

METABOLOMIC ANALYSIS OF SUSPENSION CULTURED CELLS OF SEAGRASS *CYMODOCEA ROTUNDATA*

Submitted in partial fulfilment of the requirements for the award of

Bachelor of Science degree in
BIOTECHNOLOGY

By

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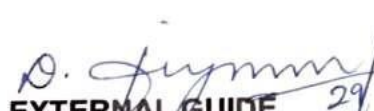
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This is to certify that the Project Report is the bonafide work of **MEENAKSHI. S (40770029)** who carried out the project entitled "**METABOLOMIC ANALYSIS OF SUSPENSION CULTURED CELLS OF SEAGRASS CYMODOCEA ROTUNDATA**" under my/ our supervision from December 2022 to May 2023.


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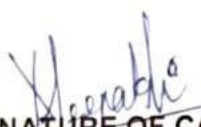

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DECLARATION

I, MEENAKSHI. S, hereby declare that the Project Report entitled "**METABOLOMIC ANALYSIS OF SUSPENSION CULTURED CELLS OF SEAGRASS CYMODOCEA ROTUNDATA**" done by me under the guidance of **Dr. S. USHA NANDHINI** (Internal) at DEPARTMENT OF BIOTECHNOLOGY, Sathyabama Institute of Science and Technology, and **DR. D. JEYAPRAGASH (External)** at **CENTRE FOR OCEAN RESEARCH**, Sathyabama Institute of Science and Technology, Chennai is submitted in partial fulfilment of the requirements for the award of Bachelor of Science degree in Biotechnology.

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ABSTRACT

Metabolomics is one of the new field of "omics" approach and youngest of the triad of system biology, emerged as a complementary tool to functional genomics that together with well-established technologies for high-throughput DNA sequencing (genomics), mRNA expression profiling (transcriptomics), and protein profiling (proteomics). Metabolomics strategies have been divided into two distinct approaches, untargeted and targeted, each with their own inherent advantages and disadvantages. Targeted metabolite analysis aims to determine the absolute concentration of metabolites using specified extraction protocols with an adapted separation and detection methods. Untargeted metabolomics allows for the investigation of metabolic phenotypes involving several hundreds to thousands of metabolites. Seagrasses are marine angiosperm. It comprises one of the most abundant and important ecosystems in the marine environment with high primary productivity. Seagrasses comprise a group of 72 species of submerged marine angiosperms growing in marine shallow coastal waters and estuaries in all continents except Antarctica.

Present study applied a GC-MS based metabolomics approach to investigate the systematic identification and characterization of metabolic changes in *Cymodocea rotundata*. This analysis identified the presence of 125 metabolites in the SCC of *C. rotundata* and they were mapped to general biochemical pathways and these metabolites spanned 77 primary and secondary metabolism pathways. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis of integration data showed profound biochemical changes including glutathione metabolism, glutamate metabolism, phenylalanine and tyrosine metabolism. Random forest analysis showed the top 15 metabolites and their classification. Also, present findings provide baseline information on *C. rotundata* metabolism in the marine environments. Furthermore, the metabolomics data from *C. rotundata* may be utilised to compare the metabolites associated with stress response, facilitating further research into the mechanisms underlying stress tolerance of other seagrass species in the marine environments.

KEYWORDS: Seagrass, Suspension Cultured Cells, Metabolomics, GC-MS, KEGG, Metabolites, Metabolism, *Cymodocea rotundata*.

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LIST OF ABBREVIATIONS

LC -MS – Mass Spectrometry.
GC-MS – Gas Chromatography Mass spectrometry.
NMR – Nuclear magnetic resonance.
TLC – Thin Layer Chromatography.
MS medium – Murashige and Skoog medium.
SCC - Suspension Cultured Cells.
EI – Electron Impact.
2, 4 -D - 2, 4-Dichlorophenoxyacetic acid.
KEGG – Kyoto Encyclopedia of Genes and Genomes.
PLS-DA – Partial Least Square Discriminant Analysis.
RF – Random Forest analysis.
MDA – Mean Decrease Accuracy
EtAc – Ethyl Acetate.
ChI – Chloroform.
MeOH – Methanol.
BAP - 6-Benzylaminopurine.
HRMS – High Resolution Mass Spectrometry.

LIST OF SYMBOLS

°C - Degree Celsius
<- Lesser than
% - Percentage

CHAPTER 1

INTRODUCTION

1.1 METABOLOMICS

Metabolomics is one of the new field of "omics" approach and youngest of the triad of system biology, emerged as a complementary tool to functional genomics that together with well-established technologies for high-throughput DNA sequencing (genomics), mRNA expression profiling (transcriptomics), and protein profiling (proteomics). The term metabolomics was developed by Pauling et al. (1971) and was first used in 1998 and even up to 2010 metabolomics was regarded as an emerging filed. For numerous organisms, complete genome (Arabidopsis Genome Initiative, 2000), transcriptome (Fowler and Thomashow, 2002) and proteome studies (Hajduch et al., 2005) have been described, but recently metabolome analyses using mass spectrometry (MS) - based platforms attracted attention. Since, metabolomics is an unbiased approach which acquires comprehensive information on the low molecular weight metabolite content of cell, tissue or organisms, their composition likely to be altered owing to different environmental conditions which reflects different genetic background (Olivier et al., 2008). Studies on the plant metabolome bought huge challenges to analytical technologies which have been employed in current plant metabolomics programs.

1.2 UNTARGETED METABOLOMICS

Metabolomics strategies have been divided into two distinct approaches, untargeted and targeted, each with their own inherent advantages and disadvantages. Targeted metabolite analysis aims to determine the absolute concentration of metabolites using specified extraction protocols with an adapted separation and detection methods (Dudavera N 2009).

Untargeted metabolomics allows for the investigation of metabolic phenotypes involving several hundreds to thousands of metabolites. In this approach, all signals in a mass chromatogram are processed in an unbiased way, allowing for a deeper investigation of metabolic phenotypes, but also resulting in significantly

more complex data processing and post-processing steps. Much progress has been made in this field over the past decade and yet plant metabolomics seems to still be an emerging approach because of the high complexity of plant metabolites and the number one challenge of untargeted metabolomics, metabolite identification.

1.3 SEAGRASS

Seagrasses are marine angiosperm. It comprises one of the most abundant and important ecosystems in the marine environment with high primary productivity (Kim et al., 2013). Seagrasses comprise a group of 72 species of submerged marine angiosperms (Short et al., 2011) growing in marine shallow coastal waters and estuaries in all continents except Antarctica (Short et al., 2007). Seagrasses are paraphyletic group of marine hygrophilous angiosperms which evolved three to four times from land plants back to the sea. It was adapted to live exclusively in an estuarine or in the marine environment and nowhere else (Papenbrock, 2012). Seagrasses provide an invaluable ecosystem by acting as breeding and nursery ground for a variety of organisms and promote commercial fisheries. Owing to the higher productivity, structural complexity and biodiversity in seagrass beds shed light to researchers to describe seagrass community as marine representation of the tropical rainforests (Subhashini et al., 2013).

1.3.1 CYMODOCEA ROTUNDATA

Cymodocea rotundata is an ecologically important tropical pioneer seagrass species distributed in the Indo-Pacific region. It is also called as smooth ribbon grass. It fast growing which means it has the ability to grow 7 to 15cm long and is believed to play a role in habitat recovery.

1.4 METABOLOMICS OF SEAGRASS

The metabolome of seagrasses may differ with respect to geographical location, substrates and other physiological factors includes wide fluctuations in the salinity which are prone to synthesize novel metabolites with defined physiological, biochemical, defense and ecological roles (Ross et al., 2008). Preliminary suggestions confirmed that seagrasses have pharmaceutically potent bioactive

secondary metabolites that are directed to prove to be a lead molecule for drug discovery (Athiperumalsami et al., 2008; Kumar et al., 2008).

In recent years, the plant-based drug discovery gained importance with the development of anti-infectious and anti-cancer drugs which contributes to undescribed bioactive molecules (Saklani and Kutty, 2008). However, the important plant derived drugs are obtained commercially by the extraction from their respective plants. Currently, the seagrass habitats are vanishing due to environmental and geopolitical instabilities and so making it very difficult to procure specialised metabolites that have been left undiscovered. Plant cell culture is considered as a promising alternative approach for producing the bioactive compounds that are challenging to be obtained by chemical synthesis or plant extraction (Kloewe et al., 2008). Nevertheless, the variations in the culture conditions such as media composition, hormone concentration, pH, and the light intensity may influence the metabolic variations with respect to the natural environments.

Though, information on the chemistry of seagrasses is scattered in the literature, some interesting aspects on their metabolomics fields of research and the metabolic variations between wild and suspension culture have not been sufficiently addressed or have not been re-examined with the latest available techniques. Despite the increasing demands of plant secondary metabolites, it becomes important to focus on the discovery of plant-based drug which is being used for the treatment of numerous diseases.

The aim objective of this study is to perform the metabolomic analysis of SCC of *C. rotundata*. This research work has been undertaken as no such studies have been done for the seagrass species *C. rotundata* as per the literature review, as this species is dominant in the Gulf of Mannar and Palk Bay. Considering the regulations, this research work has been delineated to study the metabolomics analysis suspension cultured cells (SCC) of seagrass *Cymodoceaceae rotundata*.

CHAPTER 2

LITERATURE SURVEY

1. Danaraj Jeyapragash et.al., 2021 Metabolomics is one of the new field of "Omics" approach and the youngest triad of system biology, which provides a broad prospective of how metabolic networks are controlled and indeed emerged as a complementary tool to functional genomics with well-established technologies for genomics, transcriptomics and proteomics. Though, metabolite profiling has been carried out for decades, owing to decisive mechanism of a molecule regulation, the importance of some metabolites in human regimen and their use as diagnostic markers is now being recognized. Plant metabolomics therefore aims to highlight the characterization of metabolite pool of a plant tissue in response to its environment. Seagrasses, a paraphyletic group of marine hydrophilous angiosperms which evolved three to four times from land plants back to the sea. Seagrasses share several analogous acquired metabolic adaptations owing to their convergent evolution, but their secondary metabolism varied among the four families that can be considered as true seagrasses. From a chemotaxonomic point of view, numerous specialized metabolites have often been studied in seagrasses.

This paper focus on the metabolome of seagrasses in order to explore their bioactive properties and the recent advancements adopted in analytical technology platforms and to study the non-targeted metabolomics of seagrasses using OMICS approach.

2. Manish Kumar Patel et al., 2021 Metabolomics is now considered a wide-ranging, sensitive and practical approach to acquire useful information on the composition of a metabolite pool present in any organism, including plants. Investigating metabolomic regulation in plants is essential to understand their adaptation, acclimation and defense responses to environmental stresses through the production of numerous metabolites. Moreover, metabolomics can be easily applied for the phenotyping of plants; and thus, it has great potential to be used in genome editing programs to develop superior next-generation crops. This review describes the recent analytical tools and techniques available to study plants metabolome, along with their significance of sample preparation using targeted and non-targeted

methods. Advanced analytical tools, like gas chromatography-mass spectrometry (GC-MS), liquid chromatography mass-spectroscopy (LC-MS), capillary electrophoresis-mass spectrometry (CE-MS), fourier transform ion cyclotron resonance-mass spectrometry (FTICR-MS) matrix-assisted laser desorption/ionization (MALDI), ion mobility spectrometry (IMS) and nuclear magnetic resonance (NMR) have speed up precise metabolic profiling in plants. Further, we provide a complete overview of bioinformatics tools and plant metabolome database that can be utilized to advance our knowledge to plant biology.

3. Ric CH De Vos et.al, 2007, Untargeted metabolomics aims to gather information on as many metabolites as possible in biological systems by considering all information present in the data sets. Here we describe a detailed protocol for large- scale untargeted metabolomics of plant tissues, based on reversed phase liquid chromatography coupled to high-resolution mass spectrometry (LC-QTOF MS) of aqueous methanol extracts. Dedicated software, MetAlign, is used for automated baseline correction and alignment of all extracted mass peaks across all samples, producing detailed information on the relative abundance of thousands of mass signals representing hundreds of metabolites. Subsequent statistics and bioinformatics tools can be used to provide a detailed view on the differences and similarities between (groups of) samples or to link metabolomics data to other systems biology information, genetic markers and/or specific quality parameters. The complete procedure from metabolite extraction to assembly of a data matrix with aligned mass signal intensities takes about 6 days for 50 samples.

4. Danaraj Jeyapragash et.al., 2017, Study investigated the metabolomics analysis of seagrass for acquiring diverse group of metabolites which offers various prospects for novel, financially significant constituents conveyed to the spotlight. GC-MS analysis revealed the presence of thirty-five compounds includes metabolites, flavonoids, sugars, amino acids and plant hormones. Rosmarinic acid (RA) is a pharmacologically active phytoconstituent that has so far been reported on the family of Boraginaceae and Lamiaceae. Present finding records the presence of RA from tropical seagrass *Halodule pinifolia* of Cymodoceaceae family collected from Vellar

estuary and is the first report on a tropical seagrass distributed in India. The existence of RA was identified by co-TLC, HPLC-PDA-MS and further it was confirmed by isolation of RA through prep-TLC and structural characterisation via NMR analysis. Study has explored a newer marine source, *H. pinifolia* for RA, which has an emerging potential preclinical chemical entity, and its biosynthesis can be enriched by plant tissue culture.

5. Richard K. F. Unsworth et.al, 2018, Seagrasses, flowering marine plants that form underwater meadows, play a significant global role in supporting food security, mitigating climate change and supporting biodiversity. Although progress is being made to conserve seagrass meadows in select areas, most meadows remain under significant pressure resulting in a decline in meadow condition and loss of function. Effective management strategies need to be implemented to reverse seagrass loss and enhance their fundamental role in coastal ocean habitats. Here we propose that seagrass meadows globally face a series of significant common challenges that must be addressed from a multifaceted and interdisciplinary perspective in order to achieve global conservation of seagrass meadows. The six main global challenges to seagrass conservation are (1) a lack of awareness of what seagrasses are and a limited societal recognition of the importance of seagrasses in coastal systems; (2) the status of many seagrass meadows are unknown, and up-to-date information on status and condition is essential; (3) understanding threatening activities at local scales is required to target management actions accordingly; (4) expanding our understanding of interactions between the socio-economic and ecological elements of seagrass systems is essential to balance the needs of people and the planet; (5) seagrass research should be expanded to generate scientific inquiries that support conservation actions; (6) increased understanding of the linkages between seagrass and climate change is required to adapt conservation accordingly. We also explicitly outline a series of proposed policy actions that will enable the scientific and conservation community to rise to these challenges. We urge the seagrass conservation community to engage stakeholders from local resource users to international policymakers to address the challenges outlined here, in order to secure the future of the world's seagrass ecosystems and maintain the vital services which they supply.

6. Unnikrishnan Kuzhiumparambil et.al., 2017, Seagrasses are monocotyledonous marine flowering plants that are considered lungs of the sea and are the most intense carbon sinks on the planet, delivering a range of ecologically and economically valuable biological services. In this study, we report the chemical fingerprint of *Zostera muelleri* using an untargeted metabolomic approach. High- performance liquid chromatography-mass spectrometry (HPLC-MS) and gas chromatography-mass spectrometry (GC-MS) were performed to study the metabolic profile of *Z. muelleri*. A total of 98 metabolites belonging to various chemical classes including flavonoids, phenolics, lipids, fatty acids, sugar alcohols and amino acids were identified, including two characteristic marker compounds of the genus, zosteric acid and rosmarinic acid. Chromatographic profiling yields a comprehensive map for the chemical constituents of *Z. muelleri*, and this method can be used as an effective and convenient approach to gain insights into the chemical composition of other seagrasses.

7. Manoj Kumar et.al, 2016 Metabolomics is a rapidly emerging discipline within functional genomics which is increasingly being applied to understand biochemical phenotypes across a range of biological systems. Metabolomics measures all (or a subset) metabolites in a cell at a specific time point, reflecting a snapshot of all the regulatory events responding to the external environmental conditions. Although metabolomics and systems biology approaches have been applied to the study of terrestrial plants, few marine macrophytes have been examined using these novel technologies. Marine macrophytes (including seaweeds and seagrasses) are marine ecosystem engineers delivering a range of ecologically and economically valuable biological services; however, they are under threat from a wide range of anthropogenic stressors, climate variation, invasive species and pathogens.

Investigating metabolomic regulation in these organisms is crucial to understand their acclimation, adaptation and defence responses to environmental challenges. This review describes the current analytical tools available to study metabolomics in marine macrophytes, along with their limitations for both targeted and non-targeted workflows. To illustrate recent advances in systems biology studies in marine

macrophytes, we describe how metabolites are used in chemical defence to deter a broad range of invasive species and pathogens, as well as metabolomic reprogramming leading to acclimation or adaptive strategies to environmental and anthropogenic stresses. Where possible, the mechanistic processes associated with primary and secondary plant metabolism governing cellular homeostasis under extreme environments are discussed. Further, we provide a comprehensive overview of an in-silico plant metabolome database that can be utilized to advance our knowledge from a system biology approach to marine macrophytes. Finally, functional integration of metabolomics with the allied "omics" disciplines of transcriptomics and proteomics, as well as the emerging discipline of "fluxomics" are discussed in the context of developing biological system networks, the identification of unknown gene/protein functions and the analysis of metabolic pathways in marine plants exposed to stress.

8. Sophie Alvarez, Michael J. Naldrett, 2021, untargeted metabolomics enables the identification of key changes to standard pathways, but also aids in revealing other important and possibly novel metabolites or pathways for further analysis. Much progress has been made in this field over the past decade and yet plant metabolomics seems to still be an emerging approach because of the high complexity of plant metabolites and the number one challenge of untargeted metabolomics, metabolite identification. This final and critical stage remains the focus of current research. The intention of this review is to give a brief current state of LC-MS based untargeted metabolomics approaches for plant specific samples and to review the emerging solutions in mass spectrometer hardware and computational tools that can help predict a compound's molecular structure to improve the identification rate.

9. Harald Hasler-Sheetal et.al., 2015 We investigated the response, adaptation and tolerance mechanisms of the temperate seagrass *Zostera marina* to water column anoxia. We exposed *Z. marina* to a diurnal light/dark cycle under anoxia and assessed the metabolic response by measuring the metabolome with gas chromatography coupled to mass spectrometry (GC-MS). During anoxia and light

exposure, the roots showed an altered metabolome whereas the leaves were only marginally affected, indicating that photosynthetically derived oxygen could satisfy the oxygen demand in the leaves but not in the roots. Nocturnal anoxia caused a biphasic shift in the metabolome of roots and leaves. The first phase, after 15 h under anoxia and 3 h of darkness showed a fast increase of lactate, pyruvate, GABA (γ -aminobutyric acid), succinate, alanine and a decrease in glutamate and glutamine. The second phase, after 21 h under anoxia and 9 h of darkness showed a decrease in lactate and pyruvate and an increase in alanine, GABA and succinate. This reprogramming of the metabolome after 21 h under anoxia indicates a possible mitigation mechanism to avoid the toxic effects of anoxia. A pathway enrichment analysis proposes the alanine shunt, the GABA shunt and the 2-oxoglutarate shunt as such mitigation mechanisms that alleviate pyruvate levels and lead to carbon and nitrogen storage during anoxia. This work demonstrates the applicability of metabolomics to assess low oxygen stress responses of *Z. marina* and allows us to propose an anoxia recovery model.

10. Pon. Subhashini, S. Raja, T. Thangaradjou, 2014, Seagrasses are known for their secondary metabolites of great commercial value and this study provides the first report on the establishment of cell suspension culture for a seagrass. Basal medium supplemented with 3% sucrose, 2,4-dichlorophenoxy acetic acid (2,4-D – 2.0 mg l⁻¹) and N6-benzylaminopurine (BAP – 1.0 mg l⁻¹) was used for callus induction, and same medium with 2,4-D (2.5 mg l⁻¹) was used for establishing cell suspension cultures of *Halodule pinifolia*. A growth curve was observed with a lag phase until day 12, a log phase from day 12 to day 24, and a stationary phase from day 24 to day 36 with an average absolute cell growth rate of the suspension culture of 0.425 biomass d⁻¹ and a doubling time (td) of 51 h. The growth curve was derived with a Michaelis constant, K_m (half the time required to attain the maximum cell cycle frequency) of 1.7597 h⁻¹ and a V_{max} (maximum cell cycle frequency) of 0.0016 h⁻¹, and the relationship between the cell growth and substrate hydrolysis was significant ($r^2 = 0.9206$). The histomorphological characterization of the established cell culture revealed different stages of the suspension of cells from a granular form of the suspension to a fine milky form. Consistent reproducibility of the growth pattern was observed in the suspension culture over one year so that this technique

promises to be a step forward to the in vitro production of potential natural products from seagrass, since it is more convenient for large scale production in bioreactors.

CHAPTER 3

AIM AND OBJECTIVE

AIM:

To perform metabolomic analysis of seagrass *Cymodocea rotundata* cellular suspension using "OMICS" approach.

OBJECTIVE:

- To induce callus and establish suspension cultured cells (SCC) of Seagrass *C. rotundata*.
- To analyse the metabolomic content of cellular suspension using GC – MS based metabolomic approach.
- To derive the heatmap from the metabolomic data of *C. rotundata* suspension cultured cells.
- To analyse and interpret the random forest analysis of top 15 metabolites classified from SCC of seagrass *Cymodocea rotundata*.

CHAPTER 4

MATERIALS AND METHODS

4.1 COLLECTION OF PLANT MATERIAL

Live and healthy seagrass *Cymodocea rotundata* were collected during the low tide from Olaikuda, Palk Bay region, Rameswaram by December 2022. Then the samples were washed with the native estuary water to remove epiphytes and extraneous matter. Then they were washed with the autoclaved seawater for various times. To prevent metabolite alterations driven by enzymatic reactions, the samples were instantly frozen using liquid nitrogen. After washing with distilled water those samples were shade dried for 4-5 days and further, they were made into fine course powder for further process. Seagrass sample were stored in the laboratory until consequent metabolite extraction.

4.2 CALLUS INDUCTION AND SUSPENSION CULTURED CELLS (SCC)

Induction of callus from *C. rotundata* was performed according to JEYAPRAGASH DANARAJ et al., 2018, with slight modifications. Leaves and rhizomes of *C. rotundata* were used as explant for callus induction. The explants were surface sterilized with 70% ethanol for 3 min and 0.6% sodium hypochlorite for 30 seconds followed by thorough washing with sterile seawater for 4-5 min.

These surface sterilized explants were transferred to sterile solidified MS medium (Murashige and Skoog, 1962; 15 mL) in culture tubes (125 × 125 mm; Borosil, India). The basal medium containing MS nutrients was supplemented with 4% sucrose, 0.8 % agar as gelling agent and the plant growth regulators (1.5 mg/L 2, 4 - dichlorophenoxy acetic acid and 1 mg/L 6-benzylaminopurine). Medium was prepared using autoclaved seawater (30 ppt), while the pH of medium was adjusted to 5.6 with 1 N HCl and autoclaved at 121 °C for 15 min. All the cultures were incubated at 23 ± 2 °C under cool white fluorescent light (35 mol m⁻¹ s).

Calli developed after two weeks at the edges of explants were excised and transferred to the fresh medium. Callus was maintained by sub-culturing after every

24 days in a fresh medium with the above same combination. The cellular suspension of *C. rotundata* was initiated by transferring the friable calli grown in MS agar medium to MS basal liquid medium with a salinity of 30 ppt, supplemented with 4 % sucrose, 2,4-D (1.5 mg L^{-1}) and BAP (1 mg L^{-1}). Histomorphological characterization of *C. rotundata* suspension cultured cells was examined using the light and fluorescence microscope.

4.3 METABOLITE EXTRACTION SCC OF *C. ROTUNDATA*

Metabolites from the SCC of *C. rotundata* were extracted according to JEYAPRAGASH DANARAJ et al., 2018, with slight modifications. Extraction process was carried out using three different solvents viz. chloroform (Chl), ethyl acetate (EtAc) and methanol (MeOH). SCC (10 g) of *Cymodocea rotundata* sample is shade-dried and is made into fine powder. Weigh 5g of the sample for each solvent. Dried seagrass samples were macerated with the respective solvents using motor and pestle. They were transferred to 100ml conical flask and add ~25ml of respective solvents to it. The extracts were then kept in the water bath for incubation at 40°C . for 1.5 hours. The incubated extracts were filtered using Whatman No.1 filter paper and collected.

4.3.1 ROTARY EVAPORATION

The filtrate collected using Whatmann filter paper was collected and the extract is concentrated by rotary evaporation. The extracts were poured into the vacuum flask (water bath at 45°C and cooling temperature at 10°C .) and is run at 100rpm. The crude extracts of the samples were obtained for the three different solvents viz. chloroform, ethyl acetate and methanol. Qualitative and quantitative analysis is carried out after diluting with respective solvents.

4.4 DERIVATIZATION

The derivatization is typically done to change the analyte properties for a better separation and also for enhancing the method sensitivity. Prior to GC –MS analysis derivatization is performed which may improve the capability of compound identification.

Derivatized extracts yield shelf lives of multiple years, eliminating the need to frequently re-prepare reference standards.

Before derivatization, the samples were placed in vacuum for 30 minutes to eliminate any trace of humidity. Derivatization was performed by using an aliquot of different solvent extracts by adding methoxamine hydrochloride (10mg/mL) in pyridine and shaking at 30 °C for 1 hr.

4.5 GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS

GC-MS was performed using Agilent 7890A- 5975C GC-MS system coupled with an HP-5MS capillary column with (30 m × 0.25 mm) fused silica coated with 0.25 mm CP-SIL 8 CB low beads. 1 µL of derivatized sample were injected in to the GC column using TriPlus auto sampler at a constant flow rate of helium of 1 mL min⁻¹. The injector was operated in a split less mode and maintained isothermally at 230 °C. Temperature for the MS transfer line to quadrupole was set at 280 °C, the electron impact (EI) ion source at 230 °C and the MS QUAD at 100 °C. Compound elution from plant extracts were set based on the following oven temperature program: injection temperature was set at 70 °C, followed by a 7 °C/min oven temperature gradient final to a 325 °C and then hold for 3.6 min at the same temperature. The temperature in the GC-MS system was equilibrated for 1 min at 70 °C before injecting the next sample. Ions are generated by 70 eV electron beam at an ionization current of 2.0 mA and the spectra was recorded 2.91 scans per second with a scanning range of 50-600 m/z. (Jeyapragash Danaraj et.al, 2020).

4.6 STATISTICAL ANALYSIS

All the experiments were performed in triplicates and the metabolomics data obtained from GC-MS was normalized for statistical analysis. Using Origin software (version 9.0), the one-way analysis of variance (ANOVA) was utilised for determining the significant differences between the means of tested groups by considering $p < 0.05$ as statistically significant. Using the software Metaboanalyst, total metabolites involved in the meatabolic pathways were investigated. A multivariate analysis known as Partial least square discriminant analysis (PLS-DA) was carried out for metabolomics data

using Origin 9.0 software. To determine the metabolites of *C.rotundata* of different solvent extracts hierarchical cluster analysis and heat map analysis was performed. For the classification and identification of top metabolites from the seagrass *C. rotundata* random forest analysis was used. In this study this analysis procured the most. (J. Danraj et al., 2020).

CHAPTER 5

RESULTS AND DISCUSSION

5.1 CALLUS INDUCTION OF *C. ROTUNDATA*

Though numerous studies have been reported on the biotechnological aspects of seagrasses, metabolomics analysis of in-vitro cells of seagrasses has not yet been studied. Hence, the present study succeeded with the metabolomics analysis of in-vitro cells of seagrass *Cymodocea rotundata*. An efficient and standardized protocol reported by Subhashini et al. (2014) was followed for callus induction and cellular suspension of *C. rotundata* with slight modifications. In the present study the induction of callus from *C. rotundata* was observed from the inter-nodal rhizome segments during 12th day after inoculation, when the medium supplemented with hormone concentration of 2,4-D (1.5 mg L^{-1}) and 6-BAP (1 mg L^{-1}). It was reported that the combination of auxin and cytokinin promote more calli than auxin or cytokinin alone (Cheng et al., 2006) and also been reported that the type and concentration of plant hormone alter the induction and growth of cells under in-vitro conditions (Mantel et al., 1983). The callus induced on 12th day might be due to the media preparation using sterile seawater with a salinity of 30 ppt which resembles the native forum for the explants. The calli induced was obtained from the inter-nodal segments of *C. rotundata* on the 12th day which was observed to be friable, yellowish and irregular and remained as same even after sub- culture. Salinity plays a vital role in the growth and productivity of seagrasses. Constant sub- culture of the friable calli was performed in the same medium in order to maintain the stock to establish cellular suspension. The initial sub – culture was performed by transferring the grown calli (30 days old) in Murashige- Skoog medium. Further subculture was carried out using the 21 days grown calli. (J. Danaraj, et al.)

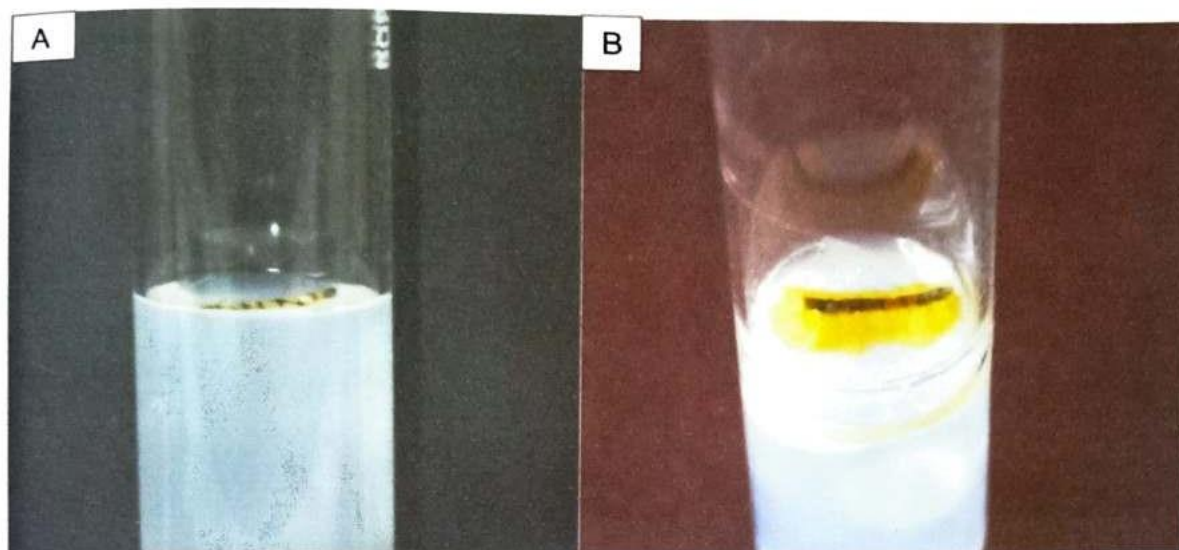


Figure 5.1 Callus induction from *C. rotundata*. A - Explant; B - Calli growth on 24th day.

5.2 SUSPENSION CULTURED CELLS

Suspension Cultured cells of *C. rotundata* can be established by transferring friable calli (after 12th subculture; each of 24 days) to MS basal liquid medium which has a pH of 5.6. The suspended friable calli in the liquid basal medium is easily broken and it can be dispersed to form larger clumps of cells till 5th day after inoculation. Upon further agitation, the disintegrated clumps turn into small cell aggregates which can be accomplished by a granular stage on 24th day. The repeated subculture after every 24 days resulted with the appearance of fine milky suspension culture. Findings of Wang and Smart (1999) and Subhashini et al. (2014) supports the present study that the addition of 2, 4-D stimulates the cells to grow continuously. Thick cell suspension was formed within 21 days after inoculation of callus.

This cellular aggregates of *C. rotundata* is slowly fragmented, forming pale yellow clumps. It was cautiously removed using sterile scalpel blade, as these clumps of cells multiply to form slimy layers in the walls of Erlenmeyer flask during agitation. Cell growth was demonstrated in liquid MS medium supplemented with a hormone concentration of 2, 4-D (1.5 mg L^{-1}) and 6-BAP (1 mg L^{-1}). Repeated sub culture resulted in the formation of milky white suspension culture which were maintained as stock. The cell aggregates of *C. rotundata* were observed under light microscope.

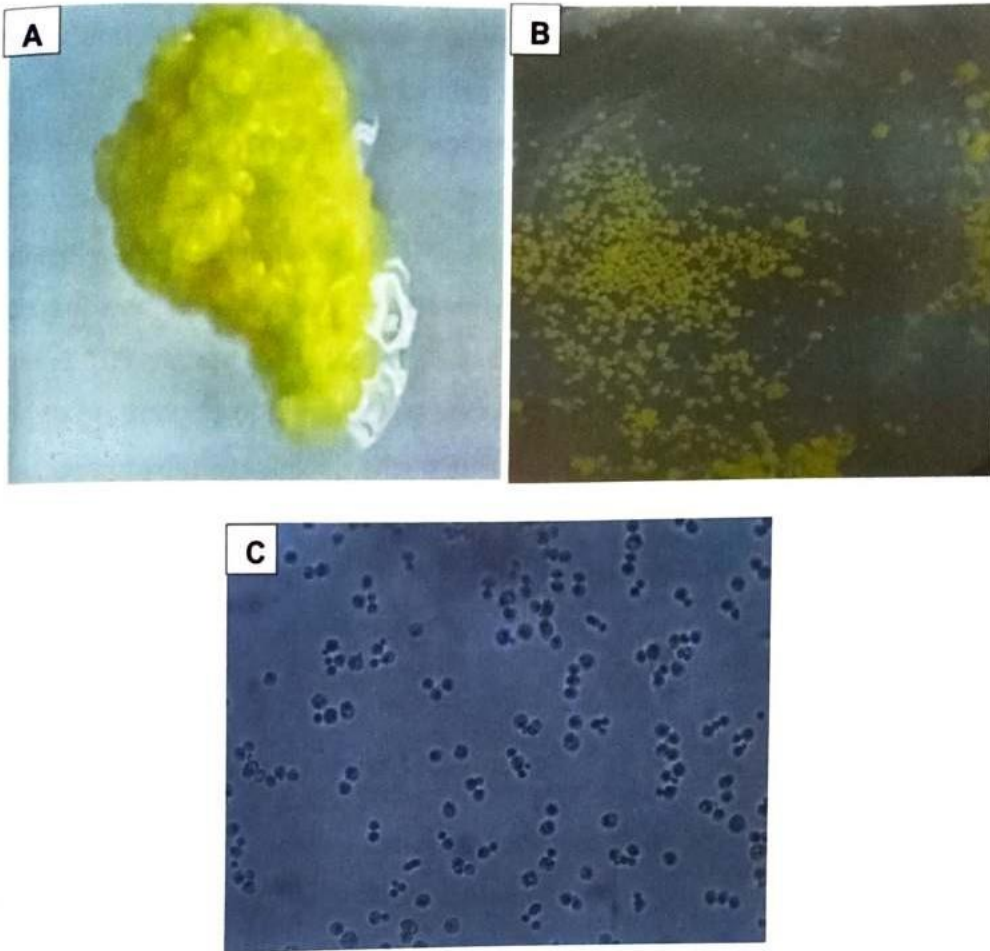


Figure 5. 2. A - Subculture of callus; B - Cellular suspension; C - Microscopic Observation.

5.3 METABOLOMIC ANALYSIS FOR SCC OF *C. ROTUNDATA*

The total ion chromatogram for all samples demonstrated a strong signals, large peak capacity and reproducible retention time indicating the reliability of metabolomics analysis. 125 metabolites were identified from cellular suspension of *C. rotundata*. The typical total ion chromatogram for each solvent extracts of SCC of *C. rotundata* was matched with an inbuilt GC- MS library and the compounds identified were matched with HMDB, KEGG and PubChem database. Metabolites which showed no differences between experimental groups were excluded and obtained 103 metabolites out of 125 in SCC respectively. These metabolites were found to be the part of primary and secondary metabolisms which includes organic acids, carbohydrates, lipids, amino acids, cofactors, prosthetic groups, electron carrier, nucleotides, peptides and plant hormones. The SCC extracts comprised of 63 organic acids, 14 amino acids, 4 flavonoids, 13 sugars, 5 plant growth regulators and 4 vitamins respectively (J. Danaraj, et al).

5.4 GC- MS ANALYSIS OF *C. ROTUNDATA*

To obtain the GC - MS derived metabolomic data, XCMS online and Metaboanalyst 4.0 software were used. Normalization was carried out for SCC experimental groups to make the data suitable for statistical analysis and in order to adjust the data for any experimental errors during the analytical run.

The chromatographic data were normalized in a row-wise manner to reduce systemic variance and to improve the performance for downstream statistical analysis. To reduce the complexity of metabolomics data and visualize samples grouping, supervised multivariate data analysis method Partial least square- discriminant analysis (PLS-DA) was performed using GC- MS normalized data derived from different solvent groups of SCC. The PLS-DA score plot and loadingplot of different solvent groups from wild and SCC was represented in Fig 5.3.

Results obtained from PLS – DA indicated that there were clear differences observed for the three different solvent groups in SCC. MeOH wild extract of *C. rotundata* could be separated in the PLS-DA score plot with EtAc and Chl extracts, while Chl extracts of SCC could be separated in the PLS-DA score plot with EtAc and MeOH extracts, despite minor overlap of EtAc with Chl sample.

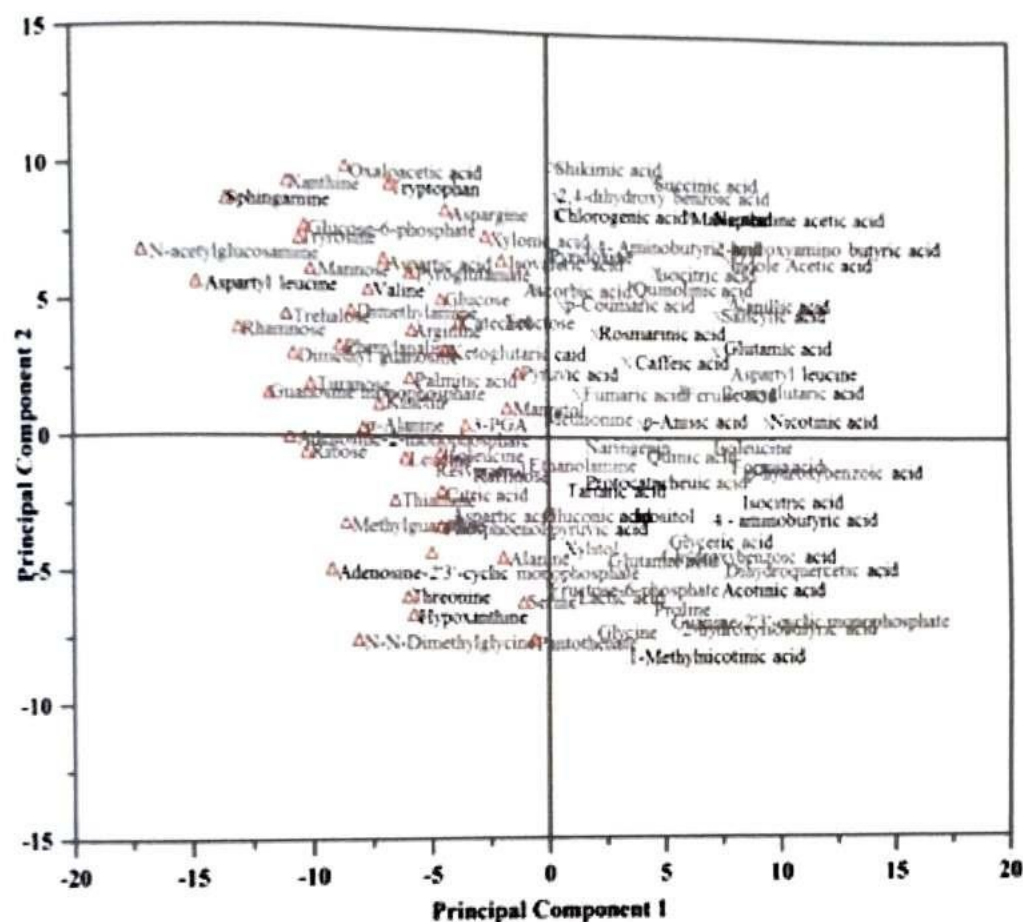


Figure 5.4 Partial least square-discriminant analysis (PLS-DA) of chloroform (Chl), Ethylacetate (EtAc) and Methanol (MeOH) extracts of *C. rotundata* SCC (Loading plot).

5.5 HEAT MAP HIERARCHICAL ANALYSIS

Heat map hierarchical clustering was performed to determine the cluster analysis of metabolite distribution with respect to different solvents. A heat map is a two-dimensional representation of data in which values are represented by colours.

Hierarchical cluster analysis of SCC sample was performed and the resulting dendrogram of six biological replicates of different solvent extracts showed that, EtAc and Chl extracts found clustered together than MeOH from SCC respectively. Also, our results showed that Chl and MeOH extracts were found SCC of *C. rotundata*. Thus, results obtained showed that the six biological replicates of MeOH extraction in SCC dominated with a greater number of metabolites such as organic acids, includes p-anisic acid, caffeic acid, p-coumaric acid, rosmarinic acid, protocatechuic acid, and flavonoid includes naringenin and vitamins than the Chl and EtAc extracts, whereas Chl and EtAc extracts dominated with lipids and amino acids respectively. (J. Danaraj, et al.)

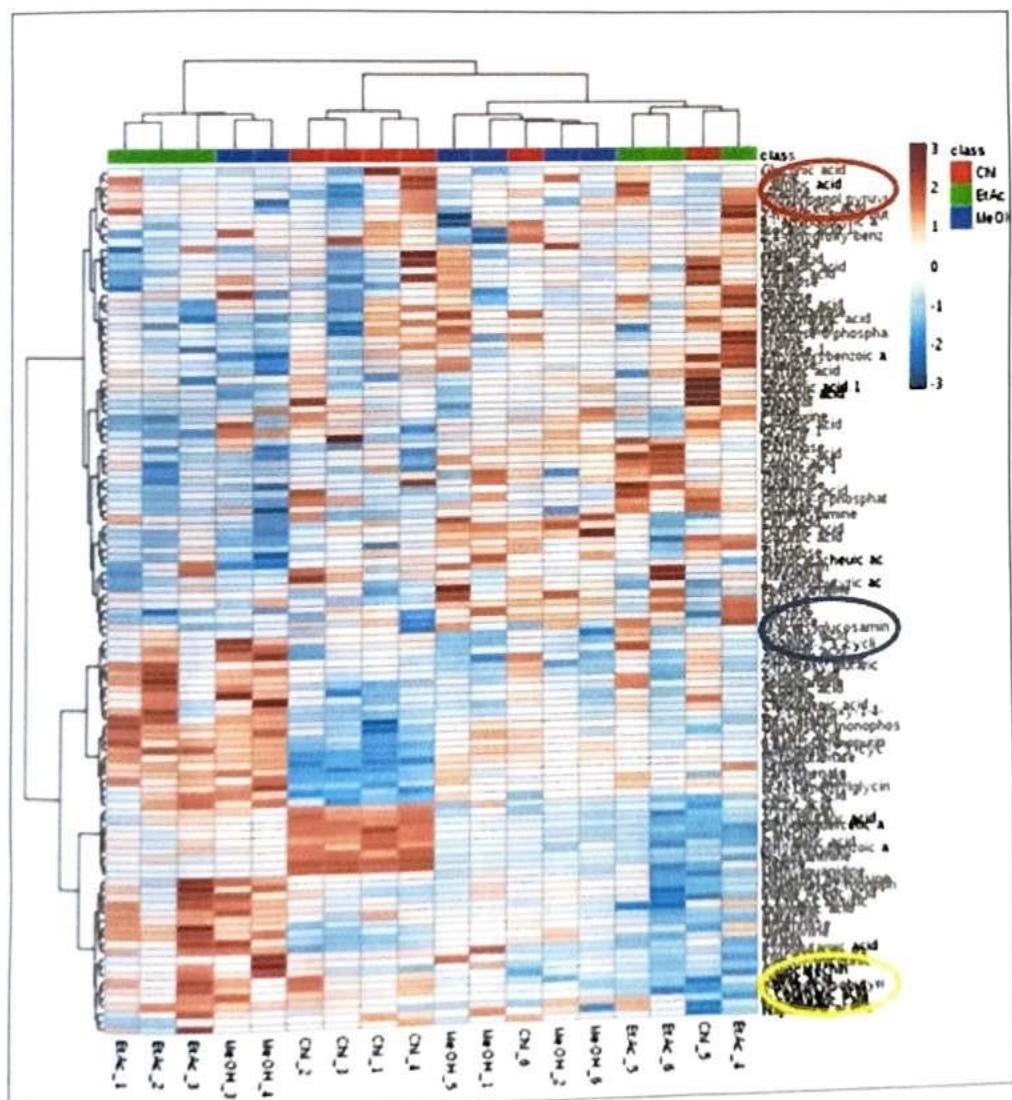


Figure 5.5 Heatmap hierarchical cluster analysis of different metabolites for different solvent extracts of *C. rotundata*.

5.6 ENRICHMENT PATHWAY ANALYSIS

Metaboanalyst software was used to investigate the list of important metabolites in the dataset, using the 'pathway analysis' option. Secondary metabolisms involving organic acids, amino acids accounts for 76.23 % of the total metabolites which are mainly participated in the pathways. Bestowing to annotations in Kyoto Encyclopaedia of Genes and Genomes (KEGG Compound Database, and in the PlantCyc, the identified metabolites were mapped to general biochemical pathways. The metabolites derived from the different solvent extracts of SCC C. rotundata undergoes 73 metabolisms in SCC.

Some of the major metabolism occurred in the SCC sample include tyrosine and phenylalanine metabolism, citric acid metabolism, glycolysis, gluconeogenesis, respectively. These pathways are mapped to most of the primary and secondary metabolisms.

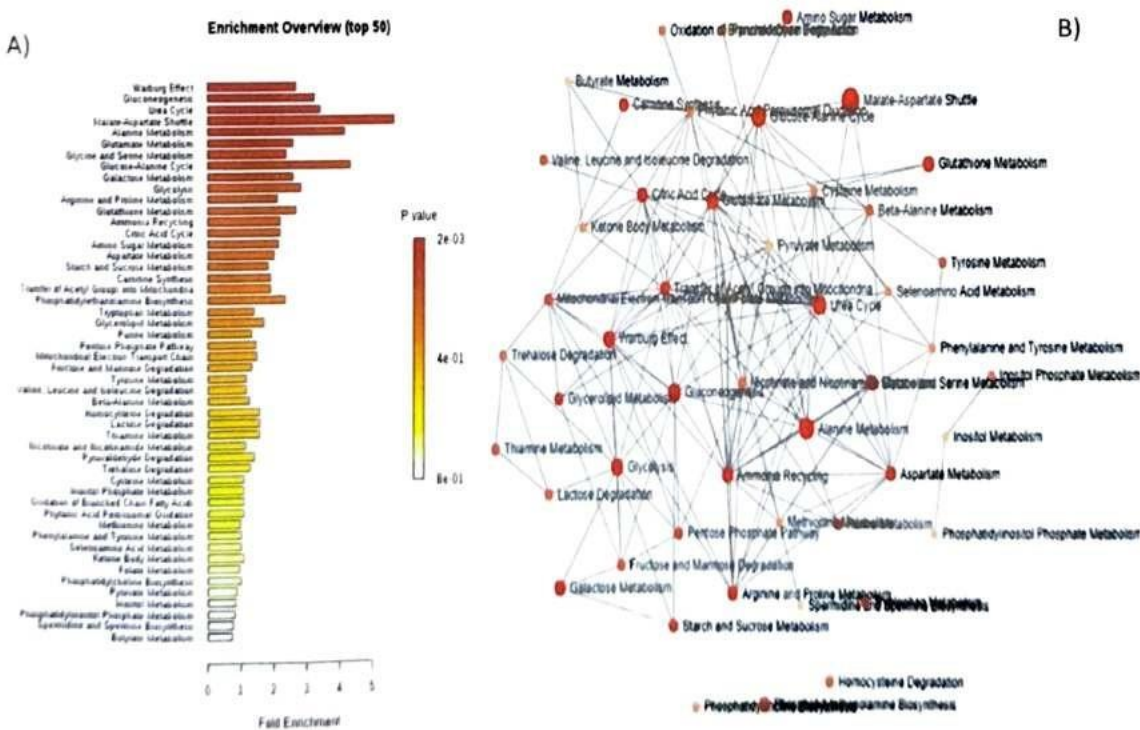


Figure 5.6 Enrichment pathway analysis for the extracts of C. rotundata. A. Enrichment overview for the top 50 metabolites; B. Enrichment metabolic interaction pathways of the metabolites.

5.7 SPECIFIC PATHWAYS RELATED TO FUNCTIONAL EFFECTS

Random forest (RF) analysis was performed to assess the compounds which contributed most between the different solvent extracts of SCC. Organic acids and amino acids are inclined to dominate the separation of different solvent extracts in SCC and the probability obtained was 81 % in SCC. Mean Decrease Accuracy (MDA) were used to identify the metabolites (variables) that contributed greatest in SCC of *C. rotundata*. The variability of organic acids such as p-anisic acid, pcoumaric acid, caffeic acid, vanillic acid, rosmarinic acid, protocatechuic acid, 4- hydroxybenzoic acid and naringenin were low in SCC of *C. rotundata* and contributed most in the sample. (J. Danaraj, et al.)

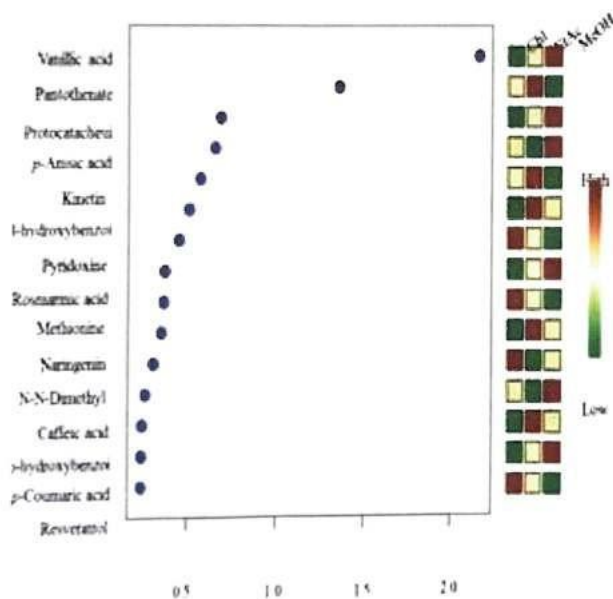


Figure 5.7 Random Forest analysis of top 15 metabolites classified from the extracts of *C. rotundata*. Coloured boxes indicate the relative concentrations of the corresponding metabolite in each group under current study (red, up-regulation; green, down-regulation).

A diverse group of metabolites that features to various bioactive potential agents against various pathogens and oxidative stress in the marine environment can be

harboured from seagrasses. Many studies have reported that seagrass shows defensive mechanism against various stresses at morphological and physiological levels, but there are no reports to explore the metabolic changes of seagrass *C. rotundata* using metabolomics.

Here we have reported a comprehensive analysis of metabolomics in the SCC of *C. rotundata* using three different solvents namely, CH₂Cl₂, EtAc and MeOH, each of with six biological replicates were used since the number of replicates should be even higher in order to derive in-depth statistical analysis of datasets emerging from the analysis. The results of the present study identified 107 metabolites in SCC of *C. rotundata*, which could cause many metabolite variations mainly sugars, flavonoids, aminoacids and organic acids. It was noted that the metabolites profiled from SCC of *C. rotundata* were found intracellular.

Some of the metabolites derived from the SCC of *C. rotundata* found similar to that accumulated in wild seagrass, most compounds produced by SCC were dependent on the environmental factors maintained under in-vitro conditions. Ning et al. (2018) identified 197 metabolites with a significant difference between the three lines of non-transgenic and multi transgenic polar cambium in which the three major metabolic groups aminoacids, lipids and carbohydrates accounted for more than 70 % of the identified metabolites. In contrast, the present study primarily involves secondary metabolism which accounted for 70 % than primary metabolisms. Li et al. (2013) identified 104 metabolites which significantly differs among different soyabean cultivars, with large number of these metabolites includes amino acid, carbohydrates and lipids, whereas Michaletti et al. (2018) reported that the content of amino acids, organic acids and sugars change in response to water deficiency in two spring-wheat cultivars. Levandi et al. (2008) used capillary electrophoresis (CE) in order to compare the difference of metabolites between three Bt (*Bacillus thuringiensis*) transgenic lines and common maize found that some metabolite significantly differs in their contents.

The level of metabolite accumulation like the accumulation of glucose, galactose and fructose in SCC extracts of *C. rotundata* found out to be enhanced, suggesting that photoassimilates were stored as hexoses in SCC. Since, the plants have evolved with intricate mechanisms can respond to and defend various stress factors and was reported that photoassimilates were stored in the form of hexoses and complex sugars in plants during stress conditions which found to act as osmoprotectant which plays a

crucial role in osmotic adjustments or protection of cellular constituents (Kieffer et al., 2008). Many plants have been shown to accumulate the aminoacids content when they undergo stresses, which can affect the regulation of ion transport, synthesis and activity of antioxidant enzymes, modulating the stomatal opening and sequestration of toxic ions in the cytosol or vacuole (Rai et al., 2002). The present study showed that the content of amino acids in SCC found to be lower than organic acids and sugars which resemble the study of Wang et al. (2015). Proline is one among the aminoacids detected in SCC of *H. pinifolia* which acts as an osmolyte and reactive oxygen species scavenger was found to increase during stress conditions, which would positively enhance the plant survival in its natural and harsh habitats (Verbruggen and Hermans, 2008; Szabados and Savoure, 2009; Burg and Ferraris, 2009). It has also been observed that organic acids such as p-anisic acid, caffeic acid, p-coumaric acid, vanillic acid, rosmarinic acid, protocatechuic acid, 4-hydroxybenzoic acid and flavonoid naringenin found accumulated at a higher concentration in SCC samples of *C. rotundata*. Rosmarinic acid was synthesized by phenylalanine and tyrosine metabolism, which acts as an ester of caffeic acid accumulated at a maximum concentration than other organic acids studied

This study also mapped the identified metabolites involved in different metabolic pathways using KEGG and the Plant Cyc metabolic database. The analysis integrating metabolomics data demonstrated that SCC caused significant variations in glutathione metabolisms, amino-sugar metabolism, phenylalanine and tyrosine metabolism and oxidative phosphorylation related pathways. The SCC of *C. rotundata* have undergone glutathione metabolism (GSH), as it could play a crucial role in stress tolerance in plant kingdom.

CHAPTER 6

SUMMARY AND CONCLUSION

Metabolomics is one of the new field of "omics" approach and youngest of the triad of system biology, emerged as a complementary tool to functional genomics that together with well-established technologies for high-throughput DNA sequencing (genomics), mRNA expression profiling (transcriptomics), and protein profiling (proteomics). Studies on the plant metabolome brought huge challenges to analytical technologies which have been employed in current plant metabolomics programs.

Untargeted metabolomics allows for the investigation of metabolic phenotypes involving several hundreds to thousands of metabolites. In this approach, all signals in a mass chromatogram are processed in an unbiased way, allowing for a deeper investigation of metabolic phenotypes, but also resulting in significantly more complex data processing and post-processing steps. Much progress has been made in this field over the past decade and yet plant metabolomics seems to still be an emerging approach because of the high complexity of plant metabolites and the number one challenge of untargeted metabolomics, metabolite identification.

Seagrasses are marine angiosperm. It comprises one of the most abundant and important ecosystems in the marine environment with high primary productivity. Climate change, now being the world's major issue and is a main factor contributing to the loss of this seagrass ecosystem. Studies related to this will facilitate for the conservation of the seagrass.

This study reports the metabolomics responses by GC-MS for SCC extracts of seagrass *C. rotundata* showed different but overlapping metabolic alterations mainly on sugars, amino acids and organic acids. These findings may provide useful information to understand the metabolite networks, their variations and metabolic pathways involved in the *C. rotundata* grown under both natural and artificial environment. GC-MS based metabolomics data obtained in this study may also be used as baseline information to study the metabolites associated with stress response which would facilitate further dissection of the mechanism of stress tolerance of other seagrass species in the marine environments.

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