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Metabolite profiling for six 'B' vitamins using LC-MS in tomato genotypes at different stages of fruit maturity

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ABSTRACT

Vitamins are essential nutrients in food crucial for maintaining good health. Tomato, being a widely consumed vegetable, provides a good quantity of vitamins. Metabolite profiling of vitamins at different stages of fruit maturity in a crop helps identify the right stage for better quality. Based on preliminary screening for quality parameters, tomato lines rich in TSS, antioxidants, lycopene and beta-carotene were selected for the present study. Eight genotypes and a wild species were profiled for 'B' vitamins at three different stages of fruit maturity, viz., green, breaker and ripe stage. A simple and sensitive liquid chromatography-tandem mass spectrometric (LC-MS/MS) method for simultaneous determination of six 'B' vitamins was developed and validated by us. Among the genotypes studied, IIHR-249-1 recorded higher niacin, pantothenic acid and biotin content. Pyridoxine content was higher in the hybrid, Arka Rakshak. The wild species, LA-1777 *(Solanum habrochaites*) was found to be rich in pantothenic acid, riboflavin and thiamine. Content of most of the vitamins increased with ripening of the fruit. IIHR-249-1 and LA-1777 were found to be rich in 'B' vitamins, earlier reported to be also rich in antioxidants and lycopene. These genotypes can be used for improving the nutritive value of tomato under crop improvement programmes, through conventional breeding or biotechnological approaches.

Key words: Tomato, B vitamins, LC-MS/MS-MRM, fruit ripening, green stage, breaker stage

INTRODUCTION

Tomato is rich in lycopene, β-carotene, phenols and flavonoids, having moderate amounts of Vitamin C (Stewart *et al*, 2000; Beutner *et al*, 2007). It is also a good source of Vitamin E, thiamine, niacin, pyridoxine, folate, vitamin K and dietary fibre (USDA, 2006). With high levels of health-promoting bioactive compounds and antioxidants, tomato fruit has also been identified as a functional and nutraceutical food (Agarwal and Rao, 1998; Canene-Adams *et al*, 2005).

Vitamins are nutrients essential in our diet. Eight of the water-soluble vitamins are known as Vitamin B-complex group. Thiamine (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin (B_7), folic acid (B_9) and cyanocobalamin (B_{12}) constitute Vitamin B-complex. The B vitamins function as coenzymes helping the body obtain energy from food. These are also important for good vision, a healthy skin, nervous system and red blood cell formation. Various methods like microbiological assays, spectrophotometric assays, capillary electrophoresis, TLC, HPLC and a few LC-MS based methods have been used for estimation of 'B' vitamins (Chen *et al*, 2006). Tomato is a very widely consumed vegetable globally and is moderately rich in vitamins, but it needs to be improved for content of 'B' vitamins - very crucial for maintaining optimal health. Metabolite profiling of the tomato fruit for vitamins can help improve its quality.

The present study was undertaken to study variations in the profile of 'B' vitamins in eight selected genotypes that included hybrids, varieties, an elite germplasm line and a wild species, at three different stages of fruit ripening. We also developed a simple, sensitive and reliable LC-MS/MS method for quantification of six 'B' vitamins, namely, thiamine, riboflavin, niacin, pantothenic acid, pyridoxine and biotin.

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MATERIAL AND METHODS

Plant material

Based on initial screening of a large number of tomato lines for quality parameters, a total of nine genotypes of tomato including released varieties, commercial hybrids, cherry tomato, an elite germplasm line and a wild species were used for profiling 'B' vitamins, as follows:

- Commercial hybrids: Arka Ananya, Arka Samrat and Arka Rakshak
- Varieties**:** Arka Ashish and Arka Vikas
- Cherry tomato lines: IIHR-2864 and IIHR-2866
- Wild species: *Solanum habrochaites* (LA-1777)
- Elite germplasm line: IIHR-249-1

Plants were raised in the field under irrigated conditions as per the standard package of practices. 'B' vitamins were assessed in fruits harvested at three different stages of ripening, viz., green stage (45 days post-anthesis), breaker stage (55 days post-anthesis) and red ripe stage (60 days post-anthesis, when the fruit is completely red or orange, yet firm). Samples were collected from three different plants in all the genotypes.

Reagents

The water-soluble vitamin standard thiamine hydrochloride (≥99%), riboflavin (99%), niacin (99.5%), calcium D-pantothenate (98%), pyridoxine hydrochloride (99%) and biotin (≥99%) were procured from Sigma Chemical Co., USA. Standard vitamin solutions were prepared in 0.01N HCl. Butylatedhydroxy toluene (BHT) and ammonium formate were procured from Himedia, India. Amino acid standard mixtures at a concentration 2.5µmoles per ml, *o*-phthalaldehyde (OPA) reagent and formic acid were obtained from Sigma. Amino acid standard solutions were prepared in 0.1N HCl. Sodium phosphate [monobasic and dibasic (anhydrous)], sodium hydroxide and boric acid were obtained from Merck, India. Organic solvents used as a mobile phase for liquid chromatography were of chromatographic/MS grade.

Equipment

Acquity UPLC-H class, coupled with Acquity TQD-MS/MS from Waters, USA with ESI source, was used for determinating water-soluble vitamins. The instrument was equipped with a degasser, quaternary pump, automatic injection system, with a diode array detector and a temperature control compartment for the analytical column. The detection system allowed simultaneous detection at various wavelengths and MRM for individual masses. The overall system-control and data acquisition were monitored by Mass Lynx™ software.

Extraction of water-soluble vitamins

Extraction of water-soluble vitamins was done as per methods previously reported, with some modifications (Santos *et al*, 2012; Zand *et al*, 2012). During extraction it was assumed, that the samples were protected from direct exposure to light, to avoid degradation of the vitamins. In brief, 3-4 fruits of tomato from each of the genotypes were homogenized for one minute in a mixer-blender. From the homogenized mixture, 10g were weighed and then extracted with 40ml of 10 mM ammonium formate/methanol 50:50 (v/v) containing 0.1% BHT. After shaking for 5 minutes to achieve good sample-dispersion in the extraction liquid, the samples were incubated in a water bath at 70°C for 40 min. After cooling down to room temperature, the samples were centrifuged at 14000g for 10 min and the volume made up to 50ml with 10mM ammonium formate. Finally, the supernatant was filtered through a 0.2µm nylon filter and injected into an UPLC-MS/MS system.

LC and MS-MS conditions

Separation, identification and quantification of the six 'B' vitamins was performed using UPLC coupled with tandem mass spectrometry detection, using Multiple Reaction Monitoring (MRM) mode. The column used was UPLC BEH C_{18} (2.1 x 50mm, 1.7µm; Waters, USA) with security guard column Vanguard BEH-C_{18} (2.1 x 5mm, 1.7µm; Waters, USA). The column oven was maintained at 25°C, with the sample injection volume being 3.0µl. Eluted vitamins were monitored using a PDA detector and TQD-MS/MS (Waters, USA), where LC-MS conditions were optimized for analysis of the vitamins. The binary mobile phase consisted of an aqueous phase of 0.1% formic acid in water (A) and organic phase of methanol (B). The initial flow was composed of 95% of A and 5% of B, and was held for 1.0 min. The gradient was gradually changed to 30% of A and 70% of B over a period of 6 min. then hold for 0.5 min. The system was then returned to 95% A and 5% B for 12 min. Flow rate was maintained at 0.1 ml/min. Simultaneous determination of the six 'B' vitamins through LC-MS/MS by MRM method is depicted in Fig. 1.

MS-MS method validation

Multiple reactions monitoring (MRM) detection mode was employed for analysis of 'B' vitamins. Details on precursor ions, collision induced product ions and the optimised cone voltage and collision energies for each of the vitamins under ESI^{+ve} mode are presented in Table 1.

MS-MS parameters such as capillary voltage, extractor voltage and RF lens volts were set at 3.2kV, 4V and 0.1V respectively. Nitrogen gas-flow for desolvation and cone were set at 550 and 50 l/h, with desolvation and ion-source temperatures at 350°C and 135°C respectively. Evaluation parameters used for validating the methodology for determination of the vitamins were: linearity, detection, quantification limits and repeatability. Regression line equations, correlation coefficients (r), LOD and LOQ for each vitamin are shown in Table 2. Linearity was determined by constructing calibration curves, and five injections (n= 5) were made at each level, resulting in mean coefficient of variation for the injections of 1.6 to 4.5%. Fresh fruits of tomato were used for validating the method of extraction and estimation of vitamins.

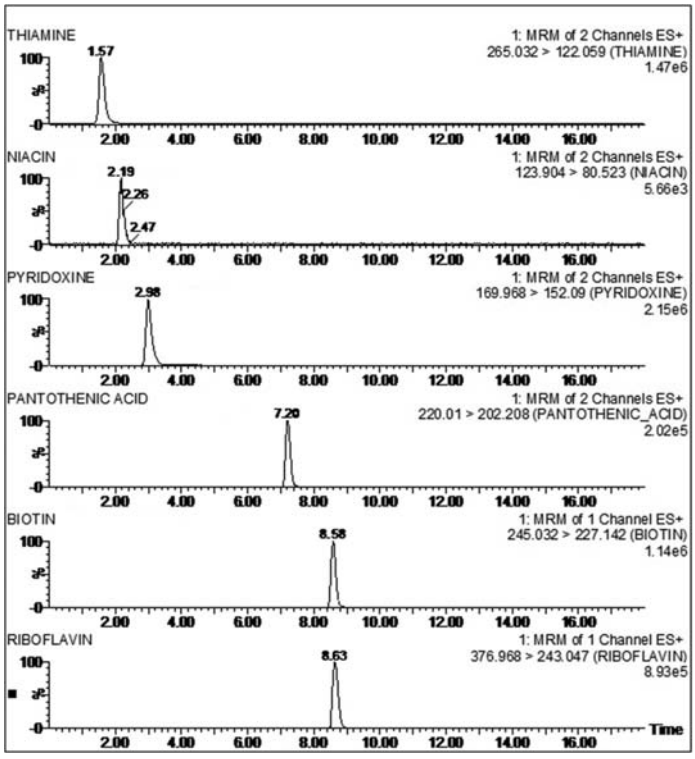

Fig. 1. LC-MS chromatograms of 'B' vitamins

Recovery obtained for all the six 'B' vitamins ranged between 85 and 90%. The recovery studies were carried out by estimating the six 'B' vitamins in the spiked and non-spiked samples. Recovery was calculated from the difference between the spiked and un-spiked samples, and was expressed as percentage.

HPLC analysis of free amino acids

Amino acids, viz., glutamic acid, alanine and valine, which are precursors for biosynthesis of most of the 'B' vitamins, were analyzed using high performance liquid chromatography (HPLC) (Bartolomeo and Maisano, 2006). The chromatograph used was LC-10A system (Shimadzu, Kyoto, Japan) connected to a UV-visible detector (10 A) with binary pump, and controlled by Shimadzu Class VP

Table 1. MRM of 'B' vitamin standards

'B' Vitamins	Formula mass	Parent ion (m/z) $[M+H]^+$	Daughter ions	Cone voltage	Collision energy (CE)	Ionisation mode
Thiamine (B_1)	264	265.03	122.06 (Q)[a]	20	16	ESI+
		265.03	144.02	20	14	ESI+
Riboflavin (B_2)	376	376.97	243.05	40	24	ESI+
Niacin (B_3)	123	123.9	80.523 (Q)[a]	34	20	ESI+
		123.9	77.47	34	18	ESI+
Pantothenic acid (B_5)	219.03	220.01	202.21	28	12	ESI+
		220.01	124.16 (Q)[a]	28	20	ESI+
Pyridoxine (B_6)	169	169.97	152.09 (Q)[a]	24	12	ESI+
		169.97	134.04	24	20	ESI+
Biotin (B_7)	244	245.03	222.14	26	14	ESI+

[a] "Q" taken as quantifier ion

Table 2. Calibration curves, LOD and LOQ for B vitamins

Compound	RT	Standard curve	Correlation coefficient (r)	LOD[a] (ng/µl)	LOQ[b](ng/µl)	Linear range tested (ng/µl)
Thiamine	1.60	Y = 4014X – 3229.6	0.999	0.50	1.53	1.92 - 15.6
Riboflavin	8.62	Y = 4493X + 1.3825	0.998	0.51	1.55	1.0 - 8.0
Niacin	2.15	Y = 212.14X + 27.2	0.997	0.62	1.87	2 - 16
Pantothenate	7.20	Y = 2593X - 66.54	0.999	0.19	0.59	1.6 - 12.8
Pyridoxine	3.02	Y = 47530X + 19652	0.996	0.5	1.50	2.14 - 17.12
Biotin	8.58	Y = 46818X + 1968.2	0.999	0.34	1.02	1.0 - 8.0

[a]LOD= Limit of detection (S/N=3)
[b]LOQ= Limit of quantitation (S/N=10)

Workstation software. The column used was Agilent Eclipse AAA (5µm, 4.6 X 150mm) with the guard column fitted with a C_{18} cartridge (Cat. no. 4287, Phenomenex). The binary mobile phase consisted of 40mM $Na_2H_2PO_4$/ Na_2HPO_4 (1:1) buffer at pH 7.8 (A) and Acetonitrile/ Methanol/Water (45:45:10) (Merck Ltd, India) (B) with flow-rate of 2.0ml/min.

Sample extraction and derivatization

Extraction of amino acids was done as per a previously reported method using Methanol:Chloroform (70:30 v/v) mixture (Marur *et al,* 1994; Pratta *et al,* 2011). Derivitization of amino acids was done using *o*-phthalaldehyde, before injection into the column, using a 20µL loop (Rheodyne, Rohnert Park, CA, USA). The column and guard column were thermostatically controlled at 32°C. The instrument was run in a gradient mode, and detection was monitored on UV absorbance at 338nm wavelength. Retention time for glutamate, alanine and valine were 5.13, 11.28 and 15.17 min respectively. Amino acids were quantified by running their standards under a gradient elution programme. The initial flow was composed of 100% of A, and was held for 1.9 min. The gradient was gradually changed to 43% of A and 57% of B, over a period of 2 min. The system was then changed to 100% B at 24 min; at 27 min, the system was returned to 100% A. The flow rate was maintained at 2.0 ml/min.

Statistical analysis

Analysis of Variance (ANOVA) was carried out for guaging the statistical significance of differences among genotypes. Results were analyzed by Two-way ANOVA (with replications), using Microsoft Excel software. Mean values were compared using least significant difference (LSD) at 1% probability. Mean values were calculated from three independent experiments in all the cases. Pearson correlation coefficient (r) among all the variables was calculated using Microsoft Excel software. Principal Component Analysis (PCA) using StatistiXL software (version 1.8) was made to assess the importance of each source of variation (genotype and ripening stage) in categorizing the results obtained to estimate metabolic changes occurring during fruit ripening.

RESULTS AND DISCUSSION

The B vitamins detected in tomato were: niacin, pyridoxine, pantothenic acid, riboflavin, thiamine and biotin (Table 3). Content of all the B vitamins differed significantly among genotypes and at different stages of fruit maturity, at $P \leq 0.01$.

Among the genotypes tested, highest values for niacin and pyridoxine were recorded at the ripe stage in Arka Vikas (0.350mg kg^{-1} fw) and Arka Rakshak (0.252mg kg^{-1} fw). Lowest pyridoxine content was recorded in the wild species, LA-1777 (0.010 mg kg^{-1} fw). However, pantothenic acid was highest at the ripe stage of the wild species LA-1777 (2.522 mg kg^{-1} fw), followed by IIHR-249-1 (1.295mg kg^{-1} fw); the other lines recorded this value in the range of 0.373 to 0.531mg kg^{-1} fresh weight. Maximum riboflavin content was recorded at the ripe stage in LA-1777 (0.621mg kg^{-1} fw), and was 15.2 times more than in IIHR-249-1 (0.041mg kg^{-1} fw), and 5.6 times more than that in the other seven lines (0.110mg kg^{-1} fw). Riboflavin content in the wild species was found to be 1.9 times more than the USDA reference value for tomato (0.034mg kg^{-1} fw). The highest value for thiamine was also observed at the ripe stage in LA-1777 (0.830mg kg^{-1} fw), followed by 'Arka Ananya' (0.326mg kg^{-1} fw), while, the lowest value was recorded in IIHR-249-1 (0.020mg kg^{-1} fw). The highest total biotin content was found at the ripe stage in IIHR-249-1 (0.564mg kg^{-1} fw), followed by 'Arka Vikas' (0.469mg kg^{-1} fw) and 'Arka Ashish' (0.427mg kg^{-1} fw). The wild species LA-1777 recorded the lowest biotin content (0.195mg kg^{-1} fw).

Some varieties of tomato have been earlier reported to contain a good quantity of pantothenic acid (0.42 to 0.54mg kg^{-1} fw), biotin (0.01 to 0.014mg kg^{-1} fw) and niacin (5.39mg kg^{-1} fw) (James, 1952). Thiamine content of 0.001 to 0.028mg per kg fresh weight was reported in fifteen commonly grown vegetables in southern Thailand (Taungbudhitham, 1995). However, in the present study, the highest value recorded for pyridoxine (0.252mg kg^{-1} fw) and niacin (0.350mg kg^{-1} fw) in tomato at ripe stage was less than the USDA reference value for both [pyridoxine (0.65 mg kg^{-1} fw), niacin (5.9 mg kg^{-1} fw)]. Riboflavin is naturally present in several foods and beverage, such as liver, cheese, milk, meat, eggs, peas, beans, whole-grain cereals, and wines (AMC, 2000; Capo-chichi *et al,* 2000). The variation in B vitamin content among the under study lines may be due to differences in their genetic background. Genotype IIHR-249-1, and the wild species LA-1777 were found to be rich in pantothenic acid, riboflavin, thiamine and biotin. These genotypes were also reported earlier to be rich in Vitamin C, lycopene, phenols and flavonoid with high TSS (Kavitha *et al,* 2013). These lines can be further used for improving vitamin content by introgression of wild species with lines having a good horticultural background.

Among the different stages of fruit ripening, niacin content increased with ripening in the case of IIHR-249-1

Table 3. 'B' vitamins in selected lines of tomato in (fruits harvested at three different stages of ripening, viz., Green stage (GS), Breaker stage (BS) and Ripe stage (RS)

Metabolite/	Niacin			Pantothenic acid			Pyridoxine			Riboflavin			Thiamine			Biotin		
ripening stage	mg kg^{-1} fresh weight																	
Genotype	GS	BS	RS	GS	BS	RS	GS	BS	RS	GS	BS	RS	GS	BS	RS	GS	BS	RS
IIHR-249-1	0.042	0.112	0.127	0.260	1.005	1.295	0.097	0.223	0.124	0.010	0.041	0.041	0.036	0.104	0.020	0.292	0.450	0.564
	(0.737)	(0.783)	(0.792)	(0.872)	(1.227)	(1.340)	(0.773)	(0.850)	(0.790)	(0.714)	(0.735)	(0.735)	(0.732)	(0.777)	(0.721)	(0.890)	(0.974)	(1.032)
IIHR-2866	0.038	0.000	0.117	0.521	0.442	0.460	0.192	0.220	0.080	0.050	0.041	0.111	0.058	0.051	0.138	0.144	0.652	0.370
	(0.733)	(0.707)	(0.785)	(1.010)	(0.971)	(0.980)	(0.832)	(0.848)	(0.761)	(0.742)	(0.736)	(0.785)	(0.747)	(0.742)	(0.799)	(0.803)	(1.073)	(0.933)
IIHR-2864	0.040	0.206	0.083	0.894	0.631	0.491	0.150	0.218	0.071	0.086	0.115	0.120	0.049	0.067	0.202	0.139	0.538	0.459
	(0.735)	(0.840)	(0.763)	(1.181)	(1.063)	(0.995)	(0.806)	(0.847)	(0.755)	(0.766)	(0.784)	(0.787)	(0.741)	(0.753)	(0.838)	(0.799)	(1.019)	(0.979)
A. Rakshak	0.043	0.057	0.000	0.516	0.524	0.373	0.130	0.185	0.252	0.034	0.093	0.115	0.079	0.023	0.178	0.139	0.477	0.376
	(0.737)	(0.746)	(0.707)	(1.008)	(1.012)	(0.934)	(0.794)	(0.828)	(0.867)	(0.731)	(0.770)	(0.784)	(0.761)	(0.724)	(0.823)	(0.800)	(0.988)	(0.936)
A. Ashish	0.000	0.000	0.067	0.168	0.864	0.416	0.078	0.264	0.125	0.010	0.053	0.123	0.029	0.032	0.262	0.224	0.617	0.427
	(0.707)	(0.707)	(0.753)	(0.817)	(1.168)	(0.957)	(0.760)	(0.874)	(0.791)	(0.714)	(0.744)	(0.790)	(0.727)	(0.729)	(0.873)	(0.851)	(1.057)	(0.963)
A. Ananya	0.062	0.040	0.044	1.058	0.371	0.532	0.181	0.152	0.169	0.026	0.059	0.108	0.278	0.033	0.326	0.042	0.582	0.315
	(0.750)	(0.735)	(0.738)	(1.248)	(0.933)	(1.016)	(0.825)	(0.808)	(0.818)	(0.726)	(0.748)	(0.780)	(0.882)	(0.730)	(0.909)	(0.737)	(1.036)	(0.710)
A. Vikas	0.025	0.073	0.350	0.314	0.905	0.454	0.058	0.180	0.066	0.008	0.034	0.098	0.040	0.031	0.185	0.057	0.221	0.469
	(0.724)	(0.757)	(0.922)	(0.902)	(1.186)	(0.977)	(0.747)	(0.825)	(0.752)	(0.713)	(0.731)	(0.773)	(0.735)	(0.729)	(0.828)	(0.746)	(0.849)	(0.984)
A. Samrat	0.054	0.061	0.045	0.854	0.364	0.473	0.138	0.182	0.100	0.025	0.098	0.113	0.009	0.037	0.168	0.031	0.435	0.391
	(0.745)	(0.749)	(0.738)	(1.164)	(0.929)	(0.986)	(0.799)	(0.826)	(0.775)	(0.724)	(0.773)	(0.783)	(0.714)	(0.733)	(0.817)	(0.729)	(0.967)	(0.944)
LA-1777	0.027	0.000	0.193	2.208	0.000	2.522	0.014	0.000	0.010	0.110	0.000	0.621	0.079	0.000	0.830	0.143	0.000	0.195
	(0.726)	(0.707)	(0.833)	(1.646)	(0.707)	(1.738)	(0.717)	(0.707)	(0.714)	(0.781)	(0.707)	(1.059)	(0.761)	(0.707)	(1.153)	(0.802)	(0.707)	(0.834)
Mean	0.036	0.061	0.114	0.755	0.567	0.780	0.115	0.180	0.111	0.040	0.059	0.161	0.073	0.042	0.257	0.130	0.377	0.362
CD (*P≤0.01*)																		
Genotype (G)		0.0095			0.0231			0.0061			0.0033			0.0057			0.0232	
Stage (S)		0.0055			0.0133			0.0035			0.0019			0.0032			0.0134	
G x S		0.0164			0.0400			0.0106			0.0056			0.010			0.0402	

*Values in parentheses are square root transformed

(0.042 to 0.127mg kg^{-1} fw), IIHR-2866 (0.038 to 0.117mg kg^{-1} fw), 'Arka Vikas' (0.025 to 0.350mg kg^{-1} fw) and LA-1777 (0.027 to 0.193mgkg^{-1} fw); but, it was higher in the breaker stage, and decreased with ripening in the other lines. Among the different stages of ripening, pyridoxine content was high in the breaker stage in all the lines studied, except 'Arka Rakshak' which recorded highest pyridoxine content at the ripe stage (0.252mg kg^{-1} fw). Pantothenic acid content increased with ripening in the line IIHR-249-1 (0.260 to 1.295mg kg^{-1} fw), and LA-1777 (2.208 to 2.522mg kg^{-1} fw), whereas, it was higher at the green stage, and decreased with ripening in cherry tomato lines IIHR-2866 (0.521 to 0.460mg kg^{-1} fw) and IIHR-2864 (0.894 to 0.491mg kg^{-1} fw). A similar trend was observed in IIHR hybrids 'Arka Ananya' (1.058 to 0.532mg kg^{-1} fw), 'Arka Rakshak' (0.516 to 0.373mg kg^{-1} fw) and 'Arka Samrat' (0.854 to 0.473mg kg^{-1} fw). In the case of varieties, it was high in the breaker stage, decreasing thereafter. Among the different stages analyzed, riboflavin increased with ripening in all the lines, while thiamine content increased with ripening in all the lines except IIHR-249-1 (where it decreased from green stage to ripe stage) (0.036 to 0.020mg kg^{-1} fw). Biotin content increased with ripening in IIHR-249-1 (0.292 to 0.564mg kg^{-1} fw) and in other lines; whereas, it decreased from the breaker to ripe stage in IIHR-2866 (0.652 to 0.370mgkg^{-1} fw), 'Arka Rakshak' (0.477 to 0.376mg kg^{-1} fw), 'Arka Samrat' (0.435 to 0.391mg kg^{-1} fw) and 'Arka Ashish' (0.617 to 0.427mg kg^{-1} fw).

In general, 'B' vitamin content in the ripe fruit was considerably higher than in the unripe fruit, which may be related to higher availability of carbohydrate precursors during fruit ripening (Carrari and Fernie, 2006). However, very few reports are available on accumulation of vitamins at different stages of fruit maturity. An increase in Vitamin C content as pepper fruits mature has been reported earlier (Osuna-Garcia *et al*, 1998; Bae *et al*, 2014).

Relation between 'B' vitamins and their amino acid precursors

Glutamate, alanine and valine precursors for biosynthesis of some of the 'B' vitamins were analyzed at different stages of fruit ripening. Highest glutamate content was recorded at the ripe stage in 'Arka Ashish' (417.86mg kg^{-1} fw), and lowest in 'Arka Samrat' (221.67mg kg^{-1} fw). There was an increase in accumulation of glutamate from the green to the ripe stage in most of the genotypes. Higher value for alanine was recorded at the ripe stage in LA-1777 (1505.4 mg kg^{-1} fw), which was about 5 times more than in the elite germplasm line IIHR-249-1 (317.05mg kg^{-1} fw). Alanine content increased with ripening in almost all the genotypes. Among the genotypes, highest valine content was recorded at the ripe stage in the wild species LA-1777 (159.02mg kg^{-1} fw), and the lowest in the hybrid 'Arka Samrat' (5.24mg kg^{-1} fw). Accumulation of valine was higher in the breaker stage compared to that in ripe or green stages (Fig. 2).

Correlation coefficients run between 'B' vitamins and the three precursor amino acids indicated that alanine and valine were strongly correlated to pantothenic acid (r = 0.97, r = 0.91 respectively, $P \leq 0.01$), whereas, alanine did not show any significant relationship with biotin. Glutamate showed significant correlation with pyridoxine (r = 0.86, $P \leq 0.01$). However, it did not show any significant relationship with niacin. In the present study, the higher levels of pantothenic acid and pyridoxine observed may be directly related to higher supply of precursor amino acids valine, alanine and glutamate.

As previously reported, glutamate is the principal free-amino-acid in ripe fruits of cultivated varieties of tomato, and free amino acids increase dramatically during fruit ripening, with their abundance changing differentially (Sorrequieta *et al*, 2010). Total amino acid content at red ripe stage was higher than in the mature green stage in tomato germplasm lines, and their relative content increased from mature-green to ripe stage (Forde and Lea, 2007; Pratta *et al*, 2011). Significant increase in glutamic acid and reduced levels of alanine and valine, throughout maturation and ripening was reported in various lines of tomato (Omas-Oliu *et al*, 2011). Increased glutamate content towards the end of ripening is also reported in tomato, which could be due to a cessation of chlorophyll biosynthesis, since, glutamate is also a precursor of chlorophyll (Carrari and Fernie, 2006).

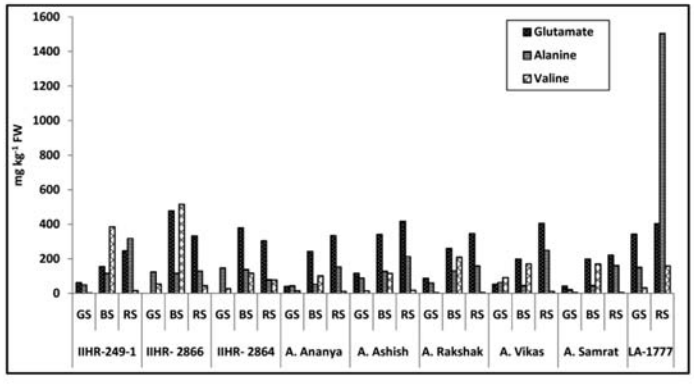

Fig. 2. Glutamate, alanine and valine in various genotypes of tomato at different stages of fruit maturity (GS: Green Stage, BS: Breaker Stage, RS: Ripe Stage)

Principal component analysis (PCA) for distinguishing genotypes and stages of fruit maturity

Principal Component Analysis (PCA) was done to understand the pattern of accumulation of 'B' vitamins at different stages of fruit maturity. As the selected genotypes were of different genetic backgrounds, PCA allowed us to understand the relation between distribution of 'B' vitamins across genotypes, and at different stages of fruit maturity.

Data on vitamins at the ripe stage in all the genotypes were subjected to PCA. PC 1 and PC 2 contributed to the wide variability of 97.4% among genotypes (Fig. 3). A biplot of PC1 and PC 2 revealed that the wild species LA-1777, and the germplasm line IIHR-249-1 were completely different from the other genotypes (hybrids, varieties or cherry tomato lines). LA-1777 was distinct from IIHR-249-1, but these two genotypes were characterized by high levels of niacin, thiamine, riboflavin and pantothenic acid. All the other genotypes grouped along with PC2 were found to contain higher levels biotin and pyridoxine, but lower levels of the other 'B' vitamins.

A second PCA was done to study accumulation of 'B' vitamins at different stages of fruit maturity. Out of six principal components (PC's), two, viz., PC 1 and PC 2, accounted for 87.8% variability among the different stages of fruit maturity (Fig. 4). In a biplot of PC 1 and PC 2, the three stages in the genotypes appeared as separate groups. The ripe stage of the genotypes was characterized by higher amounts of 'B' vitamins, particularly biotin, thiamine and riboflavin. Breaker stage was found to possess the highest amount of niacin, pyridoxine and pantothenic acid, while, the green stage was found to be associated with low levels of all the 'B' vitamins studied. A wide variability was observed between the green and the breaker stage than in the ripe stage in all the genotypes studied, indicating that biosynthesis of 'B' vitamins was primarily ripening-regulated.

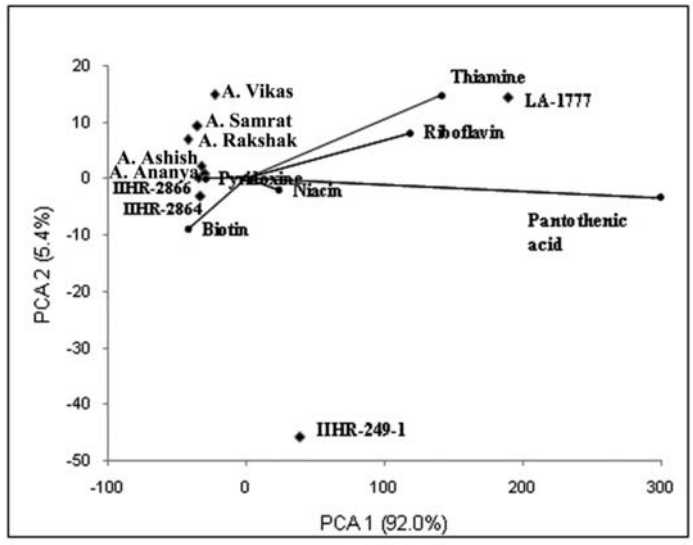

Fig. 3. Distribution of 'B' vitamins (circles) and tomato genotypes (rhombus) at ripe stage in the coordinates of Principal Components 1 and 2 (PC1 and PC2, respectively)

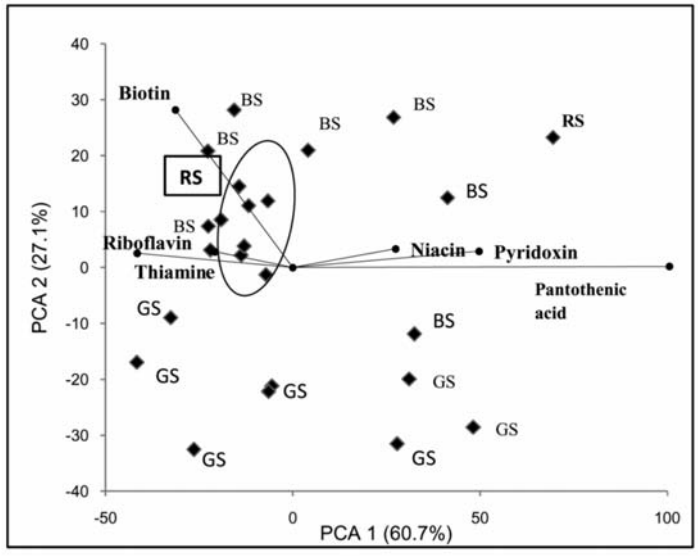

Fig. 4. Distribution of B vitamins (circles) at Green Stage (GS), Breaker Stage (BS) and Ripe Stage (RS) in the coordinates of Principal Components 1 and 2 (PC1 and PC2, respectively)

CONCLUSION

LCMS-MRM as a technique has proved to be sensitive, selective and a reliable method for individual determination of six 'B' vitamins in tomato. The elite germplasm line IIHR-249-1, and the wild species LA-1777 with high pantothenic acid, riboflavin, thiamine and biotin content; the hybrid 'Arka Rakshak', with fairly high pyridoxine serve as a good source material for improving nutritive value of the tomato for use in crop breeding or biotechnological approaches. Higher levels of alanine and valine may be indicative of higher accumulation of pantothenic acid in tomato. Ripe stage was found to be a rich source of vitamins. However, in some genotypes, niacin, pyridoxine and biotin levels remained nearly the same at breaker and ripe stages.

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