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Volatile Organic Compounds Emitted by C_3 or CAM-Induced *Mesembryanthemum crystallinum* Plants †

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Abstract: Crassulacean acid metabolism (CAM) is an adaptation of certain plants, to arid and water-stressed environments. The expression of the CAM cycle may be strongly modulated by developmental and environmental factors. *Mesembryanthemum crystallinum* is a well-known facultative halophyte, that can shift its photosynthetic carbon fixation pathway from C_3 to CAM under salinity and other abiotic stress factors. However, until now there has been no study about the volatile organic compounds (VOCs) that are emitted by *M. crystallinum* in its various life cycles, C_3, and CAM. Plants emit a part of the photosynthetically assimilated carbon into the atmosphere in the form of VOCs. Under normal conditions, isoprenoids (isoprene and monoterpenes) are the most abundant VOCs though methanol, acetaldehyde and C-6 compounds are also emitted in great quantities. Under stress conditions, the emission of these compounds generally is altered. The study of how emissions change depending of stress conditions has become a useful "*in vivo*" indicator of plant vitality and of the plant response to abiotic stresses. Within this work, we aimed to analyse the VOCs emitted from C_3 or CAM-induced *M. crystallinum* in order to evaluate the possible role that VOCs may have in the C_3/CAM transition and consequently in the adaptation of this plant to salinity. Results showed that *M. crystallinum* emits different kind of VOCs: aldehydes, hydrocarbons, ketones, alcohols and terpenoids. VOC emissions were generally higher in plants representing C_3, with only few exceptions as butanone, octanal and ethyl-hexanol that were similar in the III phase of CAM and C_3 plants. Regarding the emission of terpenoids, we could observe that whereas plants in the C_3 mode of photosynthesis emitted three types of monoterpenes: a-pinene, carene and limonene, plants in CAM state did not emit any terpenoid compound.

Keywords: common ice plant; CAM metabolism; C_3 metabolism; volatile organic compounds; salt stress

1. Introduction

Crassulacean acid metabolism (CAM) is an adaptation of certain plants, to arid and water-stressed environments. The simplest definition of CAM, first described for species of the family Crassulaceae, is that there is (1) nocturnal uptake of CO_2 via open stomata, fixation by phosphoenolpyruvate carboxylase (PEPC) and vacuolar storage of CO_2 in the form of organic acids, mainly malic acid (phase I) [1], and (2) daytime remobilization of vacuolar organic acids, decarboxylation and refixation plus assimilation of CO_2 behind closed stomata in the Calvin-cycle (phase III). Between these two phases there are transitions when stomata remain open for CO_2 uptake for a short time during the very early light period (phase II) and reopen again during the late light period for CO_2 uptake with direct assimilation to carbohydrate when vacuolar organic acid is exhausted (phase IV). A fascinating attribute of CAM plants is that the expression of the CAM cycle relative to C_3 photosynthetic fixation of atmospheric CO_2 in the light, may be strongly modulated by developmental and environmental factors [2].

Mesembryanthemum crystallinum is a well-known facultative halophyte, that can shift its photosynthetic carbon fixation pathway from C_3 to CAM (Crassulacean acid metabolism) under salinity and other abiotic stress factors [3]. In its native habitat, the Namibian Desert of Southern Africa, this plant germinates in the short rainy season and changes its mode of photosynthesis from C3 to CAM in the dry season. Further development of *M. crystallinum* is strictly influenced by progressive drought stress coupled with increasing salinity, [4]. In fact, CAM, a water-conserving mode of photosynthesis is one of the most intriguing plant adaptations to environmental stress. In recent years, *M. crystallinum* has been used as a model for studying many physiological and biochemical changes in both modes of photosynthetic carbon assimilation pathway as well as for investigation of the C_3/CAM transition in plants exposed to different factors including salinity [3,5], abscisic acid [6], excess light [7] and hydrogen peroxide [8]. In particular it has been studied the involvement of H_2O_2, and of some antioxidant enzymes (CAT, SOD) in the regulation of the C_3/CAM transition [8,9,10], as well as the redox changes in the photosynthetic electron transport carriers during this process [11].

However, until now there has been no study about the volatile organic compounds (VOCs) that are emitted by *M. crystallinum* in its various life cycles, C_3, and CAM. Plants emit a part of the photosynthetically assimilated carbon into the atmosphere in the form of VOCs. Under normal conditions, isoprenoids (isoprene and monoterpenes) are the most abundant VOCs emitted by vegetation, though methanol, acetaldehyde and C-6 compounds (hexanal, hexenal, hexanol and hexenol) are also emitted in great quantities [12]. Under stress conditions, such as salt stress, the emission of these compounds generally increases [13]. The study of how emissions change depending of stress conditions has become a useful "in vivo" indicator of plant vitality and of the plant response to abiotic stresses.

Therefore, in this context we aimed to analyse the BVOCs emitted from C_3 or CAM-induced M. *crystallinum* in order to evaluate the possible role that VOCs may have in the *M. crystallinum* C_3/CAM transition and consequently in the adaptation of this plant to salinity.

2. Experiments

2.1. Plant Material

Plants of *Mesembryanthemum crystallinum* L. were grown from seeds (collection of the Botanical Garden, Darmstadt, Germany) in soil culture under irrigation with tap water in a phytotron chamber at temperatures of 25 °C and 17 °C during the light phase and the dark phase, respectively. Irradiance was 250–300 μmol quanta m^{-2} s^{-1}. Relative air humidity ranged between 30% and 50%. After the appearance of the third leaf pair, 3 weeks after sowing, one set of plants (n = 3) was treated with 0.4 kmol m^{-3} NaCl (salt-treated), while another set of plants (n = 3) was irrigated further with tap water (controls). Twelve-day treatment of *M. crystallinum* with saline solution induced CAM, as revealed by night/day fluctuations of malate concentration in the cell sap (Figure 1). The difference between malate concentration at the beginning and at the end of the day (Δ malate) is routinely assumed a hallmark of CAM. Malate concentration in the leaf cell sap was determined using a reflectometer (RQflex 10, Merck) according to the manufacturer's instruction manual.

2.2. Gas-Exchange and VOC Emission

After 14 d of water- (control) and salt-treatment (CAM) the plants were used in the experiments (three plants per treatment). A portable infrared gas analyzer (LI-6400; Li-Cor, Lincoln, NE, USA) was used to determine CO_2 and H_2O exchange: photosynthesis (A), stomatal conductance (g_s), transpiration and intercellular CO_2 concentration all along the day in *M. crystallinum* plants in C_3 and CAM states. Measurements were carried under natural light conditions. Leaf temperature during measurements was 30 °C and the relative humidity was between 50% and 60%. To collect VOCs, the outlet of the leaf cuvette was connected to a tube filled with 200 mg Tenax. A pump was used to draw through the tube 5 L of the air flowing over the leaf inside the cuvette, at a rate of 200 mL min^{-1}. During VOC collection in the morning and midday the leaves were under a PPFD of 1000 μmol m^{-2} s^{-1}, whereas measurements in the evening (21.00, I CAM phase) were carried out under natural light conditions not to disturb the normal CAM phase in CAM plants.

Trapped compounds were thermally desorbed at 275 °C for 10 min in a Markes Unity 1 thermal desorption unit (Markes International Limited, Llantrisant, UK) under a flow rate of helium, cryofocused in a cold trap containing a 2 mm diameter × 60 mm long bed of Tenax TA backed up by Carbograph 1TDTM separated and supported at each end by quartz wool and kept at −10 °C by a Peltier cell. By rapid heating of the cryogenic trap at 300 °C, BVOCs were injected into a 30 m MS-5HP capillary column with an inner diameter of 0.25 mm (J&W Scientific USA, Agilent Technologies, Palo Alto, CA, USA), connected to a gas chromatographic–mass spectrometric unit (GC–MS–MSD 5975C) supplied by the same company. The column temperature was maintained at 40 °C for 1 min, and then increased up to 210 °C at a rate of 5 °C/min. A final temperature of 250 °C was reached using a rate of 20 °C/min. Helium was used as a carrier gas. The volatile compounds were identified based on pure standards (Rivoira, Milan, Italy) and (Sigma-Aldrich, St. Louis, MO, USA) and the NIST library provided with the GC/MS ChemStation software.

2.3. Statistical Analysis

Analyses of variance (ANOVA) were performed using VOC emissions as dependent variable, and the factor “type/phase of metabolism” as independent factor. The Fisher post-hoc testwas used to investigate the significance of different groups of means, considered significant at a probability level of $p < 0.05$ All statistical analyses were conducted using SIGMASTAT.

3. Results and Discussion

3.1. Malate Concentration

Night/day fluctuations of malate concentration in the cell sap of C_3 and CAM *M. crystallinum* plants were determined to assess the induction of CAM metabolism in salt-treated *M. crystallinum* plants (Figure 1). Whereas, in C_3 plants malate concentration was homogeneous along the day, a typical CAM rhythm of diurnal malate fluctuation was detected on day 12 of the salinity treatment. This diel rhythm in malate content can be divided into four CAM phases: I—malate accumulation; II—PEPC/Rubisco transition; III—malate decarboxylation; and IV—Rubisco/PEPC transition. Similar diel rhythms of malate levels in *M. crystallinum* leaves have been shown repeatedly [14].

3.2. Gas Exchange

The diurnal variation of A and g_s for *M. crystallinum* performing C_3 and CAM modes of photosynthesis, is shown in Figure 2. Whereas the diurnal variation of A and g_s in *M. crystallinum* C_3 plants presented maximum values at midday, the daily net CO_2 exchange and g_s patterns of CAM plants were characterized by pronounced midday depressions.

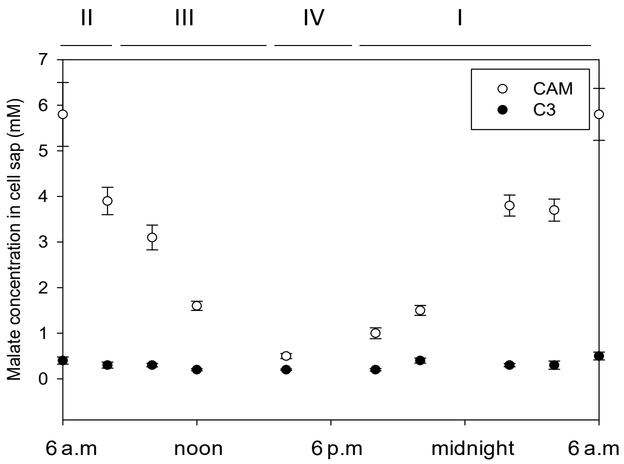

Figure 1. Night/day fluctuations of malate concentration in the cell sap of C_3 and CAM *M. crystallinum* plants. The approximate duration of four CAM phases is given above the graph. Means ± SD are presented (n = 3).

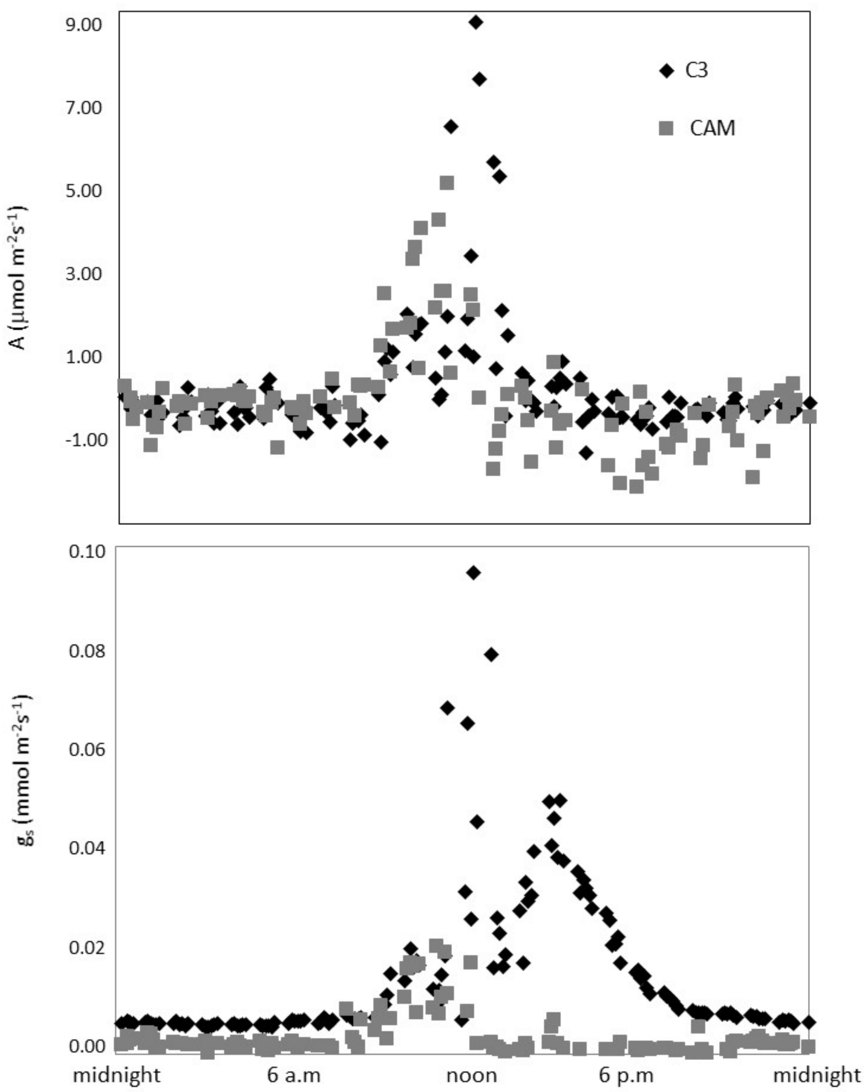

Figure 2. Daily variation of photosynthesis and stomatal conductance of *M. crystallinum* plants in C_3 and CAM metabolism.

3.3. VOCs Analysis

The collection of VOCs emitted by *M. crystallinum* CAM plants was performed during the early morning, corresponding with the second phase of CAM metabolism, when stomata were opened and CO_2 fixation took place through Rubisco and during the evening (around 21.00), corresponding to the first phase of CAM metabolism. VOCs emitted by *M. crystallinum* C_3 plants were collected during the morning (09.00–14.00). The list of the volatile organic compounds emitted by *M. crystallinum* is listed in Table 1.

Table 1. Lists of compounds emitted by *M. crystallinum*, and detected by GC-MS. Means ± SD are presented (n = 3). Different letters indicate significant statistical differences ($p < 0.05$).

	Emission Rates from *M. crystallinum* (nmol m^{-2} sec^{-1})		
Compound	**C_3**	**CAM (Phase I)**	**CAM (Phase II)**
Aldehydes			
Hexanal	0.022 ± 0.008 ^{a}	0.018 ± 0.002 ^{a}	0.004 ± 0.002 ^{b}
Octanal	0.033 ± 0.001 ^{a}	0.036 ± 0.004 ^{a}	0.002 ± 0.0002 ^{b}
Nonanal	0.014 ± 0.001 ^{a}	-	0.008 ± 0.001 ^{b}
Decanal	0.020 ± 0.0005 ^{a}	-	0.019 ± 0.011 ^{a}
Benzenoids			
Benzaldehyde	0.006 ± 0.002 ^{b}	0.015 ± 0.001 ^{a}	0.002 ± 0.001 ^{c}
Xylene	0.03 ± 0.01 ^{a}	0.005 ± 0.001 ^{c}	0.024 ± 0.004 ^{b}
Alkanes			
Nonane	0.029 ± 0.013 ^{a}	0.031 ± 0.003 ^{a}	0.0022 ± 0.0009 ^{b}
Undecane	0.007 ± 0.001 ^{b}	0.02 ± 0.002 ^{a}	0.003 ± 0.001 ^{c}
Dodecane	0.0035 ± 0.0007 ^{a}	0.0037 ± 0.001 ^{a}	0.0035 ± 0.001 ^{a}
Tetradecane	0.018 ± -0,001 ^{a}	0.0194 ± 0.0008 ^{a}	0,0032 ± 0.001 ^{b}
Alcohols			
Phenol	0.010 ± 0.007 ^{b}	0.018 ± 0.005 ^{a}	0.019 ± 0.008 ^{a}
Benzylalcohol	0.01 ± 0.0007 ^{a}	-	0.006 ± 0.002 ^{b}
2-Ethyl-1-Hexanol	0.046 ± 0.011 ^{a}	0.02 ± 0.004 ^{b}	0.002 ± 0.001 ^{c}
Terpenes			
a-Pinene	0.019 ± 0.006 ^{a}	0.021 ± 0.003 ^{a}	-
Carene	0.016 ± 0.002 ^{a}	0.009 ± 0.003 ^{b}	-
Limonene	0.128 ± 0.024 ^{a}	0.039 ± 0.01 ^{b}	-
Total			
	0.410 ± 0,033 ^{a}	0.257 ± 0.014 ^{b}	0.088 ± 0.016 ^{c}

Sixteen volatile compounds were identified, including alkanes, alcohols, aldehydes, benzenoids and terpenes. A great level of quantitative variation among the two modes of photosynthesis was observed for many of the identified volatile leaf compounds, as well for *M crystallinum* plants in the I phase and II phase of CAM metabolism. Total emission rates from C_3 plants were 1.6 and 4.6-fold-higher than from CAM plants, in phase I and phase II, respectively. Also, qualitative differences were found, as C_3 plants emitted fifteen compounds, whereas CAM plants emitted twelve compounds (different dependent on the CAM phase).

Major constituents of emissions were terpenes (0.162 nmolm^{-2}s^{-1}) and aldehydes (0.089 nmolm^{-2}s^{-1}) for C_3 plants, alkanes (0.074 nmolm^{-2}s^{-1}) and terpenes (0.071 nmolm^{-2}s^{-1}) for CAM plants in the I phase and aldehydes (0.033 nmolm^{-2}s^{-1}), alkanes (0.026 nmolm^{-2}s^{-1}) and alcohols (0.027 nmolm^{-2}s^{-1}) for CAM plants in the II phase.

C_3/CAM transition seems to be associated to a general decrease in VOC emissions, overall, regarding terpenes (carene and limonene), though the degree of reduction depends on the phase of CAM metabolism. Indeed, several individual compounds presented higher emission rates during the I phase of CAM plants than in the other cases, as benzaldehyde and undecane. Hexanal, octanal, tetradecane, nonane and α-pinene emission rates were similar in C_3 and CAM plants in the I phase. Moreover, one compound, phenol, was emitted at higher rates by CAM plants than by C_3 plants.

4. Conclusions

The data presented in this work revealed that, after salt stress, *M. crystallinum* plants emitted substantially lower VOCs in comparison to non-stressed plants. This is in contradiction to earlier experiments showing that stress in plants is usually accompanied by higherVOCs emission. However this work concerned only I and II phases of CAM. It is possible that emission of VOCs in III and IV phases takes place with a different intensity.

Author Contributions: Z.M. and I.N. conceived and designed the experiments; I.N. and M.K performed the experiments; I.N. and M.K. analyzed the data; Z.M. contributed reagents/materials/analysis tools; I.N, M.K and Z. M wrote the paper.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript: CAM: Crassulacean acid metabolism; VOC: Volatile Organic Compound.

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