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Variations in δ^{13}C Rates and Crassulacean Acid Metabolism of Six *Coleus* species

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors contributed equally for this study. Authors affirm that this work is in genuine. Both authors read and approved the final manuscript.

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ABSTRACT

Photosynthetic adaptations and biomass productivity of six *Coleus* species namely *Coleus aromaticus*, *spicatus, blumei, zeylanicus, forskholii,* and *ambonicus* were studied by determining their photosynthetic rates, Crassulacean acid metabolism (CAM) and carbohydrate levels. CAM metabolism was investigated by studying the activities of phosphoenol pyruvate carboxylase (PEPC), PEP carboxykinase and diurnal fluctuations of malate dehydrogenase along with levels of total organic acids, malic acid and citric acids. Carbohydrates such as sucrose, starch, glucose and fructose were assayed spectrophotometrically. Significant differences among the six Coleus species were observed in photosynthesis levels and CAM metabolic pathway enzymes whose activities were correlated with the aerial biomass. Our study indicates that *Coleus* is a CAM plant with nicotineamine Adenine Di nucleotide (Phosphate) (NAD (P)) as a preponderant malic enzyme and diurnal fluctuations in the levels of carbohydrates reveals that *Coleus* utilizes reservoir of starch for the synthesis of malic acid. Δ^{13}C accumulation is more in *Coleus aromaticus* and *ambonicus* whose aerial biomass values are also high indicating the correlation between carbon fixation and biomass accumulation.

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Keywords: Aerial biomass; CAM path way; carbohydrates; Coleus species; photosynthetic carbon fixation; organic acids.

ABBREVIATIONS

CAM: Crassulacean Acid Metabolism; PEPC: Phosphoenol Pyruvate Carboxylase; PEPCK: Phosphoenol Pyruvate Carboxykinase; MDH: Malate Dehydrogenase; NAD(P): Nicotineamine Adenine Dinucleotide (phosphate).

1. INTRODUCTION

Crassulacean Acid Metabolism (CAM) is one of the three forms of the photosynthetic carbon assimilation and is considered as one of the important models for probing the evolution of complex traits in response to changing environment [1]. CAM form of carbon assimilation has been characterized in 16,000 species of 328 genera in 33 families including Crassulaceae, Lamiaceae, Polypodiaceae etc. [2]. The mechanism of CAM is typically characterized by the acquisition of nocturnal CO_2 *via* protein kinase activated phosphoenol pyruvate carboxylase (PEPC), leading to the formation of organic acids mainly malic acid, which are stored in the large central cell sap vacuoles. In the subsequent light period, organic acid is released from the vacuole, decarboxylated to release CO_2 for assimilation in the Calvin cycle behind closed stomata [3]. Based on the differences in the decarboxylation of malic acid, three subtypes of CAM plants are known, NAD-ME type, NAD(P)-ME type and PEPCK type [4]. CAM plants perform nocturnal recycling of CO_2 through malic acid for the purpose of photosynthesis.

CAM plants possess high efficiency in water utilization by losing 50-100g of water per 1g of CO_2 fixed in comparison to the 250-300g of water by the C4 and 400-500 gm of water by the C3 plants respectively [5]. CAM plants contain scotoactive stomata, which opens during night facilitating the CO_2 leading to reduce photorespiration. CAM plants are also provided with various morphological, anatomical features such as thick cuticles, low surface-to-volume ratios, large cells and vacuoles with enhanced water storage capacity (succulence), reduced stomatal size and frequency, which minimizes water loss [6].

Coleus (Lamiaceae) is an important plant used traditionally in folk medicine in India due to its potential medicinal value as an expectorant, for the treatment of bronchitis, eczema and rheumatism. *Coleus aromaticus* and *Coleus zeylanicus* are aromatic perennial herbs that are native to Indonesia. *C. aromaticus* is used for chronic cough and asthma. It is considered to be antispasmodic, stimulant and stomachic and is used for the treatment of headache, fever, epilepsy and dyspepsia. *Coleus blumei* is a perennial native to Java, but now spread over many parts of tropical Africa, Europe and Asia, popular as an ornamental plant. *Coleus forskohlii*, an Indian herb, produces the labdane diterpenoid forskolin in its tuberous roots, which has been shown to be a hypertensive agent with spasmolytic, cardiotonic and platelet aggregation inhibitory activity, antiglaucomic in nature and a potent stimulator of the enzyme adenylate cyclase, which increases the levels of cAMP affecting cardiac muscle contraction, blood and intraocular pressure, cancer, eczema, rheumatism and obesity [7]. The medicinal properties of this plant have been well studied but the photosynthetic performance of this CAM plant was not studied. Previous studies have mentioned the role of environment on gas exchange and CAM mechanisms of *Plectranthus marrubioides, P. parviflorus and P. prostratus* (*Lamiaceae*) [8,9]. Here, we report the photosynthesis and CAM mechanisms of six *Coleus* species, *C. aromaticus, C. spicatus, C. blumei, C. zeylanicus, C. forskholii,* and *C. ambonicus* and their aerial biomass levels. Characterizing the *Coleus* species depending on the mechanism of CAM photosynthesis, which utilizes either malate or PEPCK and identification of starch or sucrose carbohydrate reservoir pool utilized for the carbon fixation, are few of the important aspects of the present study.

2. MATERIALS AND METHODS

2.1 Plant Material and Growth Conditions

Six *Coleus* species namely *C. aromaticus, C. spicatus, C. blumei, C. zeylanicus, C. forskholii, and C. ambonicus* were propagated in the GITAM University botanical garden, Visakhapatnam, India in 30 cm pots under 12h natural photoperiod [1600-1800 μ moles m^{-2} s^{-1}] with day/night temperatures of 30°C/23°C and

approximate relative air humidity of 60%. The plants were well watered with normal tap water. Five pots were used for each *Coleus* species and one plant is grown per pot. Third or fourth fully expanded leaf from the top of the 3-month-old plant was collected for all physiological experiments and aerial biomass measurements made by measuring the mass of the above ground plant.

2.2 Carbon Isotope Determination

Samples were prepared by drying the *Coleus* leaves in a hot air oven at 80°C for 72h. Completely dried leaves were grinded into fine powder in a mortar with pestle. 2 mg dry powder of each *Coleus* species leaf was subjected in the Flash Elemental Analyzer (NA 1112, Carlo Erba, Italy) interfaced to an Isotope Ratio Mass Spectrometer (IRMS; Delta-Plus, Thermo-Finnigan, Bremen, Germany) *via* a continuous flow device (Conflo-III). The carbon isotopic composition of plant samples ($\delta^{13}C_p$) was determined with an analytical precision of less than 0.1‰. Carbon isotope discrimination (Ä $\Delta^{13}C$) was computed according to [10] assuming the isotopic composition of atmospheric air ($\delta^{13}C_a$) to be –8‰.

$$\Delta^{13}C\ (‰) = [\delta^{13}C_a - \delta^{13}C_p]/[1 + \delta^{13}C_p/1000].$$

2.3 Extraction and Assay of Enzymes

All extractions were performed at 4°C. The leaf blades (10 g) were homogenized in a grinding medium consisting of 100 mM Tris-HCl (pH 7.8), 5 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM magnesium acetate and 1.5% PVP at 4°C. The homogenate was squeezed through four layers of cheesecloth and then centrifuged at 10000 g for 10min. The solution was filtered to remove the cellulose and washed thrice with the extraction medium. The protein was precipitated with 75% (m/v) ammonium sulphate and spun at 30000 g for 30 min and the precipitate was dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM DTT and 0.2 mM NADPH.

PEPC (EC 4.1.1.31) was assayed spectrophotometrically at 340 nm at 30°C in a final volume of 3ml following the reduction of NADH [11]. Assay buffer comprises 0.1M Tris-HCl (pH 7.8), 10 µM $MgCl_2$, 10 mM $NaHCO_3$, 5mM PEP, 0.5 µM NADH and 0.2 ml of enzyme extract. Malate dehydrogenase (EC 1.1.1.37) was assayed according to [12]. Assay buffer comprise of 0.1M Tris-HCl (pH 7.8), 0.5 µM oxaloacetic acid, 10 µM of $MgCl_2$, 0.4 µM NADH and 0.2 ml of enzyme extract. PEPCarboxykinase (EC 4.1.1.49) was assayed spectrophotometrically [13]. Carboxylation activity was measured following the oxidation of NADH by malate dehydrogenase at 340 nm using 100 mM HEPES (pH 7.0), 4% 2-mercapto ethanol, 100 mM KCl, 90 mM $KHCO_3$, 5 mM PEP, 1 mM ADP, 10 µm $MnCl_2$,4 mM $MgCl_2$, 0.14 mM NADH and 6 units of malate dehydrogenase.

NAD-ME (EC 1.1.1.38) was assayed spectrophotometrically at 340 nm [14] using 5mM malate, 25mM HEPES- KOH of pH 7.2, 2 mM NAD, 2.5 mM $MnCl_2$, 5mM DTT and 500 µM fructose bis phosphate and 0.2 ml enzyme extract in a volume of 1 ml assay mixture. NADP-ME (EC 1.1.1.40) was assayed spectrophotometrically at 340 nm [15]. Assay mixture contained 50 mM Tris-HCl (pH 7.3), 10 mM $MgCl_2$, 0.5 mM NADP and 10 mM l-malate with 0.2 ml of enzyme extract.

2.4 Titratable Acidity, Malic Acid and Citric Acid Contents

Day and night oscillations of titratable acidity were measured using fully expanded *Coleus* leaves collected during 8 A.M. and 8 P.M. [16]. 1g of leaf tissue was cut into small pieces, boiled in a beaker with 50 ml of distilled water for 30 minutes and the slurry was filtered. 2.5 ml of extract was neutralized with 5 ml of 1N NaOH for 15 minutes and 1 drop of phenolphthalein was added. The contents were titrated against 1N HCl till the solution becomes colour less [17].

Content of malic acid was determined according to [18]. 1 gram leaf sample was homogenized in a mortar and pestle with 0.6N perchloric acid. The sample was centrifuged at 15000g for 5 minutes. 1 ml of supernatant was neutralized with 80 µl of 5M K_2CO_3, centrifuged at 15000 g for 5 min. 0.5 ml of supernatant was incubated with 1.5 ml of assay buffer containing 0.5 M glycine, 0.4 M hydrazine (pH 9.0) and 0.1 ml of 40 mM NAD. The reaction mixture was pre-incubated for 30 min at room temperature before the addition of 5 µl of malate dehydrogenase and the increase in the absorbance at 340 nm due to the production of NADH was measured.

2.5 Carbohydrate Contents

The contents of starch and sucrose in the leaf tissues were estimated enzymatically according to the method of [19]. For estimation of starch,

eight leaf discs of 1 cm diameter were extracted with 80% ethanol 4-5 times at 80°C. Extracts were pooled and stored for the sucrose estimation. Ethanol extracted leaf discs were suspended in 1 cm^3 of 0.2 M KOH and boiled for 30 minutes. Tubes were cooled to room temperature and 0.2 cm^3 of 1 M acetic acid was added to each tube and incubated at 55°C for 30 minutes to hydrolyze starch. Reaction was stopped by boiling the tubes for 1 minute. 0.2 cm^3 of the extract was added to 0.2 cm^3 of distilled water and 1cm^3 of glucose enzyme reagent (Sigma). Tubes were incubated at 37°C for 20 minutes and the absorbance was read at 492 nm. For estimating the sucrose contents, ethanolic extracts were used. 0.2 cm^3 of the extract was added to 0.3 cm^3 of glucose reagent and incubated at 37°C for 30 minutes. 75 mm^3 of invertase was added and incubated at 37°C for 30 minutes. The absorbance was read at 492 nm. The reading was proportional to the original plus glucose liberated due to the invertase action on sucrose. To determine the sucrose contents, the assays were run simultaneously without invertase for glucose contents. Sucrose standards were determined by comparing the difference in the absorbance of the two samples with that obtained from sucrose standards.

The glucose and fructose contents in the 80% ethanolic extract were determined according to [20] and [21] respectively. 500 mg of leaf tissue was homogenized in 80% ethanol and centrifuged at 5,000 rpm for 10 min. For glucose estimation, 1 ml of supernatant was added to 5 ml of anthrone reagent and incubated in boiling water bath for 30 minutes. The tubes were cooled to room temperature and the absorbance was measured at 620 nm. Fructose was estimated by adding 1 ml of resorcinol reagent to 2 ml of the ethanolic extract and incubating the tubes in boiling water bath for 10 minutes. After cooling, absorbance of the sample was measured at 520 nm. Glucose and fructose concentrations were calculated using a standard curve.

2.6. Statistical Analysis

Statistical analysis of absolute values was performed by analysis of variance (ANOVA). The values are the mean ± SD for five samples in each group. The significance of the data was tested by student's *t* test. *P* values greater than 0.05 were considered as significant.

3. RESULTS AND DISCUSSION

Among the six *Coleus* species considered for this study, *C. aromaticus* and *C. ambonicus* possessed higher $\Delta^{13}C$ values and biomass yields than other species (Table 1). A significant variation in the stable isotope levels was observed among the *Coleus* species which is correlated with the PEPCarboxylase and malate dehydrogenase activities (Figs. 1 and 2). PEPCarboxykinase activities of six *Coleus* species were compared with NAD-ME and NADP-ME (Fig. 3) in which, *Coleus aromaticus* and *ambonicus* were found to possess high NADP-ME enzyme activity where as *spicatus, blumei, zeylanicus, forskholii,* have shown NAD-ME activity indicating *Coleus* plants are NAD(P)-ME type of photosynthesis carbon fixation. Day-night studies of the organic acid levels in six *Coleus* species reveals that concentrations of titrable acids, malic acid and citric acid were high during night time which were reduced during day, correlating with malate dehydrogenase activities (Figs. 4, 5 and 6). Levels of carbohydrates such as glucose and sucrose were higher than that of starch in all *Coleus* species considered for this study and the sugar levels were high during night than day time (Fig. 7). Our studies on the overall photosynthesis activities, accumulation of organic acids and carbohydrate levels have shown that *C. aromaticus* and *C. ambonicus* were showing higher photosynthetic performance and biomass yields compared with other four species.

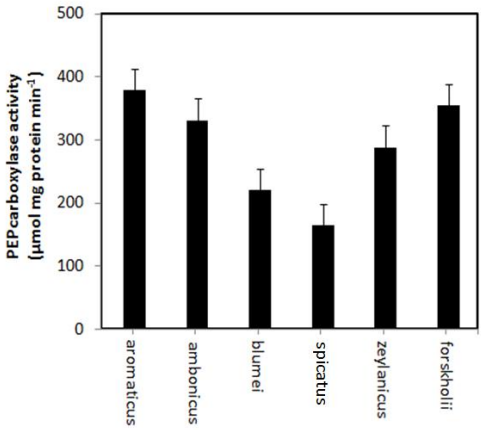

Fig. 1. PEPCarboxylase activity in 6 different *Coleus* species. Each point is an average of five independent determinations ± SE, ($t_{(4)} = 9.2$, $P < 0.05$)

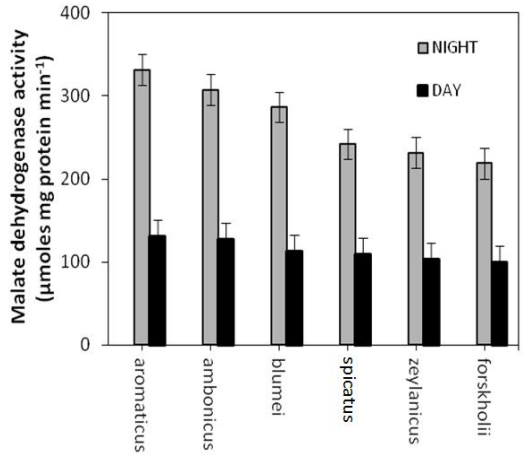

Fig. 2. Day and night activities of Malate dehydrogenase in 6 different *Coleus* species. Each point is an average of five independent determinations ± SE, ($t_{(4)} = 8.4$, $P < 0.05$)

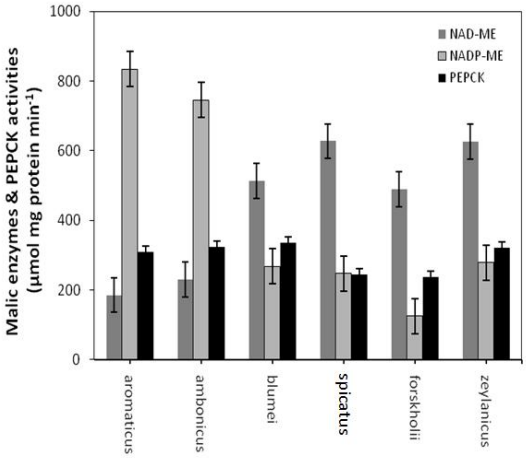

Fig. 3. Activities of malic enzymes NAD-ME, NADP-ME and PEPCK in 6 different *Coleus* species. Each point is an average of five independent determinations ± SE, ($t_{(4)} = 6.2$, $P < 0.05$)

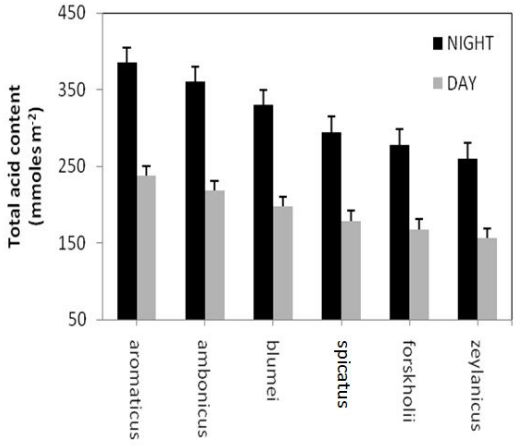

Fig. 4. Total organic acid content among six Coleus species during day and night. Each point is an average of at least five independent determinations ± SE, ($t_{(4)} = 11.2$, $P < 0.05$)

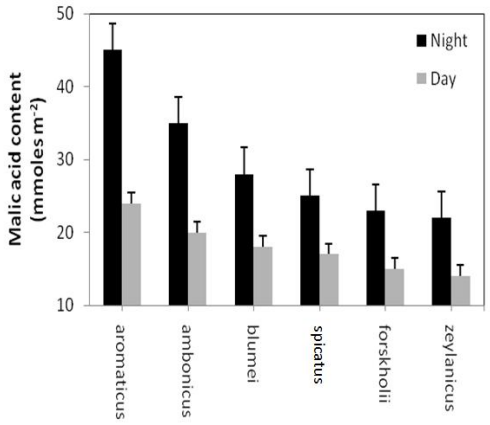

Fig. 5. Variations in the day and night Malic acid contents of six *Coleus* species. Each point is an average of at least five independent determinations ± SE, ($t_{(4)} = 4.9$, $P < 0.05$)

CAM photosynthesis is one of the remarkable examples of ecophysiological adaptations of plants to arid conditions [22]. CAM is an important elaboration of photosynthetic carbon fixation that facilitates nocturnal CO_2 fixation initially using PEPC in the cytosol leading to the formation of C4 organic acids, which gets stored in the vacuole followed by subsequent decarboxylation during day time behind closed stomata, generating an internal CO_2 source that is re-assimilated by Rubisco in the chloroplast.

In the present study, the $\Delta^{13}C$ accumulation of carbon in the leaves of six *Coleus* species was studied, whose values are ranging from -22.94‰ to -20.09‰ respectively (Table 1). $\Delta^{13}C$ values in the CAM plants *Tillandsia usneoides*, *Didierea madagascariensis* and *Microsorium punctatum* were reported to be -19.8‰ [23], -21.2‰ [24], and -22.6‰ [25] respectively. ^{13}C values in CAM plants serves as an indicator of their biomass derived from nocturnal CO_2 fixation compared to that of diurnal CO_2 fixation because PEP

carboxylase that fixes nocturnal CO_2 discriminates slightly higher against ^{13}C than Rubisco, which is responsible for the day time CO_2 uptake [26]. CAM plants gain maximum CO_2 during night by PEPCarboxylase activity; their biomass levels will be similar to that of C4 plants. The aerial biomass levels were measured in six *Coleus* species, which shows that *aromaticus* and *ambonicus* possessed high aerial biomass values than *blumei, spicatus, forskholii* and *zeylanicus* respectively (Table 1).

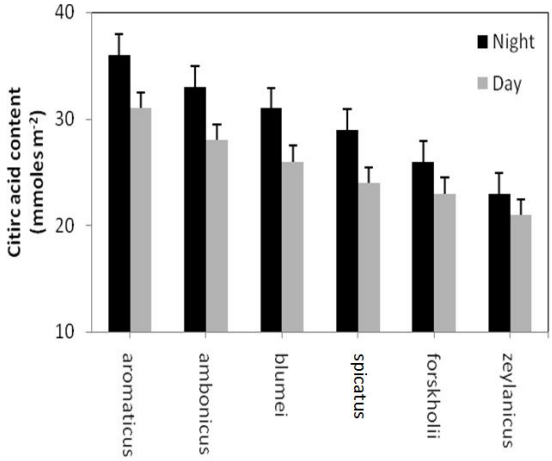

Fig. 6. Day and night variations in the citric acid contents of six different *Coleus* species. Each point is an average of at least five independent determinations ± SE, ($t_{(4)}$ = 5.6, P < 0.05)

CAM is a well known water conserving modification of photosynthesis characterized by the nocturnal CO_2 assimilation catalysed by the enzyme PEPCarboxylase leading to the synthesis of organic acids [27]. Activity of PEP Carboxylase estimated in the six *Coleus* species was depicted in Fig. 1, which shows that *C. aromaticus* and *ambonicus* were possessing high PEPC activities when compared with the other *Coleus* species.

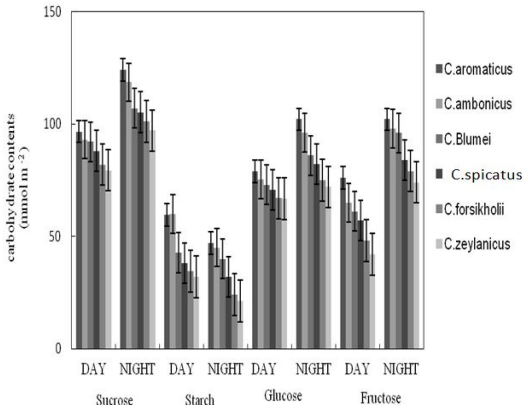

Fig. 7. Carbohydrate levels in six different *Coleus* species during day and night. Each point is an average of at least five independent determinations ± SE, ($t_{(4)}$ = 10.2, P < 0.05)

Malate dehydrogenase (MDH) is a ubiquitous enzyme, which serves in a wide range of metabolic processes of plants. MDH participation has been identified in key metabolic pathways such as Krebs cycle, photorespiration and metabolite shuttling [28]. MDH plays a remarkable role in the CAM plant's photosynthesis carbon fixation by reducing oxaloacetic acid to malic acid in CAM plants [29]. In the present study, day and night activities of MDH in six *Coleus* species were studied (Fig. 2). The night time activity of MDH was high in *aromaticus* (331.65 μ moles mg protein min^{-1}) and *ambonicus* (307.4 μ moles mg protein min^{-1}) compared to that of the other four species. The day time activities were less among all *Coleus* species without much difference in their activity (Fig. 2), which shows the limited role of MDH during day time in *Coleus*. NAD-MDH has been isolated, purified and it's day and night fluctuation has been studied in *Ananas comosus*, a PEPCK type of CAM plant [30].

Table 1. $\Delta^{13}C$ (‰) Carbon isotope levels and aerial biomass contents in 6 *Coleus* species. Each value represents an average of five independent determinations ± SE, ($t_{(4)}$ = 3.2, P < 0.05) and ($t_{(4)}$ = 7.4, P < 0.05).

Name of the plant		$\Delta^{13}C$ (‰)	Aerial biomass (g)
Coleus aromaticus	-22.94	± 0.65	793±1.12
Coleus ambonicus	-22.75	±0.81	721±0.89
Coleus blumei	-22.35	±0.10	672±0.55
Coleus spicatus	-22.06	±0.23	599±1.47
Coleus forskholii	-20.37	±0.45	591±0.84
Coleus zeylanicus	-20.09	±0.63	572±0.45

Malic acid released from the vacuoles during day time immediately undergoes decarboxylation reaction specific to NAD or NADP in malic enzyme type CAM plants, yielding pyruvate and CO_2. In PEPCK type CAM plants, malic acid will be converted to OAA prior to its decarboxylation, yielding PEP and CO_2. In the present study the activities of NADP-ME, NAD-ME and PEPCK were studied in six *Coleus* species (Fig. 3). *C. aromaticus* and *ambonicus* were possessing high rates of NADP-ME activity where as *C. blumei*, *spicatus*, *forskholii* and *zeylanicus* were exhibiting high NAD-ME activity indicating that *Coleus* is a CAM plant with NAD(P)-ME as preponderant enzyme (Fig. 3). The activities of NADP-ME were high in *Coleus*, when compared to that of NAD-ME and PEPCK. Species variation with respect to PEPCK enzyme was observed in *A. comosus* and *C. lycopodioides* with lower oxidation of malic acid [31].

Malic acid is a ubiquitous vacuolar anion, synthesized nocturnally as a result of CO_2 fixation and stored in the vacuoles. Malic acid is a central intermediary in the process of CAM photosynthesis. Nocturnal malic and citric acid accumulation decreases the osmotic potential and enhances water uptake [32]. Daytime decarboxylation of organic acids will increase internal CO_2 concentration enabling CAM plants to close stomata, at least partially and facilitating the maintenance of high photosynthesis rates [33]. In the present study the day time and night time levels of organic acids such as titrable acid number (TAN), malic acid and citric acid were estimated in six *Coleus* species (Figs. 4, 5 and 6). *Coleus aromaticus* and *C. ambonicus* accumulated high levels of organic acid Such higher levels of organic acid accumulation can be observed more in NADP-ME type CAM plants since their oxidation capacity is higher in contrast to PEPCK type plants. Nocturnal synthesis of citric acid in CAM plants might serve as the reservoir for the CO_2. High concentrations of citric acid exceeding 200 mM has been reported in CAM plants such as *Bryophyllum calycinum*, *Clusia minor*, *Clusia rosea*, *Notonia petrea* and *Cissus quadrangularis* [34].

Carbohydrates plays pivotal role in balancing the day/night CO_2 fixation of CAM plants [35]. The night time uptake of CO_2 in CAM plants is supported by the degradation of carbohydrate reserve pool for the synthesizing the PEP substrate and 20% of leaf biomass is utilized for the synthesis. Utilization of carbohydrate reserves for the synthesis of organic acids is the unique feature of CAM plants which is 20-170 times the carbohydrates catabolized for the respiration [36]. Day and night levels of carbohydrate reserves were determined in six *Coleus* species by estimating the foliar glucose, sucrose and starch contents (Fig. 7). The levels of glucose and sucrose were higher in all *Coleus* during night time than day except starch contents whose day time concentrations were higher than night time. Formation of nocturnal malate in CAM plants will reduce the carbohydrate levels as evidenced in the analysis of their sap [35]. In the present study, the levels of nocturnal starch were lower than that of glucose and sucrose indicating that starch might serve as the carbohydrate reservoir for carbon fixation in *Coleus*. Among the six *Coleus* plants considered for this study, *aromaticus* and *ambonicus* were possessing high levels of glucose, sucrose, fructose and low levels of starch. Starch is the source of malate synthesis in the members of crassulaceae and cactaceae [37,38]. In *M. crystallinum*, carbon allocated to starch is maintained at the expense of the soluble sugar pool during acclimation to salinity [39]. Deficiency in leaf starch and plastidic phosphoglucomutase (PGM) in CAM-deficient mutants of *M. crystallinum* indicated the role of starch in providing substrate for nocturnal carboxylation [8].

4. CONCLUSION

In the present study the photosynthetic performance and CAM pathway ~~of~~ *Coleus* has been demonstrated in six species among which *C. aromaticus* and *C. ambonicus* have shown to possess high photosynthetic efficiency and biomass levels~~.~~, which might be due to the presence of high NADP-ME enzyme activity when compared with *spicatus, blumei, zeylanicus, forskholii,* which are possessing NAD-ME activity. Overall *Coleus* plants following NAD(P)-ME type of photosynthesis carbon fixation. Further studies are in progress for studying the response of these plants to abiotic stress which will give more information regarding the behavior of these plants.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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