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Research article

Effect of drought stress on metabolite adjustments in drought tolerant and sensitive thyme

Mohsen Ashrafi[a], Mohammad-Reza Azimi-Moqadam[a,*], Parviz Moradi[b], Ehsan MohseniFard[a], Farid Shekari[a], Mohsen Kompany-Zareh[c]

[a] Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Zanjan, Zanjan, Iran
[b] Research Division of Natural Resources, Zanjan Agricultural and Natural Resources Research and Education Centre, AREEO, Zanjan, Iran
[c] Institute for Advanced Studies in Basic Sciences (IASBS), Gava Zang, Zanjan, Iran

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ABSTRACT

Drought is one of the most important threats to plants and agriculture; therefore, understanding of the mechanism of drought tolerance is crucial for breeding of drought tolerant plants. Here, we assessed effects of four levels of drought (90%, 55%, 40% and 25% FC) on some physiological criteria and metabolite adjustment of two different drought-responsive thyme plants (*Thymus vulgaris* as drought sensitive and *T. Kotschyanus* as drought tolerant species), using ^{1}H-NMR. Among three physiological parameters and 18 identified metabolites, species × treatment effects were significant ($P \leq 0.01$) for leaf temperature, acetic acid, citric acid, fumaric acid, malic acid, succinic acid, fructose, sucrose and serine. RWC, chlorophyll and carotenoids content, glucose, alanine and choline were affected by simple effects of species and treatment. Correlation analysis revealed that there is a different correlation between physiological parameters and metabolites in both species. This analysis also revealed that, by ignoring the correlation between malic acid and succinic acid in *T. vulgaris*, there was no significant correlation between TCA intermediate in both species. According to results, sugars, amino acid and energy metabolism were affected by drought and, among them, TCA intermediates had more alternation in two studied species so, this cycle and its intermediates probably have more prominent role than other identified metabolites in the induction of drought tolerance.

1. Introduction

Environmental stresses (biotic and abiotic stress) are serious threats to agricultural production (Nakabayashi and Saito, 2015). Drought is an important abiotic environmental stressor of plants and water deficit is typically the most limiting factor for plant growth, yield, and productivity (Barchet et al., 2014). In most areas of the world, agriculture is more influenced by drought because of changes in rainfall pattern caused by global climate change, as well as competition with growing population and industry (Sanchez et al., 2012). To increase the agricultural efficiency within the inadequate land resources, it is important to ensure higher crop yields against unfavorable environmental stresses. Understanding the reaction of plants to water-limited conditions is crucial and will pave the way for improving tolerance to drought (Reddy et al., 2004).

There are several platforms which can be used for understanding of an organism response to the environment such as molecular biology and physiology. However, these approaches provide limited data, since consider a target biomolecule and related pathways, (Klassen et al., 2017). Metabolomics is one of the most important techniques which help us to deeply and comprehensively understand the biological process and functions under different conditions (Klassen et al., 2017). Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are two mainly used analytical platforms in metabolomics (Wang et al., 2011) which both have its own advantages and disadvantages (Tian et al., 2016).

Thymus is the eight most numerous genus of Lamiaceae family with regard to the number of containing species (Stahl-Biskup and Sáez, 2002). It's been used as a medicinal, aromatic and spicy plant (Boning, 2010). Demand for thyme products is growing and is not likely to be supported by collecting from natural populations because of insufficient/irregular rainfall in traditional source areas as well as destruction of its natural habitat. Improving thyme to be cultivated under different conditions, like drought, will facilitate its future Production and conservation of its natural biodiversity (Moradi et al., 2014; Stahl-Biskup and Sáez, 2002). Based on the literature review, there is only

* Corresponding author.
E-mail address: Azimi@znu.ac.ir (M.-R. Azimi-Moqadam).

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one study that compared two different drought-responsive thyme plants (*T. vulgaris* as a drought sensitive and *T. serpyllum* as a drought tolerant species) under long-term water deficit condition using DI-FTIR mass spectrometry (Moradi et al., 2017). In the present study, because of high-throughput properties, high analytical precision and ability to monitor and quantification of impacts of drought on metabolites (Silvente et al., 2012), H-NMR based metabolomics was used to profile polar metabolites of *T. vulgaris* and *T. kotschyanus* (based on our previous work this two species differing in their tolerance to drought (Ashrafi et al., 2018) at four levels of drought.

2. Materials and methods

2.1. Plant material, growth condition, and treatment

Seeds of *Thymus vulgaris* as a drought sensitive and *T. kotschyanus* as a drought tolerant species are commercially available and were purchased from Pakan Bazr-e-Esfahan Company (Esfahan, Iran).

Seeds were sown in 10 cm diameter pots which have been filled with about 285 g soil mixture (0.5:1:1:2 ratio of perlite: sand: vermicompost: topsoil, respectively). This experiment with four biological replicates per treatment was conducted in a greenhouse with a day/night period 18/6 and mean day/night temperature 24/20 °C. Pots were daily irrigated to 95% of field capacity (FC) for two months, then four levels of watering regime including normal irrigation (95% FC), mild stress (55% FC), moderate stress (40% FC), and severe stress (25% FC) were applied. Two days after reaching to severe stress, the leaves were collected for further analysis.

2.2. Physiological measurements

Relative water content (RWC) was measured as described by Barrs and Weatherley (1962). Immediately after sampling, because of the thyme tiny leaves, the whole part of leaves was weighed (fresh weight; FW) and then soaked overnight in distilled water at 4 °C. After the cold incubation, the leaves were blotted dry and weighed (turgid weight; TW) then oven-dried at 80 °C for 48 h. Subsequently, dry weight (DW) of the leaves was measured. The leaf relative water content was calculated using equation (1).

$$\text{RWC} = (\text{FW-DW})/(\text{TW-DW})) \times 100 \text{ (Barrs and Weatherley, 1962)} \quad (1)$$

Leaf temperature was measured by an infrared thermometer (Model 8889).

Chlorophyll and carotenoids content were measured by Arnon (1949) and Lichtenthaler and Wellburn (1983), respectively. (Lichtenthaler and Wellburn, 1983) (Arnon, 1949).

2.3. Metabolite extraction and NMR spectroscopy

Plant samples were grounded in liquid nitrogen and then freeze-dried. Fifty milligrams of dried plant material was then transferred to a centrifuge tube. Afterward, 2 mL of pre-cooled water-methanol (1:1) mixture was added to the tube, vortexed for 30 s, and sonicated in an ice bath for 15 min. The sample was then centrifuged at 4 °C for 20 min. The aqueous phase was transferred to a new 2 ml tube and methanol was removed under vacuum at 37 °C for 4 h (to reduce the methanol percentage to approximately 5%); next, the supernatants were frozen at −80 °C and lyophilized in a freeze-drier for at least 24 h. Finally, 400 µL of dilution buffer (90% D_2O and 10% phosphate-buffered saline (500 mM, pH 7) and 2.5 mM maleic acid) was added to the dried aqueous fractions. Maleic acid was used as internal standards. All of the 400 µL supernatants were transferred to 5 mm NMR sample tubes. Four biological replicates of each sample were used for NMR analysis. Samples were scanned through high-resolution 1D ^{1}H NMR spectroscopy (^{1}H frequency, 400.22 MHz) generating polar metabolic profiles using a Bruker Avance 400 spectrometer (Bruker Biospin, Germany). Sample handling, automation, and acquisition were controlled using TOPSPIN 2.1 software (Bruker Biospin). For all samples, a standard ^{1}H90° pulse sequence was used, and residual water resonance was suppressed in the aqueous samples. After the probe was introduced, the samples were allowed to equilibrate for 1 min. Each spectrum was obtained as 32 k data points at a spectral width of 14 ppm and as the sum of 128 transients at a relaxation delay of 4 s.

2.4. Databases and software for metabolite identification

Standard NMR spectra of metabolites were retrieved from the Human metabolome database (HMDB) (http://www.hmdb.ca/) and the Madison Metabolomics Consortium Database (MMCD) (http://mmcd.nmrfam.wisc.edu). After the processing of 1D NMR spectra using MestReNova (Mestrelab Research, Spain), standard spectra were matched to them by considering 0.03 ppm chemical shift (Dona et al., 2016).

2.5. Quantitative ^{1}H NMR

As signal intensity is absolutely proportional to the molar concentration of metabolites in the ^{1}H NMR spectrum, ratio method was used for quantification of metabolites. Maleic acid was used as an internal standard and quantification of identified metabolites was performed using equation (2) (Fan et al., 2014)

$$m_X = m_{ST} \times \left(\frac{A_X}{A_{ST}}\right) \times \left(\frac{MW_X}{MW_{ST}}\right) \times \left(\frac{N_{ST}}{N_X}\right) \quad (2)$$

where m_X is the unknown concentration of the metabolite, m_{ST} is the known concentration of the internal standard, A_X is the total peak integral of the metabolite, A_{ST} is the total peak integral of the standard, MW_X is the molecular weight of the metabolite, MW_{ST} is the molecular weight of the standard, N_X is the number of H(s) of the metabolite responsible for its peak integral and N_{ST} is the number of H(s) of the standard responsible for its peak integral. The calculated concentration m_X of the identified metabolites is represented as mM.

2.6. Experimental design and statistical analysis

The factorial experiment in completely randomized design was used as experimental design in which water treatment and plant species were the factors. Analyses of variance (ANOVA) were carried out for physiological parameters and metabolites. Differences between means were tested using Benjamini-Hochberg corrected *t*-test. Pearson correlation coefficient was also calculated for all parameters. ANOVA, mean comparison and correlation analysis were performed using R (R Development Core Team, 2008), agricolea (De Mendiburu, 2009) and corrplot packages (Wei and Simko, 2017).

Principal component analysis (PCA), hierarchal clustering, and pathway analysis were performed using the Metaboanalyst website (Xia et al., 2015).

3. Results

3.1. Physiological measurements

There was a significant ($P \leq 0.01$) Species × treatment interaction for leaf temperature (Supplementary Table 1; Fig. 1 C).

As the drought was increased till 40% FC, leaf temperature increased in both species. After that, further increase of drought had no significant effect on leaf temperature of *T. kotschyanus* whereas *T. vulgaris* showed an increasing manner (Fig. 1 C). RWC, total chlorophyll and chlorophyll/carotenoids ratio were significantly ($P \leq 0.01$)

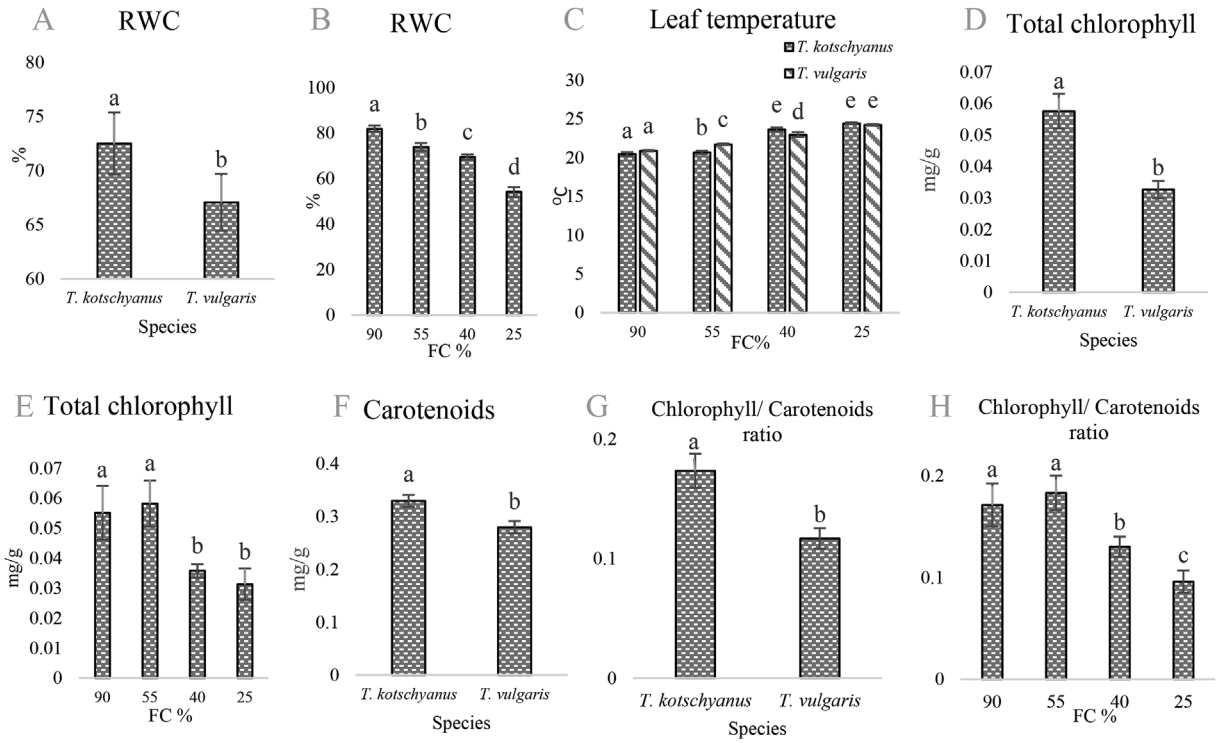

Fig. 1. Effect of species (A) and drought treatment (B) on RWC. Interaction effect of species and drought treatment on leaf temperature (C). Effect of species (D) and drought treatment (E) on total chlorophyll. Carotenoids content of both thyme species (F). Effect of species (G) and drought treatment (H) on Chlorophyll/carotenoids ratio. Each bar indicates mean and error bar represents standard errors of the mean. Bars within axes with the same letter are not significantly different according to Benjamini-Hochberg corrected *t*-test $P \leq 0.05$.

affected by species and drought treatment (Supplementary Table 1). Under all of the drought levels RWC decreased, but total chlorophyll and chlorophyll/carotenoids ratio were not affected by mild drought stress (55% FC). Total chlorophyll was decreased at 40% FC and further drought stress had no significant impact on it. On the other hands, chlorophyll/carotenoids ratio decreased by moderate and severe drought stress (Fig. 1 E and H). All of the studied physiological parameters were higher in *T. kotschyanus* than *T. vulgaris* (Fig. 1 B, C, D, F and G).

3.2. Metabolite identification

By comparing of 42 standard spectra of metabolites reported on previous works to our spectra, 18 metabolites were identified which were similar in both species (Fig. 2 and Table 1).

3.3. Statistical analysis

ANOVA results indicated that there was a significant species × drought treatment interaction for sucrose, fructose, serine, acetic acid, citric acid, succinic acid, malic acid and fumaric acid (Supplementary Table 2). The glucose, choline and alanine were under effects of simple effects of species and drought treatment and no significant changes were observed for myo-inositol, N-acetyl-alanine, threonine, valine, glycine, lactic acid and betaine in both species (Supplementary Table 2).

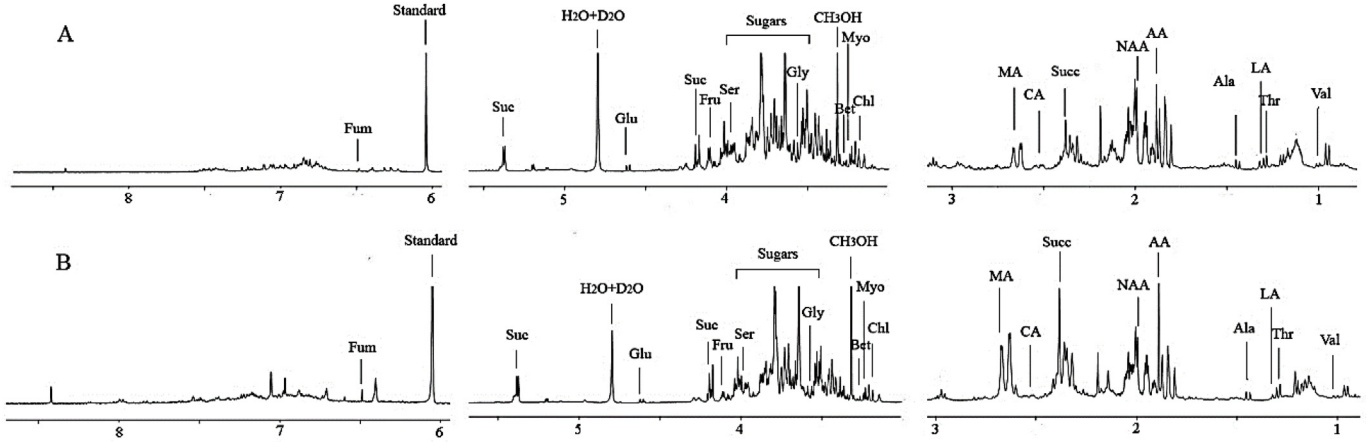

Fig. 2. 1D ^{1}H NMR spectra of *T. kotschyanus* (A) and *T. vulgaris* (B) leaf, recorded at 400 MHz, showing specific resonances of metabolites identified. Val: Valine; Thr: Threonine; LA: Lactic acid; Ala: Alanine; AA; Acetic acid; NAA: N-acetyl-alanine; Succ: Succinic acid; CA: Citric acid; MA: Malic acid; Chl: Choline; Myo; Myo-inositol: Bet: Betaine; Gly: Glycine; Ser: Serine; Fru: Fructose; Suc: Sucrose; Glu: Glucose; Standard: Maleic acid; Fum: Fumaric acid.

Table 1
Basic information of identified metabolites and their abundance in both species and 4 levels of drought.

Metabolite name	Chemical shift (multiplicity, J)	*T. kotschyanus*				*T. vulgaris*			
		90[a]	55	40	25	90	55	40	25
Acetic acid	1.90(s)	1.24[b]	1.22	0.957	0.669	1.91	0.964	0.764	1.01
Malic acid	2.36(dd, 15.3, 10.1), 2.67(dd, 15.3, 3.0), 4.30(dd, 10.1, 3.0)	11.88	14.11	12.86	11.88	11.46	16.32	13.25	17.91
Succinic acid	2.39(s)	1.69	1.83	1.77	2.35	1.36	1.81	1.59	3.08
Fumaric acid	6.51(s)	0.128	0.119	0.105	0.097	0.064	0.108	0.094	0.074
Citric acid	2.53(d,15.1),2.65(d, 15.2)	6.82	8.13	9.05	8.58	7.63	7.76	5.47	6.50
Lactic acid	1.30(d, 6.8), 4.11(q, 3.5)	2.87	2.75	2.95	2.54	2.91	3.22	2.29	2.92
Fructose	3.58(m), 3.7(m), 3.81(m), 3.90(m), 4.02(m), 4.12(d,3.8)	15.25	17.84	20.79	20.52	19.16	18.77	25.20	29.45
Sucrose	3.46(t, 9.3), 3.55(dd, 10.0, 3.9), 3.67(s), 3.74(s, 9.6), 3.8(m), 3.88(m), 4.04(t, 86), 4.21(d,8.8), 5.40(d, 3.8)	29.67	32.33	45.73	55.34	36.67	35.26	48.65	66.22
Glucose	3.22(dd,9.3,8), 3.38(m), 3.44(m), 3.51(dd, 9.8, 3.8), 3.69(m), 3.74(m), 3.81(m), 3.87(dd, 12.3, 2.2), 4.62(d, 7.9), 5.21(d,3.7)	4.31	4.94	5.79	6.55	4.88	5.16	6.38	8.14
Alanine	1.47 (d, 7.2), 3.77(q, 7.2)	8.53	9.38	12.13	12.70	10.07	10.56	14.45	13.92
Valine	1(dd, 26.7, 7), 2.27(m), 3.6(d,4.3)	3.01	3.13	3.20	3.50	2.97	3.07	3.51	3.51
Threonine	1.32(d, 6.6), 3.58(d, 4.9), 4.25(m)	3.92	3.96	4.86	4.43	3.78	4.25	4.44	4.57
Serine	3.83 (dd, 5.6, 3.8), 3.96(m)	13.94	16.81	19.96	20.98	18.39	17.35	23.32	30.83
Glycine	3.54(s)	2.72	2.62	3.81	2.50	3.07	2.97	3.08	3.24
Betaine	3.25(s), 3.89 (s)	1.09	1.73	1.53	1.50	1.51	1.50	1.76	1.57
Choline	3.19(s), 3.51(t, 4.96), 4.05 (m)	3.22	3.31	4.00	4.02	3.98	4.19	5.15	5.43
Myo-inositol	3.26(t, 9.3), 3.52 (dd, 10.0, 2.9), 3.61 (t, 9.7), 4.04(s)	1.14	1.02	1.20	1.17	1.15	1.19	1.27	1.31
N-acetyl-alanine	1.30(d, 6.8), 2(s), 4.11 (q, 3.5)	3.25	3.12	2.83	2.99	3.18	3.21	3.21	3.43
Maleic acid (Reference)	6.05(s)	5	5	5	5	5	5	5	5

[a] Drought levels.
[b] Concentration of metabolites (mM).

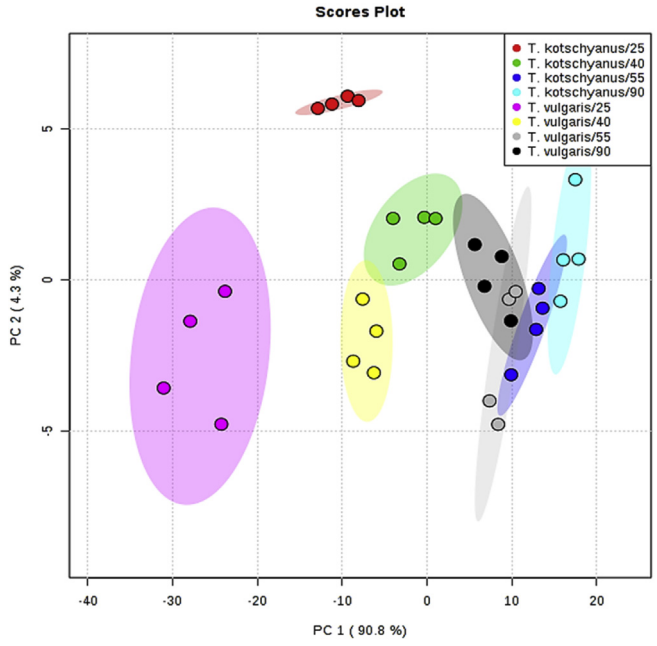

Fig. 3. Principle component analysis (PCA) of metabolites in *T. kotschyanus* and *T. vulgaris* species using PC1 versus PC2. 45 days-old plants were subjected to four levels of drought stress. 90, 55, 40 and 25 are represents drought levels as percent of FC.

PCA was able to separate species and drought treatment samples from each other. Score plot also indicated that by increasing of drought strength, samples were more separated (Fig. 3).

The hierarchal clustering separated species and drought levels (Fig. 4). As shown in Fig. 4, all samples were separated in two groups so that control and mild drought stress of both species are in the same group and moderate and severe drought stress in another group. This figure also indicating that differences between treatments are larger in *T. vulgaris* than *T. kotschyanus*.

3.4. Effects of drought treatment on metabolites concentration

All of the drought treatment levels, in *T. kotschyanus*, significantly increased sucrose concentration whereas in *T. vulgaris* only moderate (40% FC) and severe drought significantly increased it (Fig. 5 A). In *T. kotschyanus*, fructose was increased under moderate stress, but in *T. vulgaris* this metabolite had similar manner as observed for sucrose (Fig. 5 B). Glucose was affected similarly by drought in both species. Its concentration was increased under moderate and severe drought stress (Fig. 5 C).

In *T. kotschyanus*, serine was increased by enhancement of drought severity till moderate stress and then more increase of drought severity had no significant effects on this metabolite concentration. *T. vulgaris* had different respond so that serine concentration was not significantly affected by mild drought stress whereas its concentration increased under moderate and severe drought stress (Fig. 5 D). Alanine was similarly affected by drought, in both species. It was observed that mild stress had no significant effects on alanine concentration but an increase was observed under moderate drought stress and then severe stress had no significant effect on its concentration (Fig. 5 E).

Among two identified amines, only choline was affected by drought stress and species. Choline concentration was significantly increased by moderate and severe drought stress and *T. vulgaris* had a higher concentration of it than *T. kotschyanus* (Fig. 5 F and G).

Among organic acids, in both species, only lactic acid was not affected by drought stress. In *T. kotschyanus*, acetic acid was decreased only on severe drought stress but any significant change was observed in *T. vulgaris* (Fig. 5 H). Citric acid concentration of *T. kotschyanus* was increased by increase of drought stress till 40% FC and then no significant change was observed under severe drought stress. In *T. vulgaris*, citric acid concentration was decreased under mild and moderate drought but it was increased at severe drought stress (Fig. 5 I). In *T. kotschyanus* fumaric acid had a decreasing manner, by increase of drought severity, but it was significantly decreased only at severe drought stress. In *T. vulgaris*, in contrast of *T. kotschyanus*, fumaric acid had an increase at mild drought stress than control and severe drought stress significantly decreased its concentration than mild drought stress but no significant change was observed for other drought levels (Fig. 5 J).

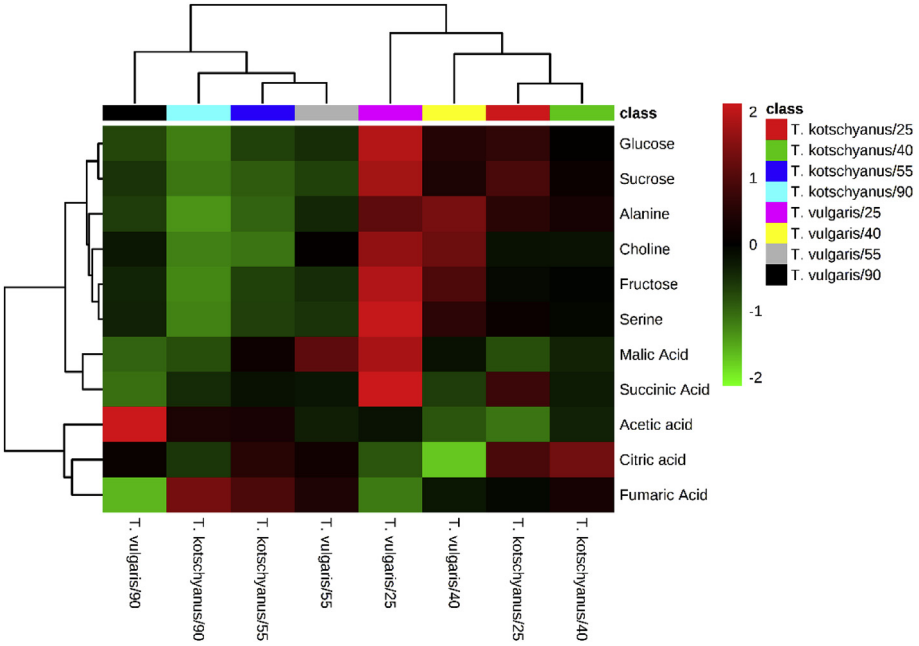

Fig. 4. Differences in primary metabolite profiles under drought treatment. Metabolites significantly affected by drought and species are shown. Heatmap colors indicate concentration of each metabolite. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Malic acid concentration of *T. kotschyanus* was increased only at mild drought stress than control and then its concentration was decreased significantly at severe drought stress than mild drought stress whereas its concentration in *T. vulgaris* was increased at mild and severe drought than control and moderate drought stress. There were no significant differences between control and moderate drought stress (Fig. 5 K).

It was observed that, in *T. kotschyanus*, succinic acid concentration elevated only at severe drought stress whereas in *T. vulgaris* its concentration was increased at mild drought stress and then it had no significant change at moderate drought stress after that it was enhanced again at severe drought stress (Fig. 5 L).

3.5. Metabolic pathway/cycle affected by drought and correlation analysis

Metabolites with significant change are involved on various pathways/cycles (Supplementary Table 3) and are shown on a simplified pathway/cycle (Fig. 6).

Correlation analysis indicated that there was a significant ($P \leq 0.01$) and positive correlation between alanine, serine, glucose, fructose, sucrose and succinic acid in both species (Fig. 5 A and B). This analysis also demonstrated that, in *T. kotschyanus*, there was a significant ($P \leq 0.01$) and negative correlation between RWC and sucrose, glucose, serine, alanine and choline. In this species, RWC was also significantly ($P \leq 0.01$) and positively correlated to acetic acid. There was a negative and significant ($P \leq 0.01$) correlation between total chlorophyll and chlorophyll/carotenoids ratio to choline, alanine and sucrose. There was also a positive and significant correlation ($P \leq 0.01$) between acetic acid and total chlorophyll and chlorophyll/carotenoids ratio (Fig. 7 A). In *T. vulgaris*, RWC was significantly ($P \leq 0.01$) and negatively correlated to sucrose, fructose, glucose, serine, succinic acid and malic acid. Total chlorophyll was significantly and negatively correlated to sucrose, fructose and serine and there was a significant ($P \leq 0.01$) and negative correlation between chlorophyll/carotenoids ratio and sucrose, fructose, glucose, serine, choline and malic acid (Fig. 7 B).

In both species, leaf temperature was significantly ($P \leq 0.01$) and positively correlated to sucrose, fructose, glucose, serine, alanine and choline. A significant ($P \leq 0.01$) and negative correlation was observed between leaf temperature and acetic acid for *T. kotschyanus* only (Fig. 7 A and B).

Correlation analysis also revealed that there was no significant ($P \leq 0.01$) correlation between Krebs intermediates in both species (by ignoring correlation of malic acid and succinic acid in *T. vulgaris*) (Fig. 5 A and B).

4. Discussion

Drought as the most important abiotic stress has deleterious effects on plants; therefore developing drought tolerant varieties will help their sustainable production. Drought tolerance or sensitivity of plants depends on plant species and genotype, length and severity of drought as well as plant growth stage (Silvente et al., 2012).

Physiological results demonstrated that among these criteria, only leaf temperature could discriminate drought tolerant and sensitive species and as reported by Moradi et al. (2014) physiological criteria are not appropriate for selecting of drought tolerant species in genus *Thymus*.

The results demonstrated that both species increased sucrose, glucose and fructose concentration under drought stress. It has been reported that plant accumulates soluble sugar such as sucrose, glucose (Zandalinas et al., 2017) and fructose (Todaka et al., 2017) under drought condition. In addition to their role as a precursor on cellular respiration, sucrose and glucose, could also act as osmoprotectant but fructose has not such activity and seems to be involved in the synthesis of metabolites like erythrose-4-phosphate which contributes on phenolic compounds and lignin biosynthesis (Rosa et al., 2009). Sucrose could be accumulated either by starch degradation (Zandalinas et al., 2017) or reduction of sugar transfer from mesophyll cells to phloem as well as reduction of growth resulted from stress (Barchet et al., 2014). Sucrose is also known as a rubisco inhibitor (Barchet et al., 2014) and could act same as proline (Zandalinas et al., 2017). Increase of sucrose concentration by degradation of starch starts with the production of β-maltose from starch at chloroplasts afterward, β-maltose is transferred to the cytoplasm and converted to glucose, fructose and ultimately sucrose (Krasensky and Jonak, 2012; Zanella et al., 2016). Sucrose could be syntehsized from glucose and fructose as well as could be a precursor for production of glucose and fructose which is under

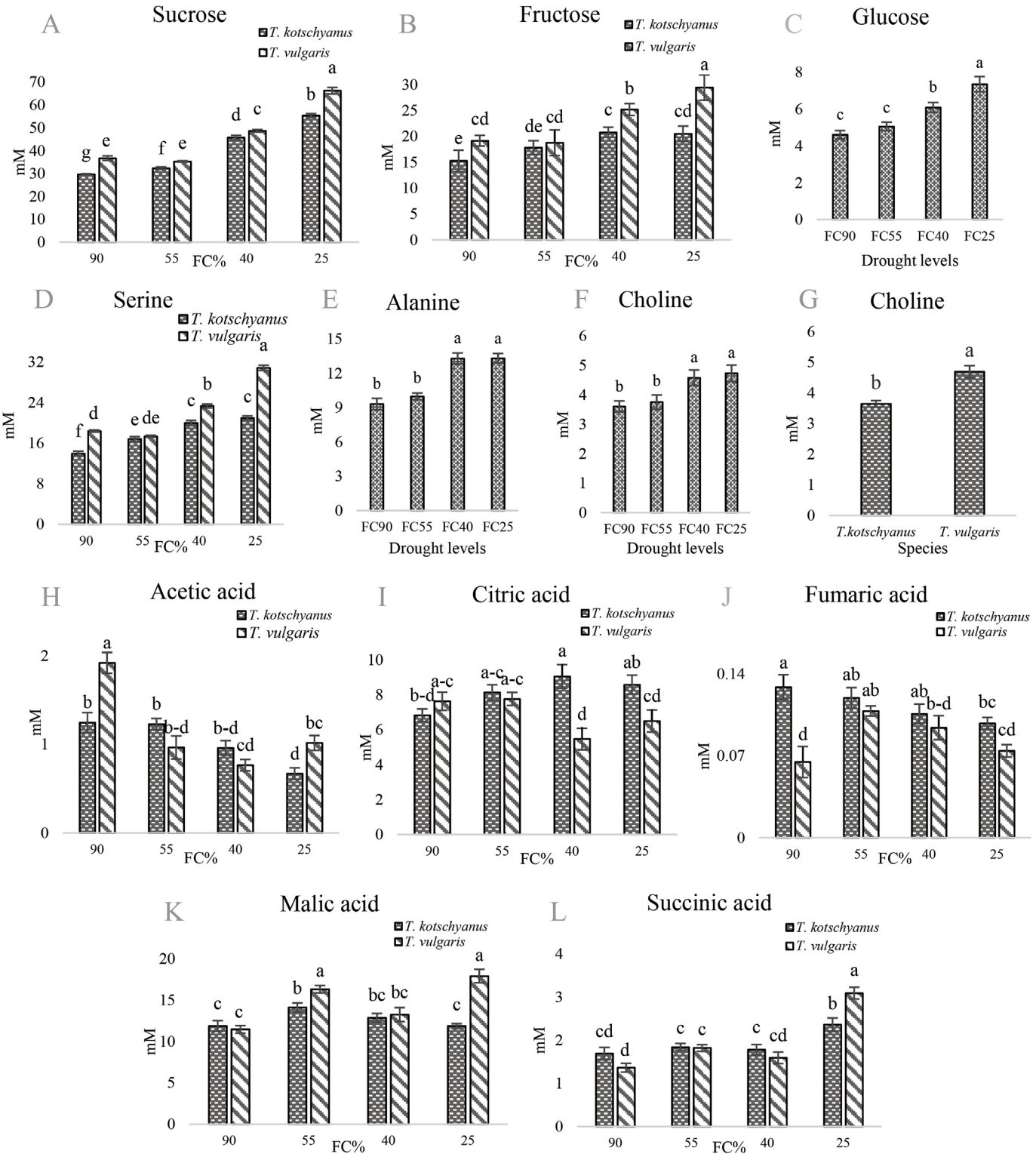

Fig. 5. Effects of drought on sucrose (A), fructose (B) and glucose (C). Each bar indicates mean and error bar represents standard errors of the mean. Effects of drought stress on serine (D) and Alanine (E). Effects of drought stress (F) and species (G) on choline concentration. Effects of drought stress on acetic acid (H), citric acid (I), fumaric acid (G), malic acid (K) and succinic acid (L) concentration of *T. kotschyanus* and *T. vulgaris*. Bars within axes with the same letter are not significantly different according to Benjamini-Hochberg corrected *t*-test $P \leq 0.05$.

environmental and developmental condition (Rosa et al., 2009).

Accumulation of some amino acids under drought stress was reported (Barchet et al., 2014; Todaka et al., 2017). This accumulation could be from de-novo synthesis or protein degradation. Zandalinas coworker (2017) suggested two main roles for amino acids accumulation which are the availability of them for protein biosynthesis and acceleration of post-stress recovery as well as osmoprotectant activity. Among six identified amino acids, alanine, valine, threonine and glycine are known to have osmoprotectant properties (Bougouffa et al., 2014). In both species, alanine and choline were similarly

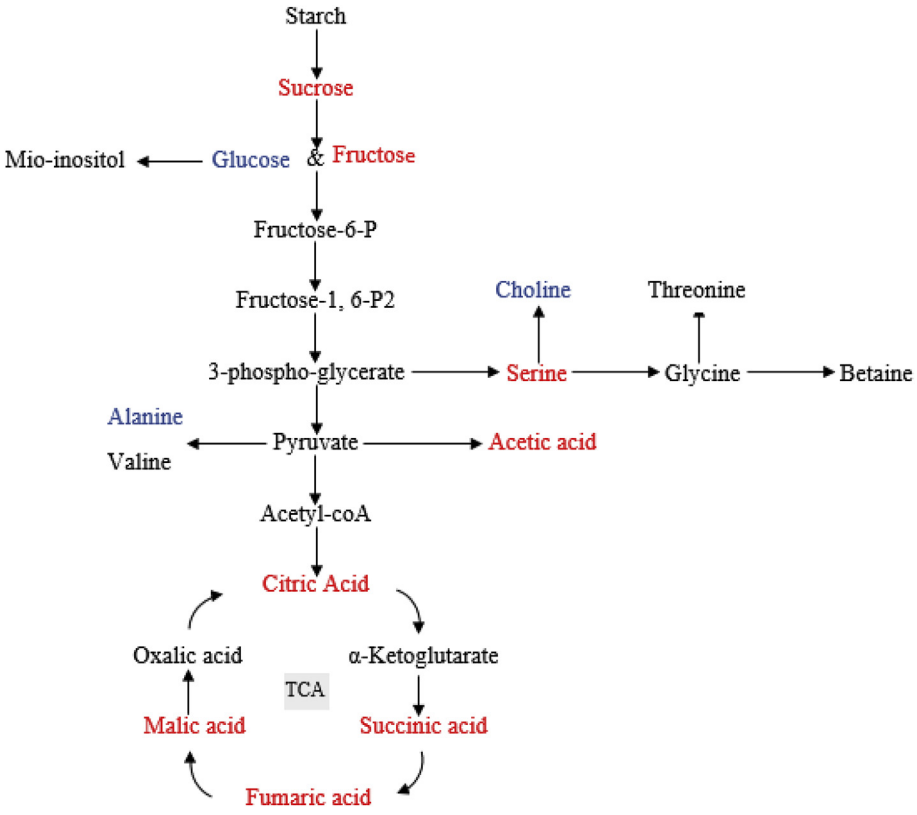

Fig. 6. Simplified pathway/cycle of *T. kotschyanus* and *T. vulgaris* metabolites that affected by drought treatment. Red: Significant effects of species × treatment. Blue: Significant effects of species or treatment. Black: Not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

increased under drought stress. Some studies reported that drought stress elevates alanine concentration (Good and Zaplachinski, 1994; Ullah et al., 2017) by synthesis of it from pyruvate (Good and Zaplachinski, 1994). Drought stress was increased Serine concentration in both species. It was previously reported that serine concentration increased by drought (Ullah et al., 2017; Zhang et al., 2017). Serine is synthesized from 3-phosphoglycerate (glycolysis pathway) and then could produce choline which is an osmoprotectant (Silvente et al., 2012). Serine is a component of some types of dehydrins, drought-inducible LEA II proteins possessing a tract of serine residues (Kosová et al., 2014), and also contributed in the synthesis of serine/arginine rich protein family which acts in alternative splicing (Duque, 2011).

According to the correlation analysis results and also previous studies, it seems that sucrose, glucose, alanine, choline as well as serine act

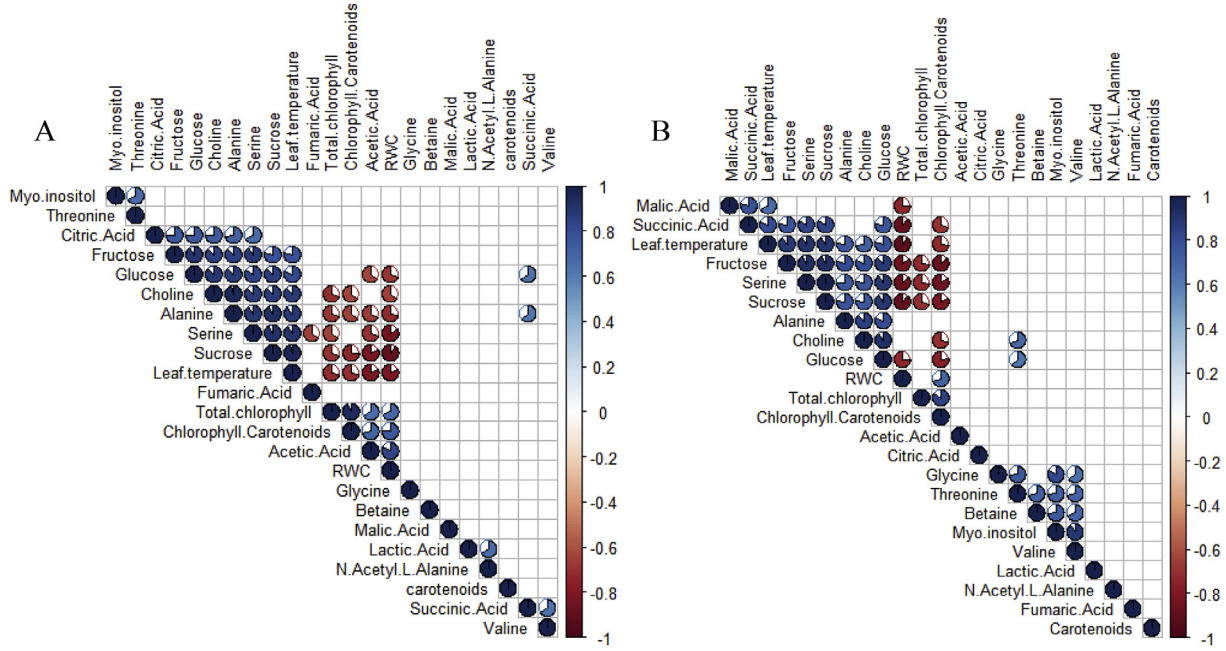

Fig. 7. Correlation matrix of metabolites and physiological parameters for *T. kotschyanus* (A) and *T. vulgaris* (B). Matrix is ordered by hierarchical clustering and Ward distance method. Color intensity and amount indicate correlation value and blank wells show no significant correlation at $P \leq 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

as the osmoprotectant in both species.

In addition to osmoprotectants, energy and carbon skeleton are also important for cell/organism stress tolerance. TCA cycle (Krebs cycle) is one of the most important cellular cycles which supplying cell energy (Sweetlove et al., 2010) and also carbon skeleton for synthesis of some amino acids (Vasquez-Robinet et al., 2008). There are contradictory reports for effects of drought on Krebs cycle and its intermediate. Some studies reported that drought increase Krebs intermediates while some other reported that it is not affected or negatively affected by drought (Araújo et al., 2012).

It has been demonstrated that Krebs intermediates have not similar flux because suppression of one of this cycle enzyme did not change the activity of other enzymes. It means that fluctuation of each intermediate is not correlated to other intermediates (Sweetlove et al., 2010). It is attributed to the relation of Krebs cycle to other cycles and pathways like synthesis/degradation of amino acids, ammonium assimilation, purines metabolism, oxalate cycle and biosynthesis of secondary metabolites (Sweetlove et al., 2010). Our results also revealed that there is no correlation between Krebs intermediates which is in accordance with Sweetlove et al. (2010).

Since the variation of Krebs intermediates had different flow than sugars and amino acids, it seems that these metabolites have a more prominent role in drought tolerance. It has been reported that suppression of succinate dehydrogenase gene in tomato (which elevate succinic acid and decrease fumaric acid levels), increased photosynthesis and biomass by mediation of an organic acid (Araújo et al., 2011). Authors also showed that there is a significant and positive correlation between succinic acid and glucose, fructose, sucrose and starch and a significant and negative correlation between succinic acid and malic acid. Results indicated that there is a significant and positive correlation between succinic acid and sucrose, fructose and glucose in *T. Vulgaris*, but in *T. kotschyanus* there is a significant and positive correlation only between succinic acid and glucose. We also observed a positive correlation between succinic acid and malic acid only in *T. vulgaris* which is contrary to Araújo et al. (2011) report.

Transformation of the tomato plant by antisense gene of *fumarase* resulted in the accumulation of fumaric acid and malic acid. This gene had a more negative effect on stomata than metabolites and reduced CO_2 and photosynthesis. Ultimately they concluded that there is a negative correlation between malic acid and fumaric acid concentration and stomatal conductance as well as transpiration. They also find that malic acid to fumaric acid ratio in guard cells is extremely effective on stomatal opening (Araújo et al., 2011). We revealed that, under drought condition, *T. vulgaris* had more soli water depletion rate as well as produce less biomass than *T. kotschyanus*. On the other hand *T. kotschyanus* could maintain its growth even under severe drought (Ashrafi et al., 2018). These observations could be explained by the increase of fumaric acid and malic acid concentration of *T. vulgaris* under drought stress and decreasing trend of this organic acid in *T.kotschyanus* (Fig. 4 J and K).

Tomato plants harboring antisense of *malate dehydrogenase* gene had more photosynthesis and biomass as well as malic acid than control. This observation was related to ascorbate because of these plants also had more ascorbate than control plants (Nunes-Nesi, 2005). Our results indicated that malic acid was increased under mild drought (in both species) and severe drought stress (in *T. vulgaris*) and other drought levels were not effective on its concentration. According to these results and observation of stunt of *T. vulgaris* under severe drought and production of less biomass than *T. kotschyanus*, under drought condition (Ashrafi et al., 2018) it seems that, in both species, growth is not correlated to malic acid, but it may be related to malic acid/fumaric acid ratio because drought stress increased and decreased this ratio in *T. kotschyanus* and *T. vulgaris*, respectively.

Inhibition of *citrate synthase* gene of tomato resulted in the reduction of Krebs intermediates and photosynthetic pigments and increase of nitrate and some amino acids (Sienkiewicz-Porzucek et al., 2008). Results indicated that photosynthetic pigments were only reduced significantly under severe drought stress which was not linked to citrate increase/decrease so, in both species, photosynthetic pigments are not likely to be related to citrate concentration.

Kim et al. (2017) reported that acetic acid has a positive correlation to the survivability of some crop plants such as wheat, rice, maize and canola. Authors suggested that increased survivability obtained by acetic acid is a result of induction of the histone deacetylase and jasmonic acid signaling pathway by the mediation of acetyl-coA (Kim et al., 2017). Under drought stress, *T. vulgaris* is more survivable than *T. kotschyanus* (Moradi et al., 2014), so *T. vulgaris* may benefits from Kim et al. (2017) proposed mechanism.

According to correlation analysis, there was a negative correlation between RWC and sucrose, glucose and serine in both species. All of these metabolites, except serine, is known as osmoprotectant (Bougouffa et al., 2014; Rosa et al., 2009) which accumulate by reduction of RWC to maintain plant water so, observation of such correlations was not unexpected. In *T. vulgaris*, RWC was negatively correlated to fructose and malic acid. According to Nunes-Nesi (2005) report, it seems that observed correlation contributed to increasing of ascorbic acid as antioxidant and photosynthesis. We observed a positive correlation between RWC and acetic acid in *T. kotschyanus*. According to experiments conducted by Moradi et al. (2014) and Kim et al. (2017), this positive correlation is likely to be related to survivability of *T. kotschyanus*. Alanine and choline were also negatively correlated to RWC of *T. kotschyanus*. Alanine is also known as an osmoprotectant (Bougouffa et al., 2014). It has been reported that accumulation of choline result in higher RWC and dry matter (Vurukonda et al., 2016). Our result indicated that *T. kotschyanus* has higher RWC that *T. vulgaris* whereas choline concentration is higher in *T. vulgaris* than *T. kotschyanus* which is in contradiction to Vurukonda et al. (2016) results.

In both species, correlation of Leaf temperature to metabolites was unlike RWC which is probably due to a negative correlation between leaf temperature and RWC.

According to the correlation analysis results, a positive and significant correlation was observed between acetic acid and photosynthetic pigments only in *T. kotschyanus*. Acetic acid by conversion to acetyl-coA incorporates in pigment biosynthesis. In both species by the increase in drought severity, photosynthetic pigments were reduced whereas acetic acid was reduced by the same trend only in *T. kotschyanus*. In *T. vulgaris*, acetic acid was reduced till moderate drought stress and after that increased. On the other hand, accumulation of malic acid or fumaric acid causing stomatal closure and reduction of biomass (Araújo et al., 2011). It seems that *T. kotschyanus* use acetic acid for production of photosynthetic pigments to maintain growth, but in *T. vulgaris*, acetic acid shift from production of photosynthetic pigments to accumulation for sustaining its survivability however, more detailed study is necessary.

5. Conclusion

As demonstrated by results, these physiological parameters are not suitable for studying drought response in *T. kotschyanus* and *T. vulgaris*. Unlike physiological criteria, metabolite profile of both species was significantly and differently affected by drought stress; therefore it could be more informative and also could help us to achieve more insight into drought tolerant mechanism in this genus.

According to the results, sugars, amino acids and energy metabolism were significantly affected by drought stress and among them, TCA cycle intermediates which is the most important cycle for providing energy and carbon skeleton have more alternation by drought than sugars and amino acids metabolism so probably it has a more prominent role in induction of drought tolerant. Finally, all of these drought-responsive metabolites are produced/consumed by different pathways/cycles; therefore if identification and manipulation of drought-responsive pathways/cycles are the ultimate goals, other Omics

technologies such as transcriptomics and Proteomics could help us to identify pathways/cycles involved in establishment of enhanced drought tolerance.

Contribution

M A, M.R A.M and P M: Design the experiment, M A: Conduct the experiments, M A and M K-Z: Data analysis, M A and M.R A.M and P M: Interpretation and analysis of results M.R A.M, P M, M K-Z, E M.F and F S: Proof reading.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.09.009.

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