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Analysis of organic acids of tricarboxylic acid cycle in plants using GC-MS, and system modeling

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Abstract

Background: Leaves of 15 plant species were collected from the catchment areas of the river Beas, Punjab, India, and analyzed for organic acids of tricarboxylic acid cycle, viz., citric acid (CA), succinic acid (SA), fumaric acid (FmA), and malic acid (MA).

Methods: Gas chromatography-mass spectrometry (GC-MS) was used to determine the content of organic acids in the leaves of plant species. Two microliters of plant sample was injected into the GC-MS, and the concentration of organic acids was quantified using standard curve.

Results: Average concentrations of these acids in the leaves of plants studied were 4.79, 0.98, 0.54, and 8.36 mg/g dw, respectively. The maximum contents of these acids were found in the leaves of *Chenopodium album* (CA = 6.42 mg/g dw), *Argemone mexicana* (SA = 1.27 and FmA = 0.73 mg/g dw), and *Rumex dentatus* (MA = 18.0 mg/g dw). Factor analysis revealed mainly two underlying factors for organic acids: Factor-1 having maximum loadings on SA and FmA and Factor-2 had maximum loadings on CA and MA. Multiple linear regression analysis of MA on other acids showed that CA and SA have positive regressions, whereas FmA has a negative regression on MA. In artificial neural network analysis, correlation between the target and output values of MA was found to be highly significant. System transfer coefficients were calculated from simulation graphs fitted to the mean values of different organic acids by using difference equations.

Conclusions: From the present study, it was found that citric acid has a maximum direct effect on the malic acid as compared to succinic and fumaric acids as revealed by path analysis. System modeling revealed that the rate of utilization of malic acid is about 9%. The present study describes a new system simulation technique in which a pathway comprising of linear transformation of biochemical constituents may be characterized in terms of its rate transfer coefficients.

Keywords: Krebs cycle, Citric acid, Succinic acid, Fumaric acid, Malic acid, Simulation, System analysis

Background

Organic acids are important metabolites of the tricarboxylic acid cycle in plants, which is the main energy-producing cycle of the cell. Analysis of organic acids is important because of their involvement in various physiological activities of plants (Bennet-Clark 1993). The organic acid contents in the leaves of plants vary with species and on the degree of development or maturation of plants, the environment, and many other factors. Since environmental or agronomic practices may affect the expression of genes in the seeds, these may also affect the organic acid contents (Kader 2008). Citric acid is a tricarboxylic acid and is mainly present in citrus fruits. It is used as a food additive to give acidity and sour taste to food and beverages (Destandau et al. 2005). Malic acid is a dicarboxylic acid, and it provides a pleasant taste and is also used as a food additive (Carocho et al. 2013; Campo et al. 2006). It plays an important role in improving the performance of muscles, reducing tiredness, as well as in improving mental clarity (Baldwin 2002). Nogués et al. (2006) reported that the leaves of

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Ranunculus glacialis contained high malic acid content. *Fumaria parviflora*, *Argemone mexicana*, *Rumex dentatus*, *Polygonum barbatum*, and *Polygonum plebeium* plants are rich sources of polyphenols as well as amino acids (Kumar et al. 2015a, b). Arias-Carmona et al. (2014) studied the contents of ascorbic acid, malic acid, oxalic acid, and citric acid in *Brassica rapa* leaves. Sánchez-Mata et al. (2012) studied the contents of citric acid, malic acid, and fumaric acid in *Foeniculum vulgare*, *Chondrilla juncea*, *Cichorium intybus*, *Taraxacum obovatum*, *Beta maritime*, and *Papaver rhoeas*. The present study was designed to study variations in the organic acid contents, i.e., citric acid (CA), succinic acid (SA), fumaric acid (FmA), and malic acid (MA) using gas chromatography-mass spectrometry (GC-MS) in the leaves of 15 plant species collected from the catchment areas of the river Beas, India. Multivariate techniques such as principal component analysis, factor analysis, multiple linear regression analysis, and artificial neural network analysis were applied to the contents of different organic acids. System simulation techniques were applied to determine system transfer functions from one acid to the next at steady state.

Methods

Study area

The river Beas originates in the southern side of the Rohtang Pass above Kullu in Beas Kund, in central Himachal Pradesh, India (32.36° N lat. and 77.08° E long.) and merges into river Sutlej at Harike (31.17° N lat. and 75.2° E long.) in the state of Punjab, India, after traversing a distance of about 470 km. Plant samples were collected from the surroundings of the river bed between the towns of Beas and Harike. Identification and authentication of the plants was done at the Botanical Survey of India, Dehradun, India.

Organic acids quantification using GC-MS

Preparation of sample

The organic acids in the plant leaves were determined by following the procedure earlier described by Sharma et al. (2016). Organic acids were extracted from 50 mg of the oven dried (80 °C, 24 h) powdered leaves of different plant species by adding 0.5 ml of 0.5 N HCl and 0.5 ml of methanol. After that, the samples were shaken for 3 h followed by centrifugation at 12,000 rpm for 10 min. To the supernatant, 300 μl of methanol and 100 μl of 50% sulphuric acid were added followed by overnight incubation in water bath at 60 °C. The mixture was cooled down to 25 °C, and 800 μl of chloroform and 400 μl of distilled water were added to it followed by vortexing for 1 min. The lower chloroform layer was used to estimate organic acids using GC-MS.

Investigation using GC-MS

For the determination of organic acids, 2 μl of plant sample, i.e., the lower chloroform layer, was injected in the system. GC conditions: Helium was used as carrier gas, the starting column temperature was 50 °C held for 1 min which was raised to 125 °C at 25 °C/min followed by additional enhancement to 300 °C at 10 °C/min, held for 15 min. Injection temperature was 250 °C, injection mode was split, gas flow in the column was 1.7 ml/min, and analytical column DB-5ms was used. MS conditions: Ion source temperature was set at 200 °C and interface temperature was 280 °C, solvent cut time was 3 min, and detector gain mode was relative. Since the sample preparation procedure resulted in the derivatization of organic acids, the studied organic acids were citric acid trimethylester, succinic acid dimethyester, fumaric acid dimethyester, and malic acid dimethyester, and their contents were estimated using standard curve.

Simulation of rate transfer coefficients

The rates of change of different components may be described by a system of linear differential equations for a constant size of the first component viz. citric acid (x_1) to malic acid (x_4).

$$\frac{dx_2}{dt} = ax_1 - bx_2$$

$$\frac{dx_3}{dt} = bx_2 - cx_3$$

$$\frac{dx_4}{dt} = cx_3 - dx_4$$

where x_i are the concentrations of the acids and a, b, c, and d are the rates of transfer from one component to the next one. The differential equations can be solved for the size of the component with respect to time. With time, the system will tend to a steady state, and the rate of change of each component will be zero:

$$\frac{dx_i}{dt} = 0$$

The system can be simulated using a set of difference equations:

$$x_2(t+1) = x_2(t) + ax_1(t) - bx_2(t)$$

$$x_3(t+1) = x_3(t) + bx_2(t) - cx_3(t)$$

$$x_4(t+1) = x_4(t) + cx_3(t) - dx_4(t)$$

where x_i (t) and x_i $(t + 1)$ are the sizes of the components at times (t) and $(t + 1)$, respectively. The matrix model representation of the difference equations given above will be as follows:

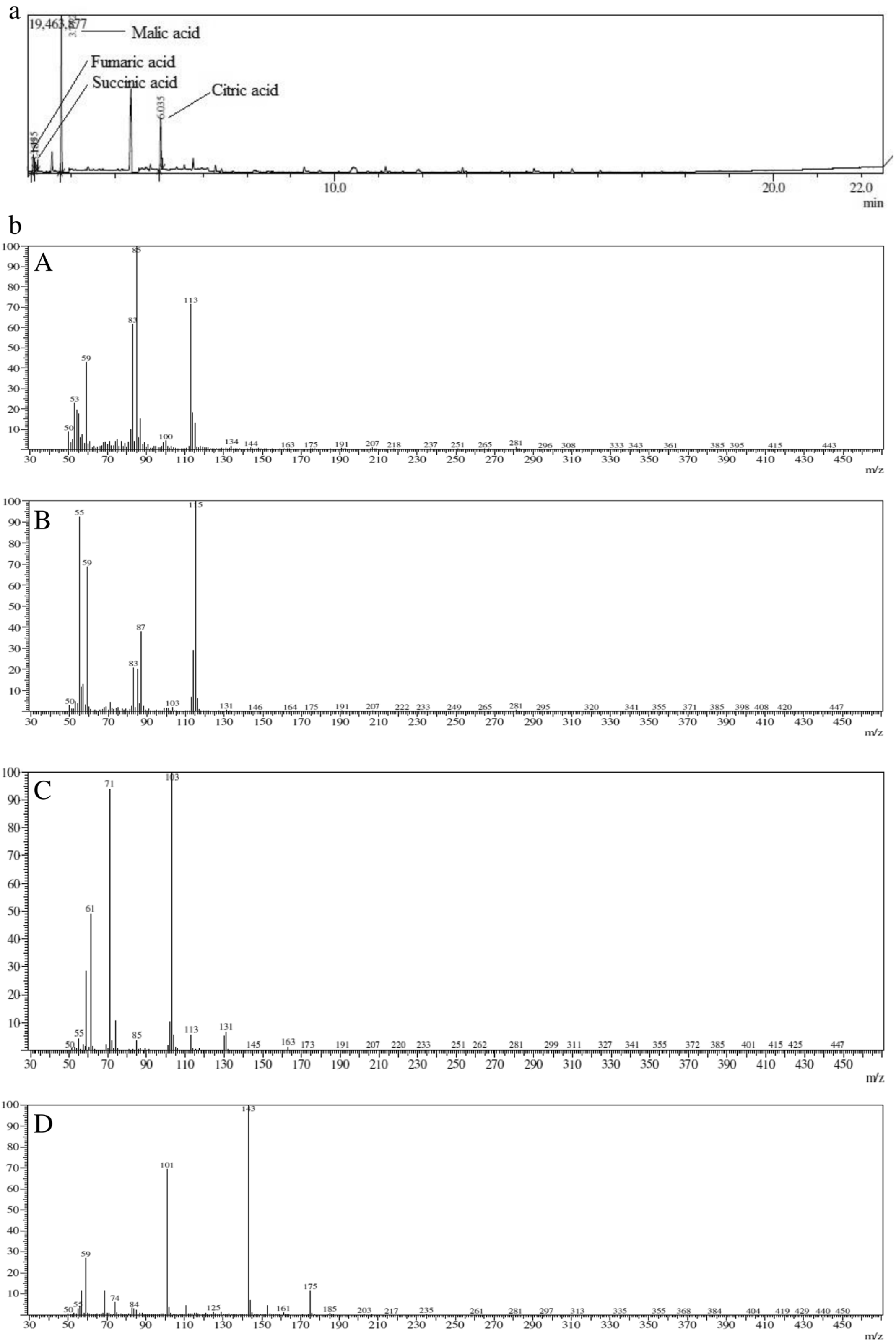

Fig. 1 **a** Total ion chromatogram of organic acid using GC-MS analysis. **b** Mass spectrums of organic acids obtained after GC-MS analysis. Fumaric acid dimethyester (*A*), succinic acid dimethyester (*B*), malic acid dimethylester, (*C*) and citric acid trimethylester (*D*)

Table 1 Mean and standard deviation (n = 3) of organic acids in the leaves of plants collected from catchment areas of the river Beas

Plant species	Family	CA (mg/g dw)	SA (mg/g dw)	FmA (mg/g dw)	MA (mg/g dw)	Total acids (mg/g dw)
Ranunculus sceleratus L.	*Ranunculaceae*	5.78 ± 0.017	0.88 ± 0.002	0.59 ± 0.006	12.3 ± 0.28	19.61
Argemone mexicana L.	Papaveraceae	4.04 ± 0.006	1.27 ± 0.013	0.73 ± 0.007	7.66 ± 0.17	13.70
Fumaria parviflora Lam.	*Papaveraceae*	3.55 ± 0.024	0.86 ± 0.002	0.44 ± 0.0002	6.19 ± 0.56	11.04
Oxalis corniculata L.	Oxalidaceae	3.85 ± 0.026	0.95 ± 0.010	0.43 ± 0.001	6.20 ± 0.42	11.43
Ageratum conyzoides L.	Asteraceae	5.77 ± 0.055	0.95 ± 0.009	0.48 ± 0.003	4.95 ± 0.30	12.16
Erigeron bonariensis L.	Asteraceae	4.32 ± 0.072	0.98 ± 0.007	0.50 ± 0.006	6.90 ± 0.21	12.69
Parthenium hysterophorus L.	Asteraceae	4.41 ± 0.042	1.01 ± 0.018	0.51 ± 0.004	9.52 ± 0.56	15.46
Chenopodium album L.	Amaranthaceae	6.42 ± 0.067	0.94 ± 0.001	0.57 ± 0.008	6.47 ± 0.02	14.40
Chenopodium ambrosioides L.	Amaranthaceae	4.61 ± 0.053	1.01 ± 0.009	0.57 ± 0.009	8.20 ± 2.11	14.39
Polygonum barbatum L.	Polygonaceae	4.55 ± 0.057	1.02 ± 0.033	0.58 ± 0.009	5.16 ± 0.45	11.31
Polygonum plebeium R.Br.	Polygonaceae	4.28 ± 0.122	0.94 ± 0.014	0.61 ± 0.010	5.41 ± 0.47	11.24
Rumex dentatus L.	Polygonaceae	6.25 ± 0.145	0.95 ± 0.004	0.55 ± 0.002	18.0 ± 1.22	25.82
Tamarix dioica Roxb. ex Roth	Tamaricaceae	5.10 ± 0.089	1.10 ± 0.013	0.70 ± 0.010	8.18 ± 0.41	15.07
Cannabis sativa L.	*Cannabaceae*	5.15 ± 0.060	0.97 ± 0.016	0.47 ± 0.008	14.61 ± 0.56	21.20
Typha angustata Chamb.	Typhaceae	3.81 ± 0.035	0.96 ± 0.022	0.44 ± 0.002	5.68 ± 0.25	10.89
Average		4.792	0.986	0.544	8.362	

CA citric acid, *SA* succinic acid, *FmA* fumaric acid, *MA* malic acid

$$\begin{bmatrix} x_1(t+1) \\ x_2(t+1) \\ x_3(t+1) \\ x_4(t+1) \end{bmatrix} = \begin{bmatrix} 1 & 0 & 0 & 0 \\ a & 1-b & 0 & 0 \\ 0 & b & 1-c & 0 \\ 0 & 0 & c & 1-d \end{bmatrix} \begin{bmatrix} x_1(t) \\ x_2(t) \\ x_3(t) \\ x_4(t) \end{bmatrix}$$

or

$$X(t+1) = MX(t)$$

where M is the rate transfer matrix and $X(t)$ and $X(t+1)$ are vectors for organic acid contents at times (t) and (t + 1), respectively. At steady state,

$$X(t+1) = X(t)$$

The system of difference equations was simulated using a self-coded software in MS-Excel to determine the rate transfer coefficients a, b, and c, by inputting the values of x_1, x_2, x_3, x_4, and d.

Statistical analysis

All the analyses were done in triplicate, and the results were expressed in mean and standard deviation values. The data were analyzed by using principal component analysis (PCA), factor analysis (FA), multiple linear regression analysis (MLR), and artificial neural network analysis (ANN) (Sokal and Rohlf 1995; Bailey 1994; Kumar et al. 2016, 2017). First-order linear difference equations were used for the simulation of the transformation of acids to a steady state. System analysis software was coded in MS-Excel. The software used were PAST, Minitab-14, and Statistica-12.

Results and discussion

The chromatogram and mass spectra of organic acids are given in Fig. 1a, b. The means and standard deviations of organic acids studied are given in Table 1. Maximum citric acid content was found in *Chenopodium album* (6.42 mg/g dw) followed by *Rumex dentatus* (6.25 mg/g dw). *Argemone mexicana* recorded maximum contents of succinic acid (1.27 mg/g dw) and fumaric acid (0.73 mg/g dw). *Rumex dentatus* recorded maximum content of malic acid (18.0 mg/g dw). Maximum content of the four organic acids was found in *Rumex dentatus* (25.82 mg/g dw) followed by *Cannabis sativa* (21.2 mg/g dw), whereas the lowest amount of total organic acids was found in *Typha angustata* (10.89 mg/g dw). The trend of variation of the organic acids in the plants studied was malic acid > citric acid > succinic acid > fumaric acid. Adamczak et al. (2012)

Table 2 Factor analysis of different organic acids

Variable	Factor-1	Factor-2	Communality
CA	0.016	*− 0.880*	0.775
SA	*0.921*	0.161	0.874
FmA	*0.922*	− 0.166	0.878
MA	− 0.012	*− 0.842*	0.709
% variance	42.5	38.4	80.9

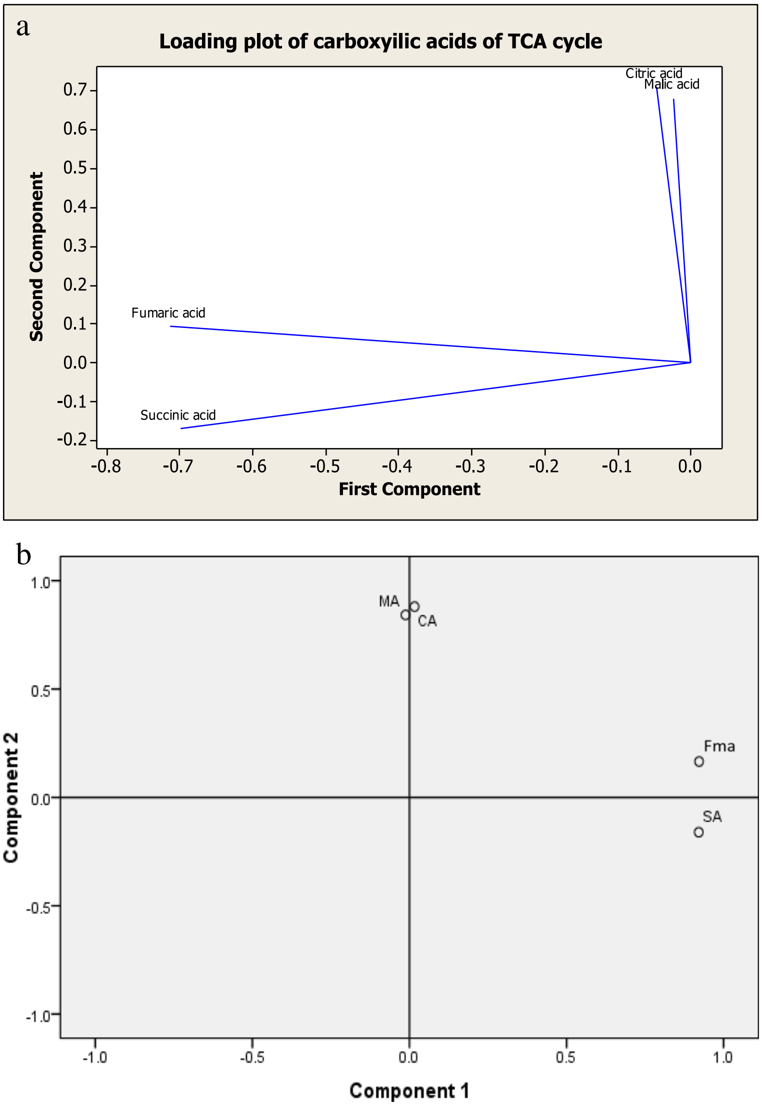

Fig. 2 **a** Loading plot of PCA for intermediate acids (SA and FmA) and source-sink acids (CA, MA). **b** Score plot of PCA for intermediate acids (SA and FmA) and source-sink acids (CA, MA)

reported citric acid contents in different species of rose, viz., *Rosa agrestis* (1.08 mg/g dw), *R. tnodora* (1.95 mg/g dw), and *R. jundzillu* (2.90 mg/g dw). Roriz et al. (2014) reported malic acid contents in *Pterospartum tridentatum* (3.23 mg/g dw), *Cymbopogon citratus* (2.23 mg/g dw), and *Gomphrena globosa* (12.33 mg/g dw). They also analyzed citric acid contents in *P. tridentatum* (5.99 mg/g dw) and *G. globosa* (2.40 mg/g dw) and fumaric acid contents in *G. globosa* (0.28 mg/g dw) and *C. citratus* (0.49 mg/g dw). Nogués et al. (2006) studied the carbon metabolism by labeling the *Ranunculus glacialis* with $^{13}C/^{12}C$-isotope ratio mass spectrometry and ^{13}C-nuclear magnetic resonance. It was observed that malic acid is not utilized by respiration, when leaves are labeled with ^{13}C-depleted CO_2 or $H^{13}CO_3^-$. However, the

Table 3 Multiple regression and path analyses of organic acids of TCA cycle

Multiple regression equation

$MA (mg/g dw) = -4.16 + 2.28 CA (mg/g dw) + 4.99 SA (mg/g dw) - 6.01 FmA (mg/g dw)$

Multiple correlation coefficient (r) = 0.5010 ($p < 0.001$)

Path analysis

Independent variables	Direct effects (DE) on MA	Indirect effects (IE) on MA				Total effects (DE + IE)
		CA	SA	FmA	Total IE	
Citric acid (CA)	0.5393	0	− 0.0204	− 0.0265	− 0.0469	0.4924
Succinic acid (SA)	0.1252	− 0.0879	0	− 0.0976	− 0.1856	− 0.0604
Fumaric acid (FmA)	− 0.1398	0.1024	0.0874	0	0.1899	0.0501

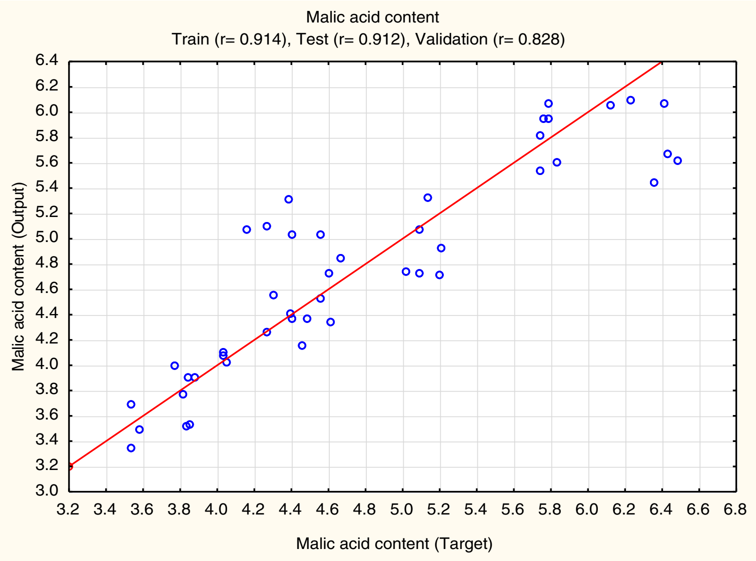

Fig. 3 Correlation between target (observed) and output (simulated) concentrations of malic acid using ANN model

biological reason for malic acid accumulation is not clear in *R. glacialis*. *Secale cereale* leaves have enhanced malic acid content when acclimated to low temperature (Crecelius et al. 2003; Streb et al. 2005).

First three components of principal component analysis accounted for 99.97% of the total variance (95.90, 3.98, and 0.097%). Factor analysis was also applied to organic acid contents (Table 2). Factor-1 accounted for 42.4% of the total variance and had maximum loadings on succinic and fumaric acids both being intermediates of the tricarboxylic acid (TCA) cycle. Factor-2 had maximum loadings on citric acid (source) and malic acid (sink) and explained 38.9% of the total variance (Fig. 2a, b). Multiple linear regression analysis (MLR) was applied to different organic acids (Table 3). From the MLR, it was revealed that citric and succinic acids contribute positively to the malic acid content, whereas fumaric acid had a negative regression with the malic acid. Beta regression coefficients (β coefficient) reveal the relative importance of different independent variables on the dependent variable (malic acid). The acid maximum contributing to formation of malic acid was citric acid, followed by succinic acid. Path analysis of the effects of different acids on malic acid content revealed that citric acid has maximum direct effect on malic acid. The total effects of succinic and fumaric acids on malic acid are negligible.

The ANN model was developed by taking malic acid as a dependent variable, and citric, succinic, and fumaric acids as independent variables using the Statistica-12 software (Fig. 3). ANN consisted of one hidden layer, *tanh* transfer function, and three neurons. The number of neurons is generally 70 to 90% of the input layer size (Boger and Guterman 1997). Correlation between target and output values of malic acid was found to be highly significant: training (r = 0.914), test (r = 0.912), and validation (r = 0.828).

The TCA cycle may be treated to be a system having four components in which the output from a component becomes an input for the next component (Fig. 4).The rate of transformation of citric acid (x_1) to succinic acid (x_2) then to fumaric acid (x_3) and finally to malic acid (x_4) may be characterized by rate transfer coefficients a, b, and c, respectively. Malic acid is then utilized for other metabolic pathways at a rate (d). Igamberdiev and Eprintsev (2016) discussed the organic acid metabolism in plants in the context of redox regulation. In a TCA cycle, two acids, citric acid and malic acid, are generally the most accumulated organic acids in plants. In the

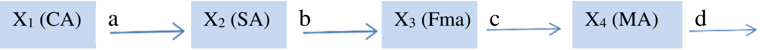

Fig. 4 Conversion of citric acid to malic acid. x_i represent concentrations of the acids and a, b, c, and d are the rate transfer coefficients

Table 4 Rate transfer coefficients of the difference equation for different organic acids

Plant species	a	b	c	d
Ranunculus sceleratus	0.12	0.82	1.23	0.059
Argemone mexicana	0.18	0.60	1.04	0.100
Fumaria parviflora	0.17	0.71	1.37	0.099
Oxalis corniculata	0.16	0.65	1.43	0.100
Ageratum conyzoides	0.08	0.51	1.03	0.100
Erigeron bonariensis	0.15	0.70	1.38	0.100
Parthenium hysterophorus	0.16	0.70	1.38	0.075
Chenopodium album	0.10	0.68	1.13	0.100
Chenopodium ambrosioides	0.15	0.69	1.23	0.086
Polygonum barbatum	0.11	0.50	0.89	0.100
Polygonum plebeium	0.12	0.57	0.88	0.100
Rumex dentatus	0.02	0.15	0.26	0.008
Tamarix dioica	0.16	0.74	1.17	0.100
Cannabis sativa	0.02	0.13	0.27	0.009
Typha angustata	0.14	0.59	1.28	0.100
Average	0.12	0.59	1.08	0.082

present paper, the transfer rates of citric acid to malic acid via succinic acid and fumaric acid were calculated. The conversion of citric acid to malic acid may be simulated in terms of system analysis. The rates of transformation of different acids in this system are represented by a, b, c, and d. Assuming that the reactions involved in the process occur at a constant rate, the concentrations of the organic acids will achieve a steady state (Easterby 1996; Fridlyand and Scheibe 1999).

Since biological systems follow homeostatic mechanisms, and have a strong resilience, it was presumed that the transformation of acids will follow a system of damped oscillations to achieve steady state within the minimum possible time. It was found by simulation that the value of x_3 (fumaric acid) shows maximum oscillations on increase in the value of the rate coefficient d. The value of d was varied for each plant species, and the variance between the simulated value of fumaric acid with respect to the steady state value was calculated. The simulated value of d at which the minimum variance with respect to the value of x_3 for 10 cycles, from the 3rd cycle (when the first formation of fumaric acid would occur) to the 12th cycle, was used to estimate the rate transfer coefficients of the difference equations (Table 4). It is seen from the transfer functions that the ratio of the concentrations of two organic acids is inversely proportional to the rates of their transfers. Figures 5 and 6 show the simulation plots for succinic acid, fumaric acid, and malic acid and the transformation coefficients from citric to malic acids for average concentrations of acids in the leaves of plants. The value of the rate constant d gives the rate of utilization of malic acid formed in TCA for energy production. It is seen from Fig. 7 that the rates of conversion of different acids are directly proportional to the rate of utilization of malic acid by the plants which helps the plants to achieve homeostasis for maintaining desired concentrations of acids in plants. The system however becomes unstable if the value of d increases to 0.13 (sustained oscillations) or 0.14 or higher (negatively damped oscillations). This would lead to changes in the compartment sizes of different acids. High contents of malic acid in the leaves of *R. dentatus* and *C. sativa* can be attributed to the low rate of utilization of malic acid (d) in these species. Difference equation and matrix models are important techniques for population growth (Poole 1974; Svirezhev and Logofet 1983), cell division, and enzyme catalyzed reactions (Thukral et al. 2001). In the present study, it has been shown that organic acids in the TCA cycle can be modeled using various techniques, viz., factor analysis, multiple linear regression, system analysis, and neural networks.

Conclusions

The present study revealed that the average values of citric, succinic, fumaric, and malic acids found in the leaves

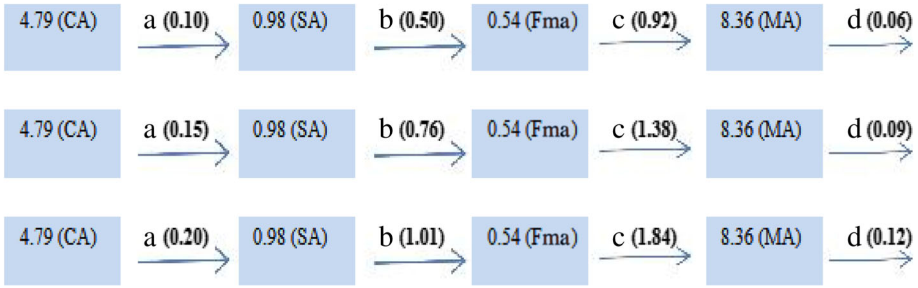

Fig. 5 Rate transfer coefficients (a, b, c) for transformation of citric acid to malic acid (using average leaves concentration) using different rates of utilization of malic acid to other pathways (d)

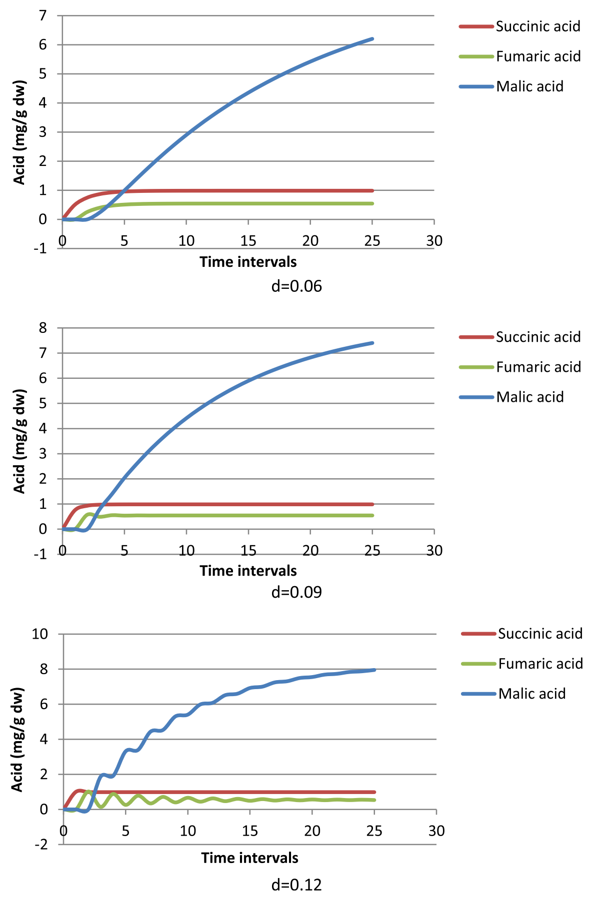

Fig. 6 Simulation of organic acid concentrations by using different rates of utilization of malic acid (d). Higher values of "d" cause oscillatory behavior, and d (0.09) stabilizes the system fast

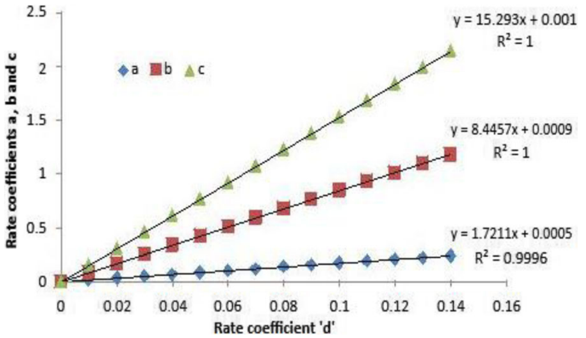

Fig. 7 Increase in rate of conversion of CA (a), SA (b), and FmA (c) with increase in rate of utilization of MA (d)

of 15 plant species were 4.79, 0.98, 0.54, and 8.36 mg/g dw, respectively. Factor analysis explained two underlying factors: Factor-1 (succinic acid and fumaric acid) and Factor-2 (citric acid and malic acid). Multiple linear regression analysis showed that citric and succinic acids are positively regressed on the malic acid, whereas fumaric acid had negative regression with the malic acid. ANN models were fitted to the data. Simulation graphs were also fitted to the average values of different organic acids by using difference equations. The system attains homeostasis by changing the values of transfer coefficients in response to the rate of utilization of malic acid.

Abbreviations

ANN: Artificial neural network analysis; CA: Citric acid; FA: Factor analysis; FmA: Fumaric acid; GC-MS: Gas chromatography-mass spectrometry; MA: Malic acid; MLR: Multiple linear regression analysis; PCA: Principal component analysis; SA: Succinic acid

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Authors' contributions

This work was carried out in collaboration between all authors. Author VK collected and analyzed the plant samples. Authors VK and AS carried out literature search and drafted the manuscript. Authors RB and AKT designed the experiment and statistically analyzed the data. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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