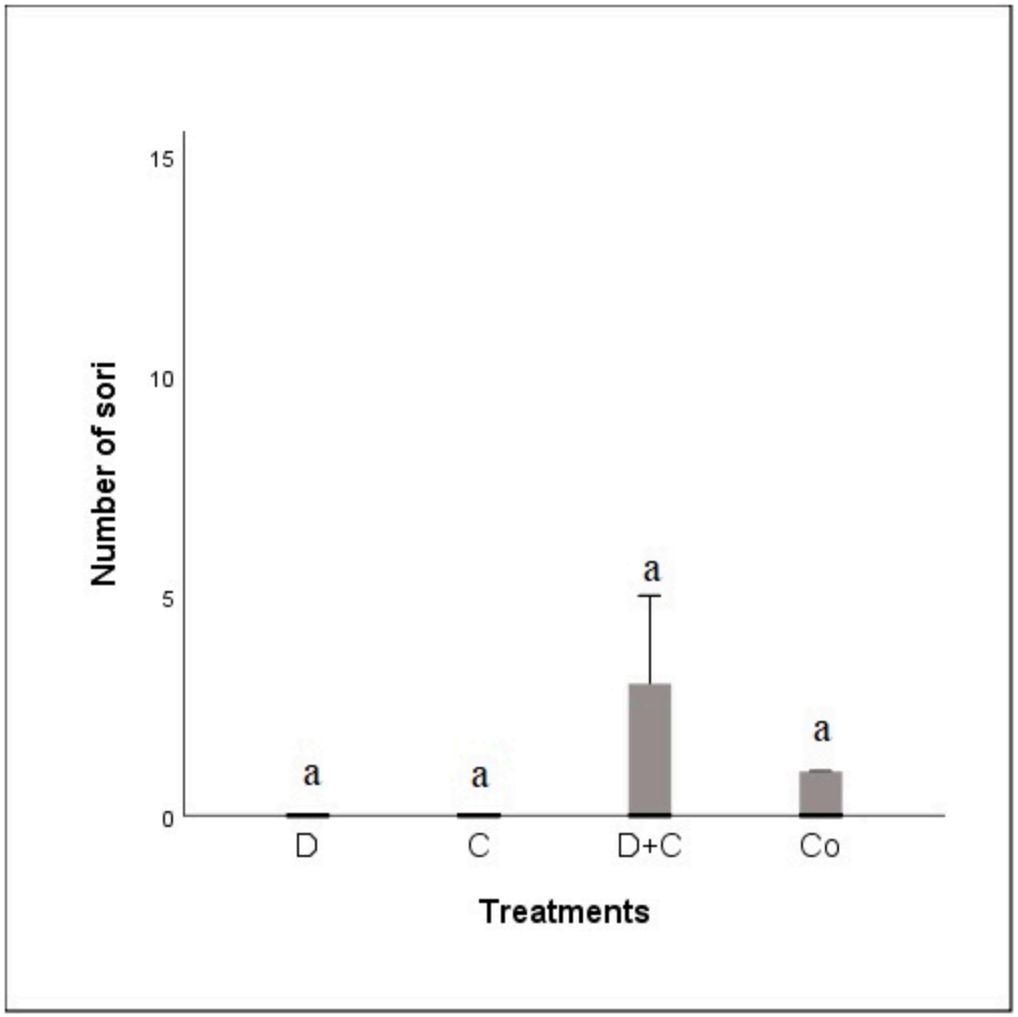

Figure 3

Number of sori produced per treatment on *S. auriculata*(Min=0; Max=13; Median=0; Standard error=2.97).