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METABOLITES EXTRACTED FROM *CAULERPA* SPP. INFLUENCE THE GROWTH OF *POSIDONIA OCEANICA* CUTTINGS: UNEXPECTED EFFECTS OF THEIR VEHICLE (DMSO)

METABOLITI ESTRATTI DA CAULERPA SPP. INFLUENZANO LA CRESCITA DI TALEE DI POSIDONIA OCEANICA*: EFFETTI IMPREVISTI DEL LORO VEICOLO (DMSO)*

Abstract - *The Mediterranean hosts a large number of allochthonous macrophytes which can replace the native species, threatening the native biodiversity and altering the ecosystem. Among the species of greatest interest are tropical algae belonging to the genus* Caulerpa *and, more specifically,* Caulerpa taxifolia *and* Caulerpa cylindracea*, both highly invasive. Algae of the genus* Caulerpa *also have the ability to compete with seagrasses, and it has been hypothesized that, at the basis of this competition, there may be allelopathic interactions mediated by bioactive substances. Among the secondary metabolites produced by algae of the genus* Caulerpa*, the most studied are the alkaloid caulerpine and the sesquiterpene caulerpenina. The aim of this research is to clarify the effects of these molecules on the growth, phenology and molecular responses* of Posidonia oceanica *cuttings acclimated under controlled conditions in mesocosm, creating the premises to clarify the metabolic pathways involved.*

Key-words: Posidonia oceanica*,* Caulerpa *metabolites, dimetil sulfoxide (DMSO)*

Introduction – Allochthonous species can replace indigenous species when introduced in an ecosystem, altering the biodiversity of native organisms. Species of greatest concern, in marine coast environment, are the green macroalgae *Caulerpa taxifolia* (M.Vahl) C. Agardh and *Caulerpa cylindracea* Sonder, tropical weeds invasive of the Mediterranean Sea, Australian and Californian seas. Few scientific data are on the role of these alien algae in altering the biodiversity of Mediterranean macrophytes and its invasive potential has not been well understood (Marbà *et al*., 2005). The *Caulerpa* genus propagates clonally showing high invasive behavior when outside their native ranges. In particular, it has been observed that the dead matte of the seagrass *Posidonia oceanica* (Linnaeus) Delile, endemic to the Mediterranean, is more sensitive than healthy meadows to the colonization by these algae (Katsanevakis *et al.,* 2010). It has been hypothesized that their invasive and ruinous potential is promoted by the secondary metabolites such as caulerpine, caulerpenin, caulerpicin and other terpenoids that algae used as the repellents to herbivores; evidences are reported on the regression of endemic communities mediated by these molecules (Katsanevakis *et al.*, 2010). The project aims, therefore, to assess the effects of allochthonous molecules purified from *C. cylindracea* and *C. taxifolia* on growth, plant phenology and molecular responses of the seagrass *Posidonia oceanica* under controlled environment in mesocosms.

Materials and methods – *Mesocosm cultivation*. Cuttings of *P. oceanica*, each composed by a plagiotropic rhizome with four shoots and roots, were sampled by scientific divers from University of Calabria (Italy) in several sites along the Tyrrhenian

coast of Calabria, where the massive presence of *C. cylindracea* had not been reported. This was to ensure that the plants had not already come into massive contact with the algae. Cuttings were prepared as described in Innocenti *et al.* (2007); the wounded area of each plagiotropic rhizome was protected with a silicone
cup filled with 2 ml of sterile seawater (Fig. 1). Then, coltures were stabilized in mesocosm at 21°C, pH 8.1 and 37 psu salinity for 7 days with the 98% of cuttings with growing leaves.

Fig. 1 - a) Wound in the rhizome of Posidonia oceanica protected by the cup with sterile sea water; b) Cuttings in a mesocosm.
a) Ferita del rizoma di Posidonia oceanica protetta dal cup con acqua di mare sterile; b) Talee in mesocosmo.

Chlorophyll extraction and measurement. 0.1g of leaves were crushed in liquid N_2; tissue powder was suspended in 80% cold acetone and incubated at 4 °C for three h and then centrifuged at 800 g for 15 min. Absorbance of 1 ml of crude supernatant was measured at 663 nm and 645 nm (7310 Jenway spectrophotometer). The concentrations of chlorophylls *a* and *b* and of the total chlorophyll were calculated according to Genot *et al.* (1994).
Caulerpa metabolites extraction and treatment. Talli of *C. prolifera* (Forsskål) J.V. Lamouroux and *C. cylindracea* were sampled at sea. Caulerpenin (CYN) from *C. prolifera* and of caulerpin (CAU) from *C. cylindracea*, were extracted according to the protocols of Carbone *et al. (2008).* The ^{1}H NMR spectroscopic analysis of the final fractions confirmed the identities and purities of the extracted metabolites with a yield of 100 mg CYN and 70 mg CAU. The purified metabolites were used in mesocom tests; as CAU and CYN are hydrophobic they were initially solubilized in 1% dimethyl sulfoxide (DMSO) and diluted at final concentration of 25 μM in sea water; 2 ml of these solutions were filled in the silicone cups and insert at the wounded area of the rhizome of each cutting; control cuttings were treated with seawater only or with 1% DMSO. For each treatment, 3 rhizomes (genets) were used, each with 3 shoots (ramets). Each treatment, both the distance in (cm) between the base of the leaves and a hole (done at the base of each leaf at time T0) was measured as leaf growth, and the leaf chlorophyll content at the end of the test (28 days) (Buia *et al.*, 2003).

Protein extraction and mass spectrometry analysis. Each shoot was cleaned of epiphytes, quickly washed in sterile distilled water, frozen in liquid N_2 and then stored at -80°C. For protein extraction leaf tissues were processed according to Piro *et al.* (2022), while protein electrophoresis and mass spectrometry analyses were performed according to Oliva *et al.* (2023).

Results – Treatment with CAU and CYN caused reduced leaf elongation, which was very dramatic in CYN-treated *P. oceanica* cuttings (Fig. 2a). Also cuttings treated with 1% DMSO showed a significant reduction in leaf elongation respect to the control in seawater. Biochemical data showed an increase in chlorophyll content in all treated cuttings compared to control cuttings; the increase was statistically significant in leaves treated with CAU and CYN (Fig. 2b).

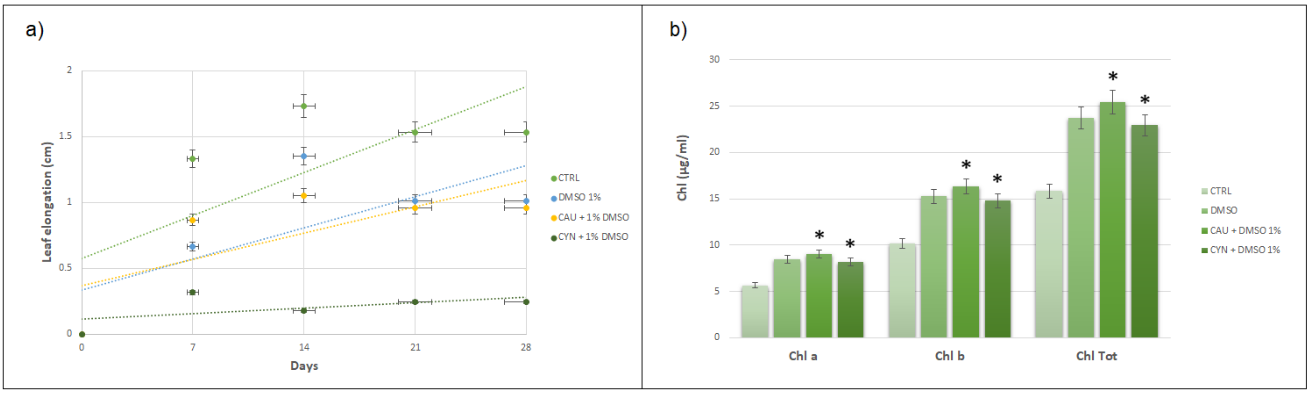

Fig. 2 - a) Leaf growth pattern of the treated cuttings. Values are the mean of three samples (±SD), $p<0.05$ for each measure; b) Chl a, Chl b, Chl tot. content in leaves after 28 days of treatment. Values are the man of three samples (±SD) *$p<0.05$

*a) Pattern di crescita fogliare delle talee trattate. I valori sono la media di tre campioni (±SD) p<0.05 per tutti i valori; b) Contenuto di Chl a, Chl b, Chl tot. in foglie dopo 28 giorni di trattamento. *p<0.05*

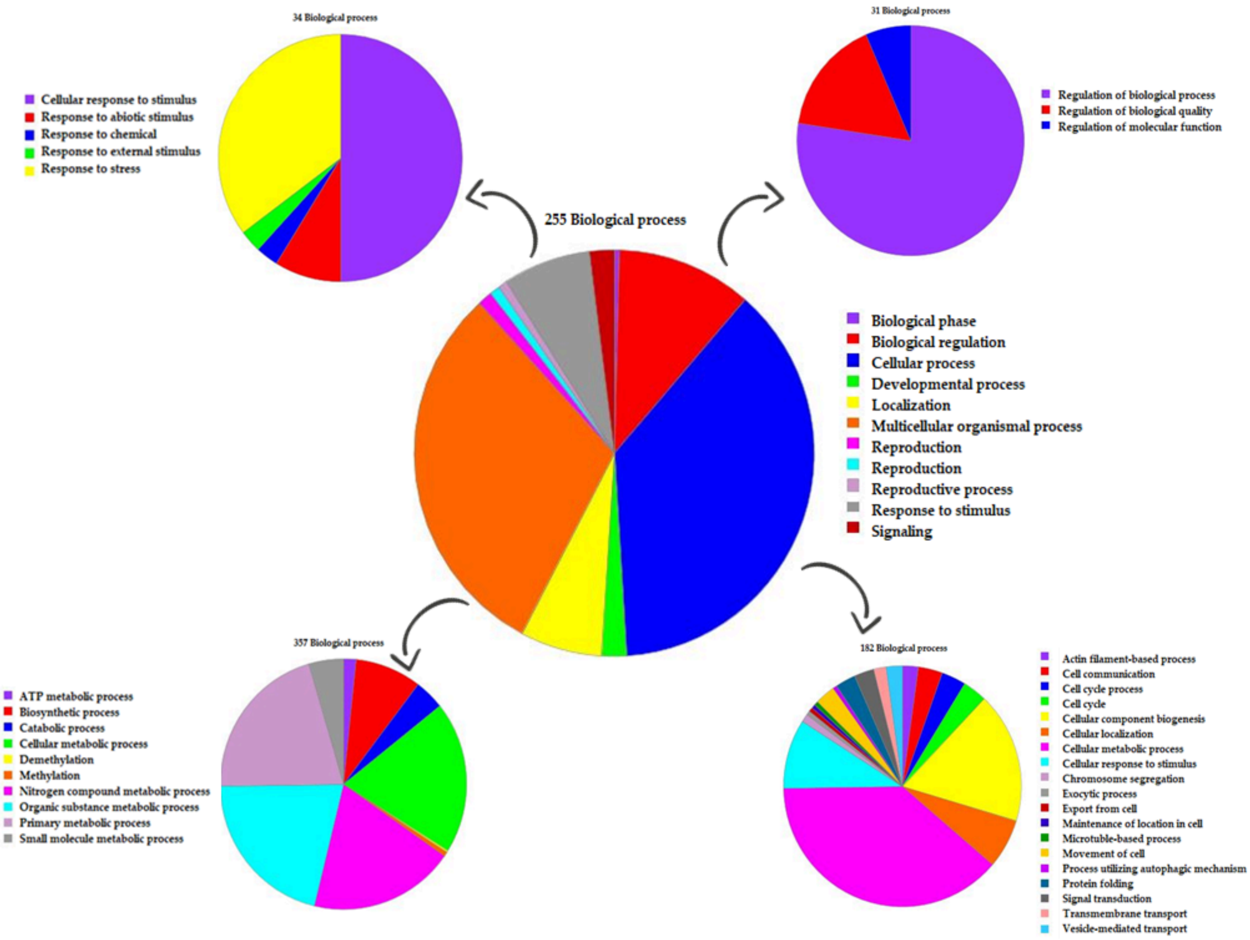

Fig. 3 – Main biological processes that were affected by the 1% DMSO treatment.
Principali processi delle proteine influenzate dal trattamento con 1% DMSO.

Analysis of differentially expressed proteins and of metabolic processes whose that proteins belong are showed in the Fig. 3. Treatment with 1% DMSO induced the up-regulation of most of the proteins in leaf tissues. Cellular metabolic process, Biogenesis and cell renewal, ATP metabolism, macromolecules metabolism, nitrogen metabolism, response to stimulus were the main affected by the DMSO treatment.

Conclusions - The systemic treatment with caulerpine and caulerpenine negatively affected the growth of leaves of *P. oceanica*, with more relevant effects in the treatment with caulerpenin; our results suggested that, in seagrasses, metabolites produced by species of the Caulerpa genus could be released and accumulated in sediments where they can come into contact with plant organs and tissues, exerting their allopathic effects. In contrast to growth inhibition, culerpine and caulerpenine induced higher chlorophyll content in leaves than control in seawater. This could be due either to an increase in chlorophyll biosynthesis, or to its accumulation. The unexpected effect of treatment with DMSO alone suggests its possible synergistic role with respect to those exerted by caulerpenine and caulerpine in inhibiting leaf growth. In marine environment, *P. oceanica* has been demonstrated to be one of the largest producer of DMSO together with its precursor, dimethyl sulfoniopropionate (DMSP) (Richir *et al.*, 2021); currently the role in plants of these molecules is still unknown. Our molecular findings suggest that DMSO strongly affects leaf cell metabolism by inducing cell biogenesis and macromolecule turnover that require considerable energy consumption. Future molecular studies will be aimed at understanding these mechanisms in detail and identifying which ones are due to treatment with *Caulerpa* metabolites.

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