Leaf proteome modulation and cytological features of seagrass *Cymodocea nodosa* in response to long-term high CO$_2$ exposure in volcanic vents

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Seagrass *Cymodocea nodosa* was sampled off the Vulcano island, in the vicinity of a submarine volcanic vent. Leaf samples were collected from plants growing in a naturally acidified site, influenced by the long-term exposure to high CO$_2$ emissions, and compared with others collected in a nearby meadow living at normal pCO$_2$ conditions. The differential accumulated proteins in leaves growing in the two contrasting pCO$_2$ environments was investigated. Acidified leaf tissues had less total protein content and the semi-quantitative proteomic comparison revealed a strong general depletion of proteins belonging to the carbon metabolism and protein metabolism. A very large accumulation of proteins related to the cell respiration and to light harvesting process was found in acidified leaves in comparison with those growing in the normal pCO$_2$ site. The metabolic pathways linked to cytoskeleton turnover also seemed affected by the acidified condition, since a strong reduction in the concentration of cytoskeleton structural proteins was found in comparison with the normal pCO$_2$ leaves. Results coming from the comparative proteomics were validated by the histological and cytological measurements, suggesting that the long lasting exposure and acclimation of *C. nodosa* to the vents involved phenotypic adjustments that can offer physiological and structural tools to survive the suboptimal conditions at the vents vicinity.

The Mediterranean submarine volcanic vents are natural sources of CO$_2$ since this gas is the main component of the volcanic emissions that have been happening for hundreds of years, forming unique and extraordinary environments in which the relative abundance of dissolved inorganic carbon (Ci) species is altered by an increase in the partial pressure of CO$_2$ (pCO$_2$), with a consequent drastic reduction in seawater pH$^{1,2}$. In these special acidic environments, marine ecosystems suffer from a drastic remodeling; while the pioneering studies on ocean acidification focused on how it negatively affects some species while favoring others$^{3}$, more recent evidence exists on the large ecological effects on herbivores, invertebrates$^{4-7}$ and on intra-community processes within seagrass meadows exposed to acidic conditions$^{8,9}$. Seagrasses have been reported to be Ci-limited in the marine realm$^{10-12}$, using CO$_2$ and bicarbonate (HCO$_3^-$) as external Ci sources for photosynthesis$^{10}$. Recent studies on ocean acidification have also aimed at resolving the question of whether seagrasses can fix an increasing amount of inorganic carbon (Ci) in the future, thus providing a way forward to their survival while alleviating the effects a more acidic seawater in their associated ecosystems$^{12}$ and references therein.$^{13-16}$.

Volcanic vents create, in the present, the necessary acidified conditions to evaluate the long-lasting effect of high pCO$_2$ exposure on acclimated populations of marine plants, which is a mandatory requirement to understand the plant’s real and sustained behavior$^{17}$. Studies conducted in naturally acidified conditions at several
volcanic sites have provided contrasting results, often suggesting species-specific responses to increased $p$CO$_2$. An ecological assessment of *Cymodocea nodosa* at a shallow acidified site at Volcano Island (Italy) revealed that the meadow is negatively affected by the environmental conditions at the low pH site, as the plant's density and biomass decreased; authors also reported a decrease in leaf area in plants acclimated to the CO$_2$ vents. This latter finding, along with similar studies, strongly suggests that the acclimation of seagrasses to the long-lasting high $p$CO$_2$ concentration encompasses several physiological and morphological adjustments. It is relevant to note that some biomechanical responses of *C. nodosa* were altered in the course of a CO$_2$ enrichment experiment and that changes in plant anatomy and cell ultrastructure have been reported for *Halodule wrightii* under ocean acidification conditions. These observations are in line to those previously observed in terrestrial plants, in which the exposure to high $p$CO$_2$ induced several anatomical alterations.

On the molecular side, a wider investigation on the gene expression profile, performed in the same population of *Cymodocea nodosa* in Vulcano island, confirmed the decrease in productivity in plants growing at the high CO$_2$ site. Contrastingly, the same study reported that productivity significantly increased with Ci availability in plants incubated with artificially CO$_2$-enriched water at a non-acidified control site, supporting the hypothesis that *C. nodosa* might in general benefit from a higher Ci availability. Taken all together, these results support the suggestion that volcanic vents may not be ideal analogues for ocean acidification studies and that the observed effects on seagrasses are not merely due to the increased CO$_2$ availability but are also influenced by other environmental factors present at these sites.

In this controversial scenario, our study aims to elucidate how a well-established natural population of *Cymodocea nodosa*, exposed to the CO$_2$ vents environment at Vulcano Island, modulates its protein metabolism and what specific modifications take place, both at the morphological and functional traits levels, associated to the long-term adaptation process.

Comparative proteomics has been previously applied to seagrasses, revealing the protein molecular dynamics for surviving under various conditions. Since the amounts of protein and transcripts corresponding to the same gene are generally loosely correlated, the advantage offered by proteomics in the present study is to reveal changes in protein accumulation induced by high CO$_2$ that cannot have been predicted from the previous transcriptomics investigation, thus contributing to elucidate the effects of a long-term exposure to naturally increased $p$CO$_2$.

**Results**

**Protein yield, proteins identification and differential accumulated proteins in leaf tissues.** A decrease of 30% in protein yield in leaf tissues of plants growing in high $p$CO$_2$ comparing to the normal $p$CO$_2$ condition was found (See the Supplementary Table 1). The SDS-PAGEs of leaf proteins provided well-resolved lanes both in normal and high $p$CO$_2$ samples. Each lane consists of about 80 different polypeptides bands, demonstrating the efficiency of the protein extraction and purification by means of the multistep protocol optimized for *C. nodosa*. Spite the same amount of leaf proteins loaded on each well, the band at 55 kDa, corresponding to the large subunit of RuBisCo, decreased in all replicates of plants living in high $p$CO$_2$ with respect to those under normal $p$CO$_2$ condition (Fig. 1). Measures from the digitalized images of the gels by the Quantity One 1-D Analysis Software (Bio-Rad Laboratories; Berkley, California) gave a mean decrease of up to 40% in the optical density of the 55 kDa band (data not shown) in the high $p$CO$_2$ samples.
Figure 2 depicts the multivariate classification (PLS-DA) of all mass spectra results from normal $p_{\text{CO}_2}$ and high $p_{\text{CO}_2}$ plants. Spectra patterns of normal (blue dots) and high (red dots) $p_{\text{CO}_2}$ plants are quite distinct. Plants collected in high $p_{\text{CO}_2}$ showed a higher degree of homogeneity in comparison to samples collected in normal $p_{\text{CO}_2}$.

The mass spectra analysis coupled with database search has identified 190 proteins in all samples (Supplementary Tables 2 and 3). The hierarchical clustering of all identified proteins is shown in Fig. 3. Under the screening criteria of a fold change greater than 2 or less than 0.60 and $p$ value < 0.05, a total of 75 proteins were identified to be differentially abundant (DAPs) by comparison between normal $p_{\text{CO}_2}$ and high $p_{\text{CO}_2}$ plants; 45 proteins resulted accumulated, while the remaining 30 proteins are depleted. All these proteins were regarded as candidate proteins associated with the high $p_{\text{CO}_2}$ adaptation and acclimation processes (Table 1).

The pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/pathway.html, accessed on 11 September 2019) identified 6 pathways ($p$ < 0.05) related to photosynthesis with enriched relative abundance, as shown in Fig. 4. Proteins involved in the light reactions of photosynthesis are the most relevantly enriched under the acidified conditions. These include the Photosystem II CP43 chlorophyll apoprotein, Photosystem II D2 protein, Photosystem II CP47 chlorophyll apoprotein, Photosystem Q(B) protein, Photosystem I P700 chlorophyll a apoprotein A2, ATP synthase subunit beta, chloroplastic, Cytochrome b6, ATP synthase subunit alpha and the chloroplastic Photosystem I P700 chlorophyll a apoprotein A1 (Table 1). In contrast, the depleted metabolic pathways were those related to carbon fixation, carbon metabolism, glycolysis/glucogenesis. The Ribulose bisphosphate carboxylase large chain and the Ribulose bisphosphate carboxylase/oxygenase activase appeared strongly depleted in acidified conditions, as also cytoplasmic and chloroplastic Malate dehydrogenase and Transketolase. The key enzymes of glycolysis
| Swiss-Prot ID | KEGG orthology (proteins) | Protein | Log FC ([high CO2] versus [normal CO2]) Normalized | FC (abs) ([high CO2] versus [normal CO2]) Normalized | DAPs ([high CO2] versus [normal CO2]) Normalized | Molecular Mass (Da) | KEGG orthology (metabolisms) | Metabolism |
|--------------|---------------------------|---------|---------------------------------------------|---------------------------------------------|---------------------------------------------|------------------|-------------------------------|------------|
| P0C365       | K02705                    | Photosystem II CP43 chlorophyll apoprotein | 13.134                                       | 8993.973                                    | STA 52,246.3                                        | ko0195 | Photosynthesis |
| P0C435       | K02706                    | Photosystem II D2 protein | 12.430                                       | 5520.685                                    | STA 39,801.0                                        | ko0195 | Photosynthesis |
| P05641       | K02704                    | Photosystem II CP47 chlorophyll apoprotein | 10.513                                       | 1461.336                                    | STA 56,276.2                                        | ko0195 | Photosynthesis |
| P0C432       | K02703                    | Photosystem Q(B) protein | 8.324                                        | 320.653                                     | STA 39,076.0                                        | ko0195 | Photosynthesis |
| P05642       | K02635                    | Cytochrome b6 | 7.938                                        | 245.255                                     | STA 24,310.3                                        | ko0195 | Photosynthesis |
| P19023       | K02133                    | ATP synthase subunit beta, mitochondrial | 7.155                                        | 142.595                                     | STA 59,216.4                                        | ko0190 | Energy metabolism |
| P06827       | K02112                    | ATP synthase subunit beta, chloroplastic | 6.426                                        | 86.00222                                    | STA 64,973.0                                        | ko0195 | Photosynthesis |
| P0C387       | K02634                    | Apocytochrome f | 6.121                                        | 63.602                                      | STA 35,580.5                                        | ko0195 | Photosynthesis |
| A5H454       | K00432                    | Peroxidase 66 | 5.701                                        | 52.048                                      | STA 33,932.3                                        | ko01100 | Lipid metabolism |
| P0C356       | K02690                    | Photosystem I P700 chlorophyll a apoprotein A2 | 4.901                                        | 29.885                                     | STA 82,672.8                                        | ko0195 | Photosynthesis |
| P12863       | K01803                    | Triosephosphate isomerase, cytosolic | 4.827                                        | 28.386                                     | STA 27,252.6                                        | ko0010 | Glycolysis/Gluconeogenesis |
| O24592       | K09840                    | 9-cis-epoxycarotenoid dioxygenase 1, chloroplastic | 4.805                                        | 27.953                                     | STA 66,007.5                                        | ko01110 | Biosynthesis of secondary metabolites |
| P0C224       | K02111                    | ATP synthase subunit alpha, chloroplastic | 4.095                                        | 17.091                                     | STA 55,721.0                                        | ko0195 | Photosynthesis |
| P0C353       | K02689                    | Photosystem I P700 chlorophyll a apoprotein A1 | 3.406                                        | 10.603                                     | STA 83,395.2                                        | ko0195 | Photosynthesis |
| Q41764       | K10363                    | Actin-depolymerizing factor 3 | 2.982                                        | 7.905                                      | A 16,013.8                                          | ko04812 | Signaling and cellular processes |
| Q8W2B7       | K13227                    | DIMBOA UDP-glucosyltransferase BX8 | 2.981                                        | 7.895                                      | A 49,926.0                                          | ko00402 | Biosynthesis of secondary metabolites |
| P46302       | K02979                    | 40S ribosomal protein S28 | 2.980                                        | 7.889                                      | A 7,467.6                                           | ko0310 | Translation |
| A1Y2B7       | no KO assigned            | Protein SUPPRESSOR OF GENE SILENCING 3 | 2.933                                        | 7.640                                      | A 67,979.5                                          | ko0195 | No assigned metabolism |
| P46252       | K02943                    | 60S acidic ribosomal protein P2A | 2.763                                        | 6.790                                      | A 11,476.7                                          | ko0310 | Translation |
| Q00827       | K00912                    | Chlorophyll a-b binding protein 48, chloroplastic | 2.675                                        | 6.386                                      | A 28,299.8                                          | ko0195 | Photosynthesis |
| A5H452       | K00432                    | Peroxidase 70 | 2.449                                        | 5.462                                      | A 33,994.0                                          | ko01100 | Lipid metabolism |
| Q9FQA3       | K00799                    | Glutathione transferase GST 23 | 2.327                                        | 5.018                                      | A 24,992.4                                          | ko00408 | Glutathione metabolism |
| B4FGS2       | no KO assigned            | Spindle and kinetochore-associated protein 1 | 2.254                                        | 4.769                                      | A 30,488.3                                          | ko0195 | No assigned metabolism |
| B6TZD1       | K08963                    | Methylthioribose-1-phosphate isomerase | 2.205                                        | 4.613                                      | A 38,735.5                                          | ko0270 | Amino acid metabolism |
| P46240       | K00799                    | Glutathione S-transferase 4 | 2.178                                        | 4.524                                      | A 24,741.1                                          | ko00480 | Glutathione metabolism |
| P11155       | K02115                    | Pyruvate, phosphate dikinase 1, chloroplastic | 2.143                                        | 4.416                                      | A 103,585.5                                         | ko00710 | Carbon fixation |
| P49101       | K06103                    | Calcium-dependent protein kinase 2 | 2.139                                        | 4.405                                      | A 58,422.9                                          | ko04131 | Exocytosis |
| B8A031       | K03644                    | Lipoyl synthase, mitochondrial | 2.126                                        | 4.366                                      | A 42,341.6                                          | ko01100 | Lipid metabolism |

Continued
| Swiss-Prot ID | KEGG* orthology (proteins) | Protein                                      | Log FC ([high CO2] versus [normal CO2]) Normalized | FC(abs) ([high CO2] versus [normal CO2]) Normalized | DAPs ([high CO2] versus [normal CO2]) Normalized | Molecular Mass (Da) | KEGG* orthology (metabolisms) | Metabolism                          |
|---------------|-----------------------------|----------------------------------------------|---------------------------------------------------|---------------------------------------------------|--------------------------------------------------|---------------------|-------------------------------|------------------------------------|
| P49094        | K01953                      | Asparagine synthetase [glutamine-hydrolyzing] | 2.126                                              | 4.364                                              | A                                                | 67,147.1            | ko00270                       | Amino acid metabolism            |
| P0C8M8        | K08852                      | serine/threonine-protein kinase CCRP1         | 2.062                                              | 4.176                                              | A                                                | 70,746.2            | ko04141                       | Protein processing in endoplasmic reticulum |
| O63066        | K10956                      | Preprotein translocase subunit SECY, chlorplastic | 2.031                                              | 4.086                                              | A                                                | 59,637.9            | ko04141                       | Protein processing in endoplasmic reticulum |
| Q8LPU4        | K11303                      | Histone acetyl-transferase type B catalytic subunit | 2.028                                              | 4.079                                              | A                                                | 53,119.5            | ko03400                       | DNA repair                       |
| C0PF72        | K00620                      | Arginase biosynthesis bifunctional protein ArgI, chlorplastic | 2.016                                              | 4.046                                              | A                                                | 48,407.3            | ko01230                       | Amino acids biosynthesis        |
| Q67EU8        | K04482                      | DNA repair protein RAD51 homolog A            | 1.998                                              | 3.994                                              | SLA                                              | 36,989.5            | ko03400                       | DNA repair                       |
| Q10717        | K16290                      | Cysteine proteinase 2                          | 1.976                                              | 3.935                                              | SLA                                              | 39,712.1            | ko01002                       | Protein degradation             |
| P41978        | K04564                      | Superoxide dismutase [Mn] 3.2, mitochondrial  | 1.914                                              | 3.770                                              | SLA                                              | 25,356.4            | ko04146                       | Oxidative stress               |
| P42390        | K13222                      | Indole-3-glycerol phosphate lyase, chlorplastic | 1.888                                              | 3.701                                              | SLA                                              | 36,691.8            | ko00402                       | Biosynthesis secondary metabolites |
| P90056        | K00413                      | Cytochrome c                                  | 1.885                                              | 3.695                                              | SLA                                              | 12,132.6            | ko00190                       | Energy metabolism              |
| P49081        | K01638                      | Malate synthase, glyoxysomal                  | 1.881                                              | 3.684                                              | SLA                                              | 62,092.2            | ko01200                       | Carbon metabolism              |
| Q9XGD5        | K00588                      | Caffeoyl-CoA O-methyltransferase 2             | 1.851                                              | 3.607                                              | SLA                                              | 29,522.0            | ko01110                       | Biosynthesis of secondary metabolites |
| P12959        | K21632                      | Regulatory protein opaque-2                   | 1.815                                              | 3.519                                              | SLA                                              | 49,812.2            | ko03000                       | Transcription                   |
| P23345        | K04565                      | Superoxide dismutase [Cu-Zn] 4A               | 1.593                                              | 3.017                                              | SLA                                              | 15,228.5            | ko04146                       | Oxidative stress               |
| Q05737        | K07874                      | GTP-binding protein YPTM2                     | 1.558                                              | 2.945                                              | SLA                                              | 22,646.2            | ko04031                       | Protein transport               |
| P06671        | K08913                      | Chlorophyll a-b binding protein, chlorplastic | 1.427                                              | 2.689                                              | SLA                                              | 28,165.7            | ko00195                       | Photosynthesis                  |
| P0C520        | K02132                      | ATP synthase subunit alpha, mitochondrial     | 1.414                                              | 2.665                                              | SLA                                              | 55,657.7            | ko01190                       | Energy metabolism              |
| Q41803        | K03231                      | Elongation factor 1-alpha                     | −7.805                                             | 223.659                                            | STD                                              | 49,574.4            | ko03013                       | Translation                     |
| Q08062        | K00025                      | Malate dehydrogenase, cytoplasmic             | −7.712                                             | 209.621                                            | STD                                              | 35,931.6            | ko01200                       | Carbon metabolism              |
| P0C510        | K01601                      | Ribulose bisphosphate carboxylase large chain | −7.099                                             | 137.062                                            | STD                                              | 53,450.7            | ko00710                       | Carbon fixation                |
| Q43298        | K04077                      | Chaperonin CPN60-2, mitochondrial             | −6.689                                             | 68.074                                             | STD                                              | 61,219.3            | ko03018                       | Protein folding                |
| P27923        | K02977                      | Ubiquitin-40S ribosomal protein S27a          | −5.271                                             | 38.628                                             | STD                                              | 17,909.5            | hsa03010                      | Translation                     |
| P14640        | K07374                      | Tubulin alpha-1 chain                         | −4.601                                             | 24.271                                             | STD                                              | 50,414.8            | ko04514                       | Citoskeleton metabolism        |
| Q02245        | K07374                      | Tubulin alpha-5 chain                         | −4.556                                             | 23.538                                             | STD                                              | 50,251.7            | ko04514                       | Citoskeleton metabolism        |
| Q9ZT00        | K19199                      | Ribulose bisphosphate carboxylase/oxygenase activase, chlorplastic | −4.483                                             | 22.367                                             | STD                                              | 48,108.8            | ko00710                       | Carbon fixation                |
| Swiss-Prot ID | KEGG orthology (proteins) | Protein | Log FC ([high CO2] versus [normal CO2]) Normalized | FC (abs) ([high CO2] versus [normal CO2]) Normalized | DAPs ([high CO2] versus [normal CO2]) Normalized | Molecular Mass (Da) | KEGG orthology (metabolisms) | Metabolism |
|---------------|---------------------------|---------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|-------------------|-------------------------------|------------|
| P09315        | K05298                    | Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic | -4.386 | 20.907 | STD | 43,208.4 | ko00010 | Glycolysis/Gluconeogenesis |
| Q75IC9        | K00615                    | Transketolase, chloroplastic | -4.094 | 17.078 | STD | 73,391.4 | ko01200 | Carbon metabolism |
| P24631        | K13993                    | 17.5 kDa class II heat shock protein | -3.6320088 | 12.398 | STD | 17,568.0 | ko04141 | Protein processing in endoplasmic reticulum |
| P08440        | K01623                    | Fructose-biphosphate aldolase, cytoplasmic | -3.430 | 10.778 | STD | 39,059.9 | ko00010 | Glycolysis/Gluconeogenesis |
| P26301        | K01689                    | Enolase 1 | -2.870 | 7.313 | D | 48,290.9 | ko00010 | Glycolysis/Gluconeogenesis |
| P04712        | K00695                    | Sucrose synthase 1 | -2.545 | 5.8369 | D | 92,129.6 | ko05000 | Starch and sucrose metabolism |
| Q43704        | K02541                    | DNA replication licensing factor MCM3 | -2.539 | 5.811 | D | 85,694.0 | ko03030 | DNA replication and repair |
| P15719        | K00051                    | Malate dehydrogenase [NADP], chloroplastic | -2.475 | 5.560 | D | 47,429.3 | ko01200 | Carbon metabolism |
| P0C1M0        | K02115                    | ATP synthase subunit gamma, chloroplastic | -2.464 | 5.516 | D | 40,131.4 | ko0195 | Photosynthesis |
| P38560        | K01915                    | Glutamine synthetase root isozyme 2 | -2.115 | 4.332 | D | 40,492.8 | ko00250 | Amino acid Biosynthesis |
| P18122        | K03781                    | Catalase isozyme 1 | -2.073 | 4.207 | D | 57,389.9 | ko04146 | Oxidative stress |
| P02582        | K06759                    | Actin-1 | -2.038 | 4.164 | D | 41,902.1 | ko04514 | Citoskeleton metabolism |
| Q6XZ79        | K00847                    | Fructokinase-1 | -2.028 | 4.078 | D | 34,861.4 | ko03000 | Starch and sucrose metabolism |
| Q9SP22        | K08057                    | Calreticulin | -1.854 | 3.616 | SLA | 48,052.8 | ko04141 | Protein folding and sorting |
| O22424        | K02987                    | 40S ribosomal protein S4 | -1.832 | 3.561 | SLA | 30,130.6 | ko03013 | Translation |
| Q195N6        | K01006                    | Pyruvate, phosphate dikinase regulatory protein, chloroplastic | -1.804 | 3.491 | D | 46,360.8 | ko00710 | Carbon fixation |
| B4G072        | K13227                    | DIMBOA UDP-glucosyltransferase BX9 | -1.710 | 3.272 | D | 50,358.7 | ko04020 | Biosynthesis secondary metabolites |
| Q9ZSV1        | K24070                    | Poly [ADP-ribose] polymerase 1 | -1.698 | 3.244 | D | 111,614.5 | ko03410 | DNA repair |
| P80607        | K13379                    | Alpha-1,4-glucan-protein synthase [UDP-forming] | -1.654 | 3.148 | SLA | 41,717.1 | ko00520 | Carbohydrate metabolism |
| B4FAT0        | K11996                    | Adenyllytransferase and sulfurtransferase MOCS3 2 | -1.514 | 2.856 | SLA | 52,564.9 | ko03013 | Translation |
| Q8S4P4        | K11430                    | Histone-lysine N-methyltransferase EZ3 | -1.498 | 2.824 | D | 102,888.1 | ko02270 | Amino acid metabolism |
| Q43272        | K00131                    | NADP-dependent glyceraldehyde-3-phosphate dehydrogenase | -1.432 | 2.698 | SLA | 53,773.0 | ko00010 | Glycolysis/Gluconeogenesis |

**Table 1.** Differential abundant proteins (DAPs) in leaf tissue of high pCO2 samples comparing with those of normal pCO2 samples. Accession number, protein name, fold change expressed as Log (2) and absorbance, protein behavior KEGG orthology, molecular mass and metabolisms have been shown. Strongly accumulated and strongly depleted proteins are reported in bold. Details on mass spectrometry parameters for peptides for each identified proteins are reported in the Supplementary Table 2 STA: Strongly accumulated; STD: Strongly depleted; A: accumulated; D: Depleted; SLA: Slightly accumulated; SLD: Slightly depleted * KEFF codes are developed in the Kanehisa Laboratories
Glyceraldehyde-3-phosphate dehydrogenase A, Fructose-bisphosphate aldolase and Enolase 1 were also depleted. The Malate synthase, that facilitates the glyoxylate cycle, the Pyruvate phosphate dikinase involved in the alternative glycolysis, the Serine-threonine protein and many proteins involved in the amino acid metabolism are enriched under acidified conditions. Also the glutathione metabolism seems to be upregulated as the Glutathione transferases are accumulated under acidified condition.

Protein folding and turnover seemed also to be affected under acidified conditions as the Elongation factor 1-alpha, Chaperonin CPN60-2 and Ubiquitin-40S ribosomal protein S27a were strongly depleted. Proteins belonging to the cytoskeleton metabolism are affected by acidification such as Tubulin alpha-1 chain, Tubulin alpha-5 chain and Actin-1 (Table 1).

The leaf blades of *C. nodosa* growing under acidified conditions were shown to be almost 15% wider (3.22 ± 0.43 mm) than those of plants living in normal pCO2 (2.76 ± 0.52 mm); epidermal cells have larger areas and thinner cell walls in high pCO2 leaves than those of normal pCO2 cells (Fig. 5). Leaves growing under acidified conditions have also larger parenchyma cells, lesser number of cells/mm2 and thinner cell wall than those of normal pCO2 cells (Supplementary Table 4).

Schematic representation of DAPs involved in different metabolic pathways/cellular processes in *Cymodocea nodosa* to cope the environmental conditions at CO2 vents is reported in the Fig. 6.

**Discussion**

The comparative proteomics data showed that the long-term exposure to high pCO2 in the vicinity of volcanic vents strongly affected the inorganic carbon assimilation in leaves of *Cymodocea nodosa* as demonstrated by the significantly decreased levels of the key carbon metabolism enzymes. These results suggest that the chronic exposure of *C. nodosa* to CO2-enriched volcanic emissions did not act positively toward carbon fixation, neither via Rubisco nor via PEPC, indicating a general depression of both inorganic carbon fixation pathways; even if the accumulation of Malate synthase and Pyruvate dikinase can pose the question whether or not seagrasses have a carbon concentrating mechanism, its existence to date is not proven and more evidence from “omics” is still required.

Our proteomic findings reinforce the results of a gene expression study carried out simultaneously on plants from the same populations, in which a significant down-regulation of the transcripts related to carbon metabolism, carbon uptake and carbohydrate metabolism were also found to be strongly down-regulated in the acidified leaves. Proteomics and transcriptomics thus demonstrate that the long-lasting exposure to the vents conditions...
Figure 5. Cytological measurements of leaf epidermis of *Cymodocea nodosa* plants growing in normal and high pCO₂ environments. Epidermal leaf cell microphotographs of *C. nodosa* growing in normal (a) and high (b) pCO₂ environments. Boxplots (± SD) showing the cell area (c) and cell wall thickness of epidermal thickness (d) of *C. nodosa* growing in normal and high pCO₂ environments.

Figure 6. Schematic diagram of differentially expressed proteins belonging to metabolic pathways/cellular processes leading to the acclimation/tolerance of *Cymodocea nodosa* in volcanic vents. The acclimation strategy combines the reduction of carbon fixation, gluconeogenesis, carbohydrate metabolism and protein synthesis with increasing photophosphorylation, cell respiration and aminoacid metabolism to maintain the high energy demand for leaf expansion and elongation of the mesophyll cell; the cell expansion is accomplished by the cell wall loosening, the vacuole enlargement and the cytoskeleton remodeling. Proteins belonging the oxidative stress response pathway, the Gluthatione metabolism and the biosynthesis of secondary metabolites were also accumulated, suggesting that potential external stress factor other than CO₂ are at play at the Vulcano submarine vents. Proteins and related KEGG codes, reported also in Table 1, are developed by Kanehisa Laboratories. 32.
lead to the overall depression of the primary metabolisms, and are the probable cause of the reduction of the net plant productivity (NPP) and plant biomass found in plants growing in the vicinity of the vents\cite{25,26}.

The metabolism of proteins is also negatively affected; previous proteomics studies of seagrasses living under several acute stressors or long-lasting disturbing factors also reported lower protein content linked to depletion of Rubisco\cite{28,30}. Here we found that the lower protein contents is mainly due to an impaired protein synthesis at the post-transcriptional level in leaf tissue of plants under acidified conditions. Also protein function and turnover seems be the machinery that require an increasing energy demand.

Despite the strong depletion of the Calvin cycle proteins, a significant positive correlation between proteins related to the light reactions of photosynthesis and the exposure to high pCO2 was found, the same happening with some proteins belonging to photosystem II and photosystem I. High levels of Chl a and significant increases in maximum electron transport rate and in compensation irradiance were previously found in \textit{C. nodosa} grown under acidified conditions at this same volcanic CO2 vent, corroborating the hypothesis that acidification promotes the photosynthetic light reactions\cite{8}. Moreover, we found that the energy metabolism from photophosphorylation and from oxidative phosphorylation are positively affected by high pCO2 exposure; the latter comes from leaf mitochondrial respiration which have been previously found to be up-regulated in the response to high pCO2\cite{32,33}. The observed depletion of the Calvin cycle proteins near the vents is likely to reflect a higher efficiency in the use of CO2 that would require a higher energy availability from the photosynthetic electron transport chain. On the other hand, a potential imbalance between the photosynthetic electron transport chain and the Calvin cycle reactions could also result in the formation of reactive oxygen species (ROS).

Increases in ROS may result from a number of other stress factors, and are usually associated with increases in the overall antioxidant capacity. The observed accumulation of antioxidant enzymes such as Peroxidases, Glutathione S-transferases, Superoxide dismutases and enzymes belonging the phenylpropanoid pathway has been well documented as defense responses of seagrasses to light stress and heavy metal toxicity\cite{27,29}. The enhanced multi-enzyme antioxidant system indicates that vents conditions results in ROS production, triggering the response to scavenge H2O2, and maintaining the cell redox status. Taken all together, our results support the previously conveyed idea that potential external stress factors other than CO2 are at play at the Vulcano submarine vents, significantly affecting the plants' metabolic balance\cite{8,34,35}.

The adaptive strategy that plants use to cope with vents conditions also involves some morphological adjustments. If at the meadow level, \textit{C. nodosa} lowered the density, biomass and below/aboveground biomass ratio at the acidified site\cite{25,26}, at plant level, no significant differences in number of leaves per shoot (total mean number of leaves per shoot was 4 ± 0.7, data not shown) was found. Interestingly, we found that plants growing closer to the vents had shorter but wider leaf blades, epidermal cells with thinner cell walls and larger parenchyma cells. These morphological differences could indicate ecotypes, eventually selected by this extreme environment, but results from the population genetics carried out on the same sampling site showed no genetic differentiation and these morphological differences found should then be considered as a phenotypical response of \textit{C. nodosa} to the pressure of the acidified environment. A similar pattern of leaf parameters was also recently described at the Vulcano CO2 vents by Vizzini et al.\cite{36}. Our data support the hypothesis that under acidified conditions the cell expansion contributed more than cell division to the leaf expansion; following this assumption, leaf blades become shorter and wider than those grown in normal pCO2 condition. Further studies conducted on \textit{C. nodosa} and \textit{Halodule wrightii} demonstrated that an elevated CO2 concentration has effects on leaf mechanical resistance such as on the leaf anatomy and cell ultrastructures\cite{27,29}. The authors reported that high pCO2-grown \textit{C. nodosa} had an increased leaf-breaking force related to leaf growth; leaf width and cross-section area were larger under acidification in \textit{Halodule wrightii}, thus indicating that increased CO2 may manifest in large part at cellular level. Here we might conclude that the morphological traits have shown a positive correlation between mesophyll cell size and pH at CO2 vents, suggesting that wider leaves have a higher capacity to buffer pH. Even if is demonstrated that exposure to elevate pCO2 alters plant structure by inducing change in rate of cell division and cell expansion in seed plants\cite{35}, further investigation needs to elucidate whether \textit{C. nodosa} growing in the vicinity of volcanic vents might ameliorate potential adverse effects on growth by means the mesophyll cell expansion and modified cell water uptake. In support of this idea, Ruocco et al.\cite{36} found that \textit{C. nodosa} exposed to high pCO2 overexpressed transcripts encoding for enzymes that play an integral role in pH homeostasis of the cells. Under this concept, mesophyll cells of \textit{C. nodosa} might couple the ions homeostasis with increased water uptake to adjust the osmotic balance.

Moreover, specific molecular rearrangements seem to validate the hypothesis that high pCO2 led to larger cell size; biosynthesis of secondary metabolites appeared to be positively related to acidification and also to hormone-mediated response such as ABA biosynthesis\cite{36,37}. The actin-depolymerizing Factor 3 coupled with the Calcium-dependent protein kinase 2 (CDPK) have been found to modulate the plant cell shape through the regulation of the actin filament network in cytoskeleton\cite{38,39} and also to have a role in the re-organization of plant cytoplasm in response to a wide range of internal and external stimuli, suggesting a direct correlation between signal transduction and actin cytoskeleton reorganization in plants\cite{38,39}. The strong depletion of tubulin and actin cytoskeleton constituents further support the suggestion that acidification affects the cytoskeleton dynamics and might trigger the modulation of cell enlargement and elongation in mesophyll cells. A pattern of thinner cell walls was also found in mesophyll epidermal cells of plants growing in the acidified site. Seagrasses possess a very different cell wall composition as well as proportion of polysaccharide and monosaccharides than terrestrial plants\cite{40}; the modified cell wall structure and metabolism lead to an increase in the polyamionic character of seagrass cell wall\cite{41}. It is well known that an acid cell wall is necessary for wall loosening to occur, thus promoting cell expansion and growth\cite{42}; this mechanism is induced by the hormone auxin during cell elongation. In seagrasses, under normal conditions, the extracellular carbonic anhydrase mediates the conversion of HCO3− to CO2 generating acid zones created by H+ extrusion from the cytoplasm to the cell wall\cite{43}; we can speculate that
in an acidified environment the increased exogenous protons could, at least partially, substitute auxin in inducing cell enlargement. The thinner cell wall found in acidified epidermal cells of C. nodosa is likely to come from the cell elongation without ex novo biosynthesis of structural wall carbohydrates, due to the impaired primary metabolism and depressed carbon fixation; the lowered biomass of plants exposed to high pCO2 environment also indicates that leaf elongation occurred mainly by means of cell expansion. Thus, cell wall seems to be a critical player in response to acidification and further studies on the cell wall metabolism of C. nodosa growing near Vulcano CO2 vents are necessary.

In conclusion, proteomic analysis and cytological features evidence some physiological and structural adaptive traits of the seagrass Cymodocea nodosa growing in the vicinity of the Vulcano CO2 vents. This adaptation strategy combines the reduction of carbon fixation and gluconeogenesis with increasing photophosphorylation and cell respiration to maintain the high energy demand for leaf expansion and elongation of the mesophyll cell. Our results largely corroborate the findings of previous metabolic and transcriptomic studies carried out in the Vulcano vents, raising additional concerns on the use of volcanic vents as proxies for future acidification conditions. On the other hand, the specificities of the volcanic emissions also raise interesting questions and allow the investigation of pertinent physiological questions.

Methods

Sites description and plant sampling. Vulcano, the southernmost island of the Aeolian Archipelago, contains the most recently active center of submerged CO2 vents systems. The most recent CO2 emissions originated from a volcanic activity on Vulcano occurred in 2002 has caused a series of gas explosions. Most of the active submerged seeps are located along southern and western shores of Baia di Levante, where dispersed underwater leaks cover a 0.13 km2 shallow area (1 m depth). Gas composition at the seeps consists of 99% of carbon dioxide and dissolved hydrogen sulphide from the seeps was undetectable at the sampling locations; seawater parameters, daily irradiance and pCO2 concentration at two sites were reported in Olivé et al.25.

For molecular analyses, C. nodosa samples (i.e., morphological individuals with two or more shoots) were collected at 5 m depth by SCUBA diving in the acidified site referred as high pCO2 environment (38°25′05″N-14°57′59″E) and in a nearby site referred as normal pCO2 environment (38°25′22″N-14°57′82″E)26. To assure the representation of the seagrass meadows in the study sites, sampling were performed along three grids of 20 × 20 m each with the internal distance between sampled plants of 4–5 m to reach a total sampling of 15 individuals at each site. Once collected, epiphytes on leaf surface were rapidly and carefully removed by a razor, then leaves were rinsed in distilled water and immediately frozen in liquid nitrogen and kept at -80 °C until the protein extraction procedure described in Mazzuca et al.27.

For the histological and cytological analyses adult leaves were selected from the 15 individuals at each sampling site, cleaned from the epiphytes, washed in sea water and fixed in 4% formalin in 0.1 M phosphate buffer pH 7.2 and stored refrigerated.

Extraction and purification of total protein from leaves. Frozen leaves were pooled forming 3 biological replicates, each composed by 5 individuals, because of the low amount of leaf tissue for each shoot. Leaf proteins were extracted by the multistep procedures28; for each extraction 1.4 g of pooled leaves were powdered in a mortar in liquid nitrogen until a fine powder was obtained. At this powder a volume of 10% TCA in acetone was added and centrifuged at 13,000 rpm for 5 min at 4 °C. Subsequently, four washes were performed in 80% acetone in water. After centrifugation the pellet containing the precipitated proteins was dried at room temperature. Approximately 0.1 g of powdered tissue was dissolved in 0.8 ml of phenol (buffered with Tris–HCl, pH 8.0, Sigma, St. Louis, MO, USA) and 0.8 ml of SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris–HCl, pH 8.0, 0.5% 2-mercaptoetanol) in a 2 ml microfuge tube. The samples were vortexed for 30 s and centrifuged at 13,000 rpm for 5 min to allow the solubilization of proteins in the phenol phase. The phenol phase was mixed with five volumes of 0.1 M ammonium acetate in cold methanol, and the mixture was stored at -20 °C for 30 min to precipitate proteins. Proteins were collected by centrifugation at 13,000 rpm for 5 min. Two washes were performed with 0.1 M ammonium acetate in cold methanol, and two with cold 80% aceton, and centrifuged at 13,000 rpm for 7 min. The final pellet containing purified protein was dried and dissolved in Laemmli 1DE separation buffer pH 6.8. Two samples were also used for the histological and cytological analyses, adult leaves were selected from the 15 individuals at each sampling site, cleaned from the epiphytes, washed in sea water and fixed in 4% formalin in 0.1 M phosphate buffer pH 7.2 and stored refrigerated.

Electrophoresis of leaf proteins, protein in-gel digestion and mass spectrometry analyses. A gel was prepared at a concentration of 10% acrylamide/bisacrylamide, according to the method of29. The ratio of acrylamide/bisacrylamide was 12.5% in the running gel and 6% in the stacking gel. All biological replicates were heated for 5 min at 100 °C and 25 μg of activated proteins were loaded on the each well in the gel. The electrophoretic run was carried out at 60 mA for the stacking gel and 120 mA in the running gel at power of 200 V. The electrophoresis ran for an average time of 1 h and 15 min. The gels were stained with Coomassie Blue overnight and subsequently destained with several changes of destaining solution (45% methanol, 10% acetic acid). Digitized images of the destained SDS-PAGEs were analyzed by the Quantity One 1-D Analysis Software (Bio-Rad Laboratories; Berkeley, California) to measure the optical densities at each lane of all biological replicates from both sites. The amount of protein at bands of 55, 25, and 10 kDa was done using the marker reference bands at
and a 150 mm separation column (Zorbax 300SB-C18, 5 µm pore size) coupled to an Agilent Technologies 1200 Technologies) for statistical analysis. Protein intensities were log2 normalized and fold-change analysis was carried out (N-fold validation, using N = 3 and 10 repeats). The PLS-DA class prediction model loading, i.e. the plot of out using a threshold of 3. Multivariate Partial Least Square Discriminant Analysis (PLS-DA) was then carried out using a hybrid quadrupole-time-of-flight (Q-TOF) mass spectrometer (6550 IFunnel Q-TOF, Agilent Technologies, CA, USA), with a nano LC Chip Cube source (Agilent Technologies, CA, USA) according to Lucini and Bernardo47. The chip consisted of a 40-nL enrichment column (Zorbax 300SB-C18, 5 µm pore size) and a 150 mm separation column (Zorbax 300SB-C18, 5 µm pore size) coupled to an Agilent Technologies 1200 series nano/capillary LC system and controlled by the MassHunter Workstation Acquisition (version B.04).

A volume of 8 µL was injected per run, loading peptides onto the trapping column at 4 µL min⁻¹ in 2% (v/v) acetonitrile and 0.1% (v/v) formic acid. After enrichment, the chip was switched to separation mode and peptides were back flushed into the analytical column, during a 60 min acetonitrile gradient (from 3 to 90% v/v in 0.1% formic acid) at 0.6 µl min⁻¹. The mass spectrometer was used in positive ion mode and MS scans were acquired over a mass range from 300 to 1700 m/z, at 4 spectra s⁻¹.

Twelve precursor ions per scan were selected for auto-MS/MS, adopting an absolute threshold of 1000 and a relative threshold of 0.01%, and enabling active exclusion after 2 spectra of the same precursor. Ramped collision energy was used for collision-induced decomposition, as a function of peptide charge. Peptide identification from MS/MS spectra, proteins inference and validation were performed in Spectrum Mill MS Proteomics Workbench (Rev B.04; Agilent Technologies). Auto MS/MS spectra were extracted from raw data accepting a minimum sequence length of 3 amino acids and merging scans with the same precursor within a mass window of ± 0.4 m/z, in a time frame of ± 30 s. Search parameters were Scored Peak Intensity (SPI) ≥ 50%, precursor mass tolerance of ± 10 ppm and product ions mass tolerance of ± 20 ppm. Carbamidomethylation of cysteine was set as fixed modification and trypsin was selected as enzyme for digestion, accepting 2 missed cleavages per peptide.

Considering that a species-specific proteome was not available, the proteome referring to viridiplantae in Uniprot was used; downloaded on April 2015, a total of 144,283 entries can be found according to this criterion. Auto thresholds were used for peptide identification in Spectrum Mill, to achieve a target 1% false discovery rate. Label-free quantitation, using the protein summed peptide abundance, was carried out after identification.

Statistical analyses. The results were directly exported to Mass Profiler Professional B.04 (Agilent Technologies) for statistical analysis. Protein intensities were log2 normalized and fold-change analysis was carried out using a threshold of 3. Multivariate Partial Least Square Discriminant Analysis (PLS-DA) was then carried out (N-fold validation, using N = 3 and 10 repeats). The PLS-DA class prediction model loading, i.e. the plot of the weight for each protein in the model within the latent vectors, was used to select those proteins being more discriminant in class prediction (those having a score of above + 0.2 rather than below -0.2). These proteins were exported from the covariance structures in the PLS-DA hyperspace and further discussed.

Preparation of samples and histological analyses. For each individual, several 5 × 5 mm pieces of six fixed leaves were cut and washed in 0.15 M phosphate buffer three times for 10 min; subsequently leaf pieces were treated with 1% osmium tetraoxide in phosphate buffer. Leaf pieces were then dehydrated trough the increasing concentration of ethanol solutions. Dehydrate samples were then imbedded in epoxy resin, obtained by mixing Epon 812-Araldite and ethanol (1: 1, v/v) for 4–5 h at 4 °C and then embedded in pure resin overnight at room temperature. The embedded samples were polymerized in an oven at 60 °C for 3 days; then each sample was cut in 0.2 mm thick sections through the ultramicrotome. The sections were transferred onto slides and stained with Methylene Blue. At least ten sections for each leaf sample were observed and photographed at 100 X magnification and digitalized using Image J open source software. A measurement bar of 100 µm as a reference scale was added to the images obtained for subsequent analysis using CellProfiler open source software (Broad Institute; country). The areas of 20 cells per section of the epidermis and parenchyma were measured and the thickness of the epidermis and parenchyma cell wall was also measured and a 100 × 100 µm scale square. The measurements obtained were divided into two datasets of all the samples of the two sites; the first dataset with the comparison of the mean values of the cell area and the second dataset with the comparison of the average values of the wall thickness. Significance of values from both datasets were made by t-student test using the GraphPad Prism 8 software.

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Author contributions
S.M. and J.S. coordinated the manuscript writing, S.M., A.P., J.S., R.S. conceived and designed the experiments, S.M., A.P., R.S., J.S., I.O., I.B. and M.M.C. participated to the sampling campaign and the field work, A.P., L.B., and I.L. performed the mass spectrometry analysis, A.P., L.B. and I.A.A. performed the bioinformatics and statistical analyses. S.M. and A.P. performed the cytological measurements. All Authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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