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Quantification of phenolic acids and antioxidant potential of inbred, hybrid and composite cultivars of maize under different nitrogen regimes

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Abstract

Maize (*Zea mays* L.) is a multipurpose crop, which is immensely used worldwide for its nutritional as well as medicinal properties. This study evaluates the effect of varying concentrations of nitrogen (N) on accumulation of phenolic acids and antioxidant activity in different maize cultivars, including inbreds, hybrids and a composite, which were grown in natural light under controlled temperature (30°C/20°C D/N) and humidity (80%), with sufficient (4.5mM) and low (0.05mM) nitrogen supply. Seeds of different cultivars were powdered and extracted in a methanol:water (80:20) mixture through reflux at 60-75°C, and the extracts obtained were subjected to high performance thin layer chromatography (HPTLC), using ethyl acetate: acetic acid: formic acid: water (109:16:12:31) solvent system for the separation of phenolic acids. Antioxidant activity of the extracts was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and H_2O_2-scavenging activity assays. At sufficient nitrogen condition, the contents of different phenolic acids were higher in the composite cultivar (8.7 mg g^{-1} d.wt. in gallic acid to 39.3 mg g^{-1} d.wt. in cinnamic and salicylic acids) than in inbreds and hybrids. Under low nitrogen condition, the phenolic acids contents declined significantly in inbreds and hybrids, but remained almost unaffected in the composite. The antioxidant activity was also the maximum in the composite, and declined similarly as phenolic acids under low nitrogen supply, showing a significant reduction in inbreds and hybrids only. Therefore, the maize composite has a potential for being used as a nutraceutical in human-health sector.

Key words

Antioxidant activity, Free radicals, HPTLC, Maize cultivars, N fertilization

Introduction

Maize (*Zea mays* L.), the third major cereal after rice and wheat, is the staple food of a huge population in Latin America, Asia and Africa. The United States, China, India and Brazil are the major producers of maize, accounting for nearly 73% of the annual global production of nearly 456 million tons. Currently, India produces about 23 million tons of maize annually. It makes about 9% of India's food basket and 5% of the world's dietary energy supply. Of late, maize has become a cynosure in biological research for its phenolic compounds such as gallic acid, vanillic acid, tannic acid, coumaric acid and caffeic acid, which exhibit activity against cancer, age-related muscular degeneration, stimulation of hepatocarcinogenesis, inflammation, and diabetes (Dykes

and Rooney, 2007; Moreno *et al.*, 2007; Pandey and Rizvi, 2009; Pandey *et al*., 2013). Corn silk, comprising the yellowish thread-like strands from the female flower of maize, has long been providing a curative therapy for cystitis, edema, kidney stones, prostate disorder, urinary infections, bedwetting, obesity, fatigue, depression, nephrotoxicity and oxidative stress (Ebrahimzadeh *et al.*, 2008; Bai *et al.*, 2010; Hu *et al.,* 2010; Sepehri *et al.,* 2011; Al-Jawad *et al*., 2012; Hasanudin *et al*., 2012).

The recommended commercial maize varieties available in the country include mainly hybrid (single or double cross) and composites (open-pollinated). Hybrids are developed through cross-pollination between elite inbred lines, whereas composites are derived from the advanced generation of random crossing between outstanding lines. Hybrids are usually developed for specific traits such as yield, days to maturity, kernel colour etc, but their seeds cannot be used for next sowing. However, composites are derived from open pollination involving phenotypically outstanding lines, which may include inbreds, hybrids, varieties that are similar in maturity, height, seed size, colour, and resistance to biotic and abiotic stresses. The primary advantage of composite variety is that the farmer can use their own seeds for three to four years.

In plants, nutrients and the environmental factors regulate biosynthesis of secondary metabolites through their influence on biomass production and the active components (Christensen *et al.*, 2006). Nitrogen, an essential macronutrient, has significant potential to affect polyphenolic accumulation in certain plants (Biesiada *et al*., 2009). The N concentration in soil is significantly depleted due to leaching, denitrification, volatilization and over-cultivation of agricultural crops. This has prompted farmers to use chemically-synthesized N fertilizers. Adequate use of organic and inorganic fertilizers increases the yield of essential oils and secondary metabolites, the main components of medicinal plants (Khalid *et al.,* 2006; Biesiada *et al*., 2009). However, since N fertilizers are very costly, as well as detrimental to the environment, there is need for developing and selecting varieties with high nitrogen-utilization efficiency (NUE) that can grow well in low nitrogen environments and synthesize relatively large amounts of polyphenolic compounds.

The present study was designed to evaluate the effect of nitrogen supply on accumulation of phenolic acids and the inherent antioxidant potential of maize cultivars. Also, a comparison was made among the inbreds, hybrids and composite varieties for high NUE, phenolic-acid content and antioxidant potential.

Materials and Methods

Plant materials and growth conditions: Seeds of different maize (*Zea mays* L.) cultivars, including inbreds (EI 116, NAI 197 and HKI 536), hybrids (BIO 9681, Vivek 5 and HKH 309) and a composite (Pratap), *were procured from the ICAR□Indian Institute of Maize Research, New Delhi,* surface-sterilized by dipping completely in 0.1% $HgCl_2$ solution for 1 min, and then washed with deionized water 4-5 times. Seeds were placed in a conical flask containing 70 ml of 1mM $CaCl_2$ solution and covered with foil. Aeration was provided to seeds for 24 hr through a serological pipette attached to a tube connected to the aeration pump**.** For germination, seeds were placed on 1mM $CaCl_2$-soaked germination paper, kept in a beaker containing $CaCl_2$ solution, covered with plastic bag and kept in incubator at 30ºC for 48 hr. The germinated seeds were kept as such on this moist germination paper until seedlings grew to a length of 5-6 cm. Seedlings with intact roots were then transferred to plastic tubs containing basal hydroponic nutrient solution. Half-strength Hoagland solution was supplied for first three days and then a full-strength solution was given after a gap of 3 days. The modified Hoagland solutions were prepared with deionized water with all the macro- and micronutrient concentrations held constant except nitrogen. The nitrogen was supplied at low (0.05mM) and sufficient (4.5mM) level as ammonium nitrate. The hydroponic solution contained 0.5mM phosphoric acid, 2.25mM $CaCl_2$, 0.75mM $MgSO_4$, 2.4mM KCl, 1mM NaCl, 0.05µM H_3BO_3, 0.01µM $MnCl_2$, 0.002µM $ZnSO_4$, 0.0015µM $CuSO_4$, 0.000075µM $NH_4Mo_7O_{24}$ and 0.074µM Fe-EDTA (Ganie *et al.,* 2015). The nutrient solution was aerated continuously with the help of aquarium pump. Plants were grown in a glass house at Hamdard University, New Delhi, under controlled temperature (30°C/20°C dark and night), relative humidity (80%) and natural light. Nutrient solution was changed every third day to replenish the nutrients and the plastic tubs were reshuffled every week to minimize the chances of biological error. The cobs were dried and stored for laboratory tests.

Extraction of plant material : Dried seeds were ground to fine powder and a 12 g of it was used for extraction in a round-bottom flask, with 80% methanol (60–75°C) for 6–7 hr using reflux condenser. The extract was concentrated in a rotavapor at 55–65°C and then reconstituted in 5ml of HPLC-grade methanol. The aliquot was filtered through 0.2mm syringe filters and the filtrate was stored at 4°C.

Assay of free radical DPPH-scavenging activity: Antioxidant potential of each seed sample and of the standard ascorbic acid was assessed on the basis of their capacity to scavenge free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Marinova and Batchvarov, 2011). About 20–200µl of each sample or standard was added to 2 ml of DPPH (HiMedia

Laboratories Pvt. Ltd., Mumbai) in methanol (0.33%) taken in a test tube. After incubation at 37 ºC for 30 min, the absorbance of each solution was determined at 517 nm, using a_UV-Visible spectrophotometer (Perkin Elmer, USA). The corresponding blank reading was also taken, and the DPPH radical-scavenging capacity was calculated by the following equation:

DPPH radical scavenging activity (%) = [Abs (C) – Abs (S)] × 100;

where, Abs (C) is the absorbance of DPPH radical + methanol, Abs (S) is the absorbance of DPPH radical + extract/standard.

The IC_{50} value *i.e.*, the concentration of the sample required to scavenge 50% of DPPH free radical was calculated by regression analysis.

Assay of H_2O_2-scavenging activity : The ability of seed extracts to scavenge hydrogen peroxide (H_2O_2) was determined by the spectrophotometric method (Ruch *et al.,* 1989). A 2mM L^{-1} H_2O_2 solution was prepared in phosphate buffer (pH 7.4). Seed extracts (20–200µg ml^{-1}) were added to H_2O_2 solution (0.6 ml) and absorbance was read at 230nm after 10 min against a blank solution containing phosphate buffer without H_2O_2. For each concentration, a separate blank sample was used for background subtraction. The H_2O_2-scavenging potential of seed extracts, expressed in terms of IC_{50} value, was calculated by the following equation:

% H_2O_2-scavenging activity = [Abs (C) – Abs (S)] / Abs (C) × 100

where, Abs (C) is the absorbance of the control, and Abs (S) is that of the extract/standard. The IC_{50} value, *i.e.*, the concentration of the sample required to scavenge 50% of H_2O_2 was calculated by regression analysis.

HPTLC analysis of phenolic compounds: Filtrate (3µl) of each of the extracts was individually applied on silica gel 60 F254 pre-coated 10x10 cm HPTLC plates, (Merck, Darmstadt, Germany) with the help of CAMAG LINOMAT-5 applicator. The plate was eluted to a distance of 9cm at room temperature (25°C) in a specific solvent system *i.e.,* ethyl acetate: acetic acid: formic acid: water (109:16:12:31, v/v/v/v). The sample was applied on 6 mm wide band, using CAMAG LINOMAT-5 automated TLC applicator with nitrogen flow providing a delivery speed of 147nl s^{-1} from syringe.

Detection and quantitation: After sample application, plates were developed in a CAMAG twin trough glass tank pre-saturated with mobile phase for 25 min. The plate was developed horizontally in CAMAG horizontal developing chamber (10cm × 10cm) at room temperature. After heating at 105°C for 4 min, the developed plates were documented with a digital camera, using illumination at 254nm, 366nm and under white light. Derivatization of dried plates was performed by spraying the hot plate with a solution of 0.5% 2-aminoethyl diphenyborinate in ethylacetate, followed by 5% polyethylene glycol in dichloromethane under fume hood. The plate was observed after 20 min under UV-254nm, 366nm lights in CAMAG UV cabinet, and the HPTLC fluorescence image was documented. The consequent digital scanning profiling was carried out with a CAMAG TLC scanner 3 fitted with WinCATS-V1.2.3 software, at a wavelength of 254nm, 366nm and under white light. Relative change (RC) was calculated as the difference of phenolic acid content values at sufficient nitrogen and low nitrogen, divided by the value at sufficient nitrogen and then multiplied by 100.

Statistical analysis : Two-way analysis of variance (ANOVA) was performed to determine significant difference between nitrogen treatment and maize cultivar on the level of phenolic acids contents and antioxidant activity. Statistical significance was determined at $p<0.05$ level, using Tukey's post hoc test. All the statistical analyses were completed using the GraphPad Instat V3.10 statistical software (GraphPad Software, Inc. La Jolla, CA USA).

Results and Discussion

Examination of the effect of nitrogen availability on maize plants grown in a glass house under low nitrogen (0.05mM) and high (4.5mM) fertilization conditions revealed that all the cultivars (inbreds, hybrids and composite) showed normal growth under high nitrogen conditions, attaining almost similar height and producing similar kernel yield. Under low nitrogen conditions, the inbreds and hybrids showed retarded growth and less kernel yield, but the composite did not exhibit any decline in growth. The difference in growth and yield levels of different types of maize cultivar substantiates the view that the synthetically-produced composites perform better than the naturally-occurring genotypes (Marquez-Sanchez and Sahagun-Castellanos, 2002; Juliann *et al.,* 2004). Because of their genetic heterozygosity, the composites were likely to have evolved a mechanism to express certain proteins important for increasing their nitrogen-utilization efficiency (NUE).

The average antioxidant potential of inbreds, hybrids and the composite was determined as a function of N fertilization. Results of assays of DPPH and H_2O_2-scavenging capacity of grain extract, expressed in terms of IC_{50} values, are presented in Table 1. Lower the IC_{50} value, higher was the antioxidant activity of the extract. Under both high and low nitrogen conditions, the antioxidant activity varied significantly ($p < 0.05$) within cultivars, the composite

Table 1 : The IC_{50} values of seeds of different maize cultivars for DPPH free radical scavenging activity and H_2O_2 scavenging activity as a function of applied nitrogen level. Each value represents mean ± S.D (n = 3). P < 0.05. * = significant, NS = Non-significant

Cultivars	IC_{50} values (mg ml^{-1})			
	DPPH-scavenging activity		H_2O_2 scavenging activity	
	Nitrogen concentration		Nitrogen concentration	
	0.05 mM	4.5 mM	0.05 mM	4.5 mM
EI 116	164 ± 9.2	85 ± 4.5*	125 ± 7.3	66 ± 3.2*
NAI 197	177 ± 8.6	96 ± 5.1*	138 ± 8.2	72 ± 4.6*
HKI 536	138 ± 7.2	76 ± 4.3*	119 ± 6.9	62 ± 3.3*
BIO 9681	98 ± 5.3	58 ± 2.8*	82 ± 4.4	53 ± 2.9*
Vivek 5	97 ± 7.3	63 ± 3.1*	76 ± 4.2	49 ± 2.6*
HKH 309	86 ± 4.2	71 ± 3.8*	85 ± 4.2	58 ± 2.8*
Pratap	61 ± 3.1	52 ± 2.6^{NS}	51 ± 2.7	43 ± 2.4^{NS}

Table 2 : HPTLC analysis of phenolic acid concentrations and relative change for different maize cultivars as a function of applied nitrogen level. Phenolic acid concentrations are expressed in mg g^{-1} dry weight. Relative change (RC) is expressed in %. The difference in concentration of phenolic acid is marked by *a* if significant, and by *b* if non-significant (P<0.05).

Standard phenolic compounds	Nitrogen concentration (mM)	Maize seed sample						
		Inbreds			Hybrids			Composites
		EI 116	NAI 197	HKI 536	BIO 9681	Vivek 5	HKH 309	Pratap
Gallic acid	4.5	2.3±0.2	2.8±0.3	3.1±0.3	5.4±0.5	4.8±0.4	5.3±0.5	8.7±0.8
	0.05	1.2±0.1^a	0.9±0.1^a	1.8±0.2^a	3.1±0.3^a	3.9±0.4^a	4.5±0.4^a	8.2±0.7^b
	RC	47.82	67.85	41.93	42.59	18.75	15.09	5.74
Vanillic acid	4.5	3.9±0.4	2.9±0.3	4.8±0.4	2.3±0.2	7.3±0.7	7.1±0.6	12.3±1.2
	0.05	1.3±0.1^a	1.1±0.1^a	3.2±0.3^a	1.1±0.1^a	5.9±0.5^a	6.1±0.6^a	11.8±1.1^b
	RC	66.66	62.07	33.33	52.17	19.18	14.08	4.06
Tannic acid	4.5	11.3±1	9.3±0.8	9.7±0.9	13.3±1.2	16.2±1.5	15.8±1.4	31.3±2.8
	0.05	6.3±0.6^a	4.3±0.4^a	5.3±0.5^a	11.6±1.1^a	15.3±1.4^a	14.9±1.4^a	29.7±2.7^b
	RC	44.25	53.76	45.36	12.78	5.55	5.70	5.11
O-Coumaric acid	4.5	7.3±0.7	2.2±0.2	6.5±0.6	8.3±0.8	9.5±0.9	11.7±1.1	23.4±2.1
	0.05	3.2±0.3^a	1.2±0.1^a	3.6±0.3^a	7.9±0.7^a	8.2±0.7^a	9.1±0.8^a	21.7±2^b
	RC	56.16	45.45	44.61	4.82	13.68	22.22	7.26
Ferulic acid	4.5	3.2±0.3	7.3±0.7	5.9±0.5	15.3±1.4	12.3±1.1	7.2±0.7	28.3±2.6
	0.05	1.1±0.1^a	4.2±0.4^a	3.1±0.3^a	13.9±1.3^a	11.8±1.1^a	5.9±0.5^a	27.1±2.5^b
	RC	65.62	42.46	47.46	9.15	4.06	18.05	4.24
Cinnamic acid	4.5	0.9±0.1	1.5±0.1	2.9±0.3	7.9±0.7	10.2±0.9	13.3±1.2	39.3±3.6
	0.05	0.1±0^a	0.2±0.1^a	0.7±0.1^a	6.5±0.6^a	9.8±0.9^a	12.9±1.2^a	38.3±3.5^b
	RC	88.88	86.66	75.86	17.72	3.92	3.01	2.54
Caffeic acid	4.5	4.1±0.4	2.3±0.2	6.4±0.6	24.1±2.2	19.3±1.8	22.6±2.1	36.3±3.3
	0.05	1.2±0.1^a	1.4±0.1^a	3.2±0.3^a	22.9±2.1^a	18.1±1.6^a	20.9±1.9^a	35.5±3.2^b
	RC	70.73	39.13	50.00	4.98	6.22	7.52	2.20
Salicylic acid	4.5	7.8±0.7	5.1±0.5	1.9±0.2	13.4±1.2	22.7±2.1	26.3±2.4	39.3±3.6
	0.05	2.3±0.2^a	3.1±0.3^a	0.3±0.04^a	12.3±1.1^a	21.5±2.1^a	25.2±2.3	37.6±3.4^b
	RC	70.51	39.21	84.21	8.21	5.29	4.18	4.32

cultivar 'Pratap' having higher activity, as compared to hybrids and inbreds (Table 1). The activity correlates directly to the total phenolic content, thus indicating that high total phenolic levels would ensure a high antioxidant capacity. The levels of nitrogen significantly ($p < 0.05$) affected the antioxidant activity in inbreds and hybrids; plants grown with sufficient nitrogen exhibited a significantly higher antioxidant potential than those grown at a low nitrogen level. The increased antioxidant activities recorded for inbreds and hybrids with sufficient N supply were related direct to total

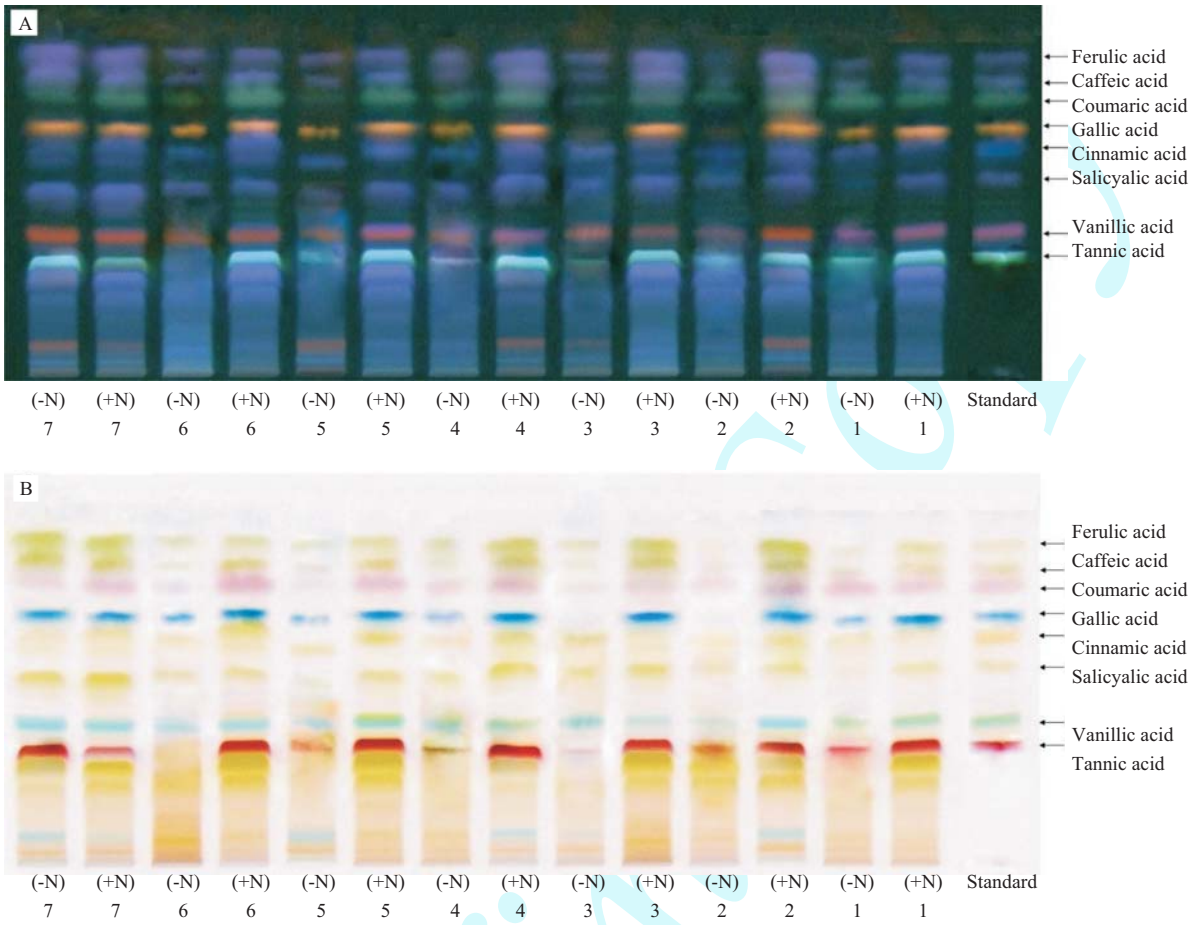

Fig. 1 : HPTLC profiles of seed extracts of different maize cultivars grown under sufficient (+N: 4.5mM) and low (-N: 0.05mM) nitrogen supply, as compared to the phenolic acid standards. The figure shows HPTLC plate after derivatization (A) at 366nm and (B) under white light. (1) EI 116; (2) NAI-197; (3) HKI 536; (4) BIO 9681; (5) Vivek 5; (6) HKH 309; (7) Pratap.

phenolic contents. However, nitrogen supply did not have a significant ($p > 0.05$) effect on the antioxidant potential of composite cultivar, as is evident from IC_{50} values for DPPH-scavenging activity (52 and 61 mg ml^{-1} under sufficient N and low N conditions respectively) and for the H_2O_2-scavenging activity (43 and 51 mg ml^{-1} under sufficient nitrogen and low nitrogen conditions, respectively) (Table 1).

Being a major component of chlorophyll, proteins, ATP (adenosine triphosphate), DNA as well as secondary metabolites such as phenolic acids, nitrogen is a vital requirement of plants. This study has demonstrated that the antioxidant activity and phenolic contents of maize kernels varied significantly in relation to nitrogen fertilization as well as cultivars. The antioxidant activity of different maize cultivars was determined as a function of cultivars and nitrogen fertilization by the assay of DPPH- and H_2O_2-scavenging activity. DPPH, a stable free radical, is scavenged by antioxidants by donation a hydrogen atom or an electron (Chang *et al.*, 2007), leading to the formation of reduced DPPH-H (1, 1 diphenyl-z-picrylhydrazine), which can be quantified easily by its decrease in absorbance at 517nm (Chang *et al.*, 2007; Shekhar and Anju, 2014). H_2O_2 itself is not toxic to cells, but it produces superoxide anion (O_2^-) inside the cell, which is a strong free radical that can damage essential macromolecules including DNA, proteins and lipids (Jabeen *et al.,* 2009; Arshi *et al.*, 2010).

The free-radical-scavenging-activity assay measures the anti-oxidative effect of any substance in the reaction medium in terms of its reducing ability. The composite cultivar possessed highest DPPH and H_2O_2-scavenging capacity, which might be attributed to the presence of higher amounts of phenolic and flavonoid compounds. The results

Table 3 : Spot colors and corresponding Rf values of standard phenolic acids

Standard phenolic compounds	Colour	Rf value
Ferulic acid	Blue	0.62
Caffeic acid	Blue	0.56
O-Coumaric acid	Green	0.53
Gallic acid	Orange	0.48
Cinnamic acid	Light blue	0.43
Salicylic acid	Blue	0.37
Vanillic acid	Pink	0.28
Tannic acid	Sky blue	0.23

further exhibited that nitrogen deficiency led to a significant decrease in the antioxidant capacity of inbreds and hybrids, but not of composite, as observed earlier also (Phuong *et al.*, 2008; Ma *et al.*, 2015). It is expected that the composite varieties can synthesize sufficient secondary metabolites because of their high NUE, and hence display a significant antioxidant potential.

Due to correlation between phenolic acids and their health-promoting activity, and in consonance with the objective to estimate the nutraceutical potential of maize varieties, a chromatographic fingerprint of water-soluble phenol compounds was developed. The variations in phenolic acids content in various seed extracts were significantly correlated to treatment and cultivar (Table 2). At sufficient nitrogen, quantities of phenolic acids were enhanced in all the cultivars, with the composite having the maximum [8.7mg (gallic acid) and 39.3mg (cinnamic acid and salicylic acid) g^{-1} d.wt.]. On the contrary, the phenolic acids content was significantly reduced under low N condition, with inbreds having the minimum [0.2mg (cinnamic acid) and 6.3mg (tannic acid) g^{-1} d.wt.]. Reduction in nitrogen supply did not affect phenolic acids content significantly in the composite cultivar, as evident from the values ranging from 8.2mg (gallic acid) to 38.3mg (cinnamic acid) g^{-1} d.wt. The phenolic acid spots obtained after spraying the HPTLC plate with 0.5% 2-aminoethyl-diphenyborinate in ethyl acetate (Fig. 1), indicated that all the sample constituents were clearly separated without any tailing and diffuseness. In 80% methanolic seed extract, the 8 spots with Rf values 0.62, 0.56, 0.53, 0.48, 0.43, 0.37, 0.28 and 0.23 (Fig. 1, Table 3), correlated to ferulic acid, caffeic acid, O-coumaric acid, gallic acid, cinnamic acid, salicylic acid, vanillic acid and tannic acid, respectively. Concentration of phenolic acids was significantly reduced under low nitrogen.

The results suggest that these secondary metabolites constitute an adaptive strategy for the plant to conserve nitrogen under low N conditions, and sufficient nitrogen conditions favor accumulation of these metabolites.

Phenolic compounds in plants are the naturally-occurring antioxidants, and their free-radical scavenging activities are thought to be important in protecting plants from many chronic diseases (Gross, 2004; Neuhouser, 2004). Polyphenolic compounds are produced by plants for a variety of reasons. Their characterization and quantification in medicinal plants require special attention (Ghasemzadeh and Ghasemzadeh, 2011) to develop reliable chromatographic fingerprints that represent the pharmacologically active and chemically characteristic components of herbal medicine and help in their identification. Phenolic acids are highly effective antioxidants that can scavenge free radicals, activate antioxidant enzymes and inhibit oxidases (Heim *et al.*, 2002). They possess a wide range of biological activities, showing anti-inflammatory, anti-allergic, hepatoprotective, antiviral, anticancer and antidiabetic actions (Middleton *et al.*, 2000; Nardini and Ghiselli 2003). In the present study, eight phenolic acids, namely, gallic acid, ferulic acid, vanillic acid, tannic acid, caffeic acid, o-coumaric acid, cinnamic acid and salicylic acid were quantified in different maize cultivars. HPTLC-fingerprinting profile can serve as a diagnostic tool to identify and authenticate the maize cultivars, based on the level of phenolic compounds (Rachna *et al.*, 2013). Composite cultivars possessed maximum phenolic acid contents due to genetic heterogeneity (Mandal, 2014). Nardjis *et al.* (2012) also reported a significant decrease in the total phenolic acid contents of maize grown under nitrogen deficiency.

In the present study, the maize cultivars showed significantly lower phenolic contents and antioxidant potential at low nitrogen level than at a sufficient nitrogen level, which might affect their food as well as medicinal value. The composite performed relatively better even under low nutrient conditions.

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