

Full Length Research Paper

HPLC analysis of water-soluble vitamins (B1, B2, B3, B5, B6) in *in vitro* and *ex vitro* germinated chickpea (*Cicer arietinum* L.)

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In the present communication, a comparative HPLC analysis of water-soluble vitamins (B-group) was carried out in *in vitro* and *ex vitro* germinated chickpea (*Cicer arietinum* L.) seeds. Quantitative analysis of *ex vitro* and *in vitro* germinated seedlings showed significant differences in vitamin contents. Maximum amount with a linear increase in B1 (thiamine HCl), B2 (riboflavin), and B3 (nicotinamide) contents was noticed up to 9 days old *ex vitro* grown seedlings. However, B5 (pantothenic acid) and B6 (pyridoxine HCl) were higher in *in vitro* germinated seedlings. Thus, vitamin production was age and culture conditions dependent, which is discussed in detail. The study revealed that the germinated chickpea grains can be used for human consumption with value addition of vitamin B-group in the diet of vegetarians.

Key words: *Cicer arietinum* L., water-soluble vitamins, *in vitro* and *ex vitro* culture.

INTRODUCTION

Chickpea (*Cicer arietinum* L) is grown in different countries of Asia, Africa, Europe, North and South America. It is mostly produced and consumed in South East Asia, Middle East and some Mediterranean countries. India accounts for 65 and 70 percent total area and production in the world, respectively. The productivity of chickpea in India is 8.55 q/ha which is higher than the mean global productivity. It is one of the most important pulse crop of India and shares 31 and 42 percent area and production of pulses in the country (Asthana et al., 1996). Chickpea is known by various names as gram, Bengal gram, chana, chole, harbara, sanagalu in Indian vernacular languages. Chickpea is consumed in all the countries of Indian subcontinent and the Middle East in various forms and dishes. It is a rich source of protein (19-20%) and a good supplement in cereal-pulse diet of vegetarians.

Pulses generally lack in vitamin A and C like cereals. However, they may contain vitamins in germinating grains (ICRISAT, 2002; Gopalan et al., 2007). The digestibility of the protein in pulses is low but can be enhanced by boiling, cooking or sprouting of seed. The vitamin content in pulses may increase in germinating grains or sprouts and may lead to value addition in food before or after cooking.

Vitamins are reported to reduce the damage by free radicals and check degenerative disease (Jacab and Sotoudeh, 2002). Vitamin supplements have ergogenic and performance enhancing effect (Clarkson, 1993; Zhao et al., 2004). Therefore, the present investigation was undertaken to determine the presence of water-soluble (Figure 1) vitamins (B-group) in germinating chickpea grains in *in vitro* and *ex vitro* conditions for possible value addition in the diet for human consumption.

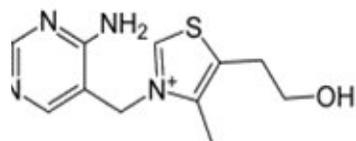
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Abbreviations: HPLC, High performance liquid chromatography; RSD, standard relative deviation; DMRT, Duncan multiple range test.

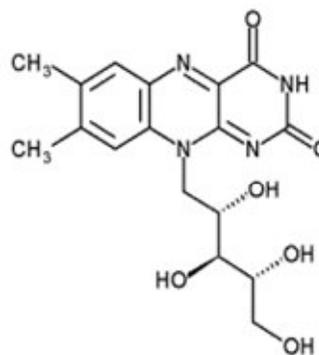
MATERIALS AND METHODS

Plant material

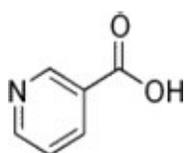
Chickpea seeds were purchased from a public supermarket at Qu-



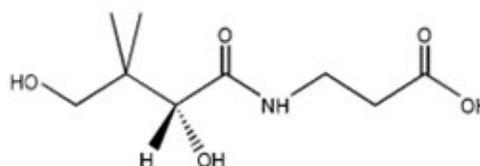
Thiamine B1



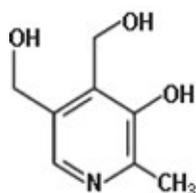
Riboflavin (B2)



Nicotinamide B3



Pantothenic B5



Pyridoxine B6

Figure 1. Chemical structure of water-soluble vitamins.

sais in Dubai and sown in *in vitro* and *ex vitro* conditions. In *in vitro* conditions, the seeds were placed for germination in Petri plates on cotton wet with 50 ml basal nutrient medium and incubated at $25 \pm 2^\circ\text{C}$ under 16-h photoperiods with cool white fluorescent illumination ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$ PFD). In *ex vitro* conditions, the seeds were sown in the well-prepared seed bed with appropriate moisture. The data for water-soluble vitamin contents were recorded after two days of sowing in both the conditions and continued up to 11 days.

Reagents and solvents

All the chemicals and reagents used were of analytical grade viz., HPLC methanol (Merck), acetonitrile HPLC (Merck), glacial acetic acid (BDH), triethylamine (RDH), orthophosphoric acid (BDH), pic₆ (hexane sulphonic acid sodium salt (Merck). Water was distilled and deionized by using Millipore direct q system.

Table 1. The important parameters for the calibration curves.

Vitamin		$y = ax + b$	r	Concentration range (g/ml)
B1	Thiamine HCl	$Y = 7.09e+004x - 5.59e+005$	0.9964	1-5.86
B2	Riboflavin	$Y = 9.80e+004x - 1.13e+005$	0.9977	5-25
B3	Nicotinamide	$Y = 1.6e+005x - 2.37e+004$	0.9932	1-5.01
B5	Panthenic Acid	$Y = 1.57e+005x + 1.2e+004$	0.9953	5-10
B6	Pyridoxine HCl	$Y = 1.66e+005x - 1.99e+004$	0.9996	1 - 6.05

a = Slope; b = intercept; r = correlation coefficient.

Standard preparation

Standard stock solution for vitamin B1 (thiamine HCl) was prepared by dissolving 26.7 mg of thiamine hydrochloride in 25 ml of double distilled water. Standard stock solution for vitamin B2 (riboflavin) was prepared by dissolving 6.9 mg of riboflavin in 100 ml of extraction solution because the extraction solution has limit to dissolve 7 mg of riboflavin. Standard stock solution for vitamin B3 (nicotinamide) was prepared by dissolving 41.5 mg of nicotinamide in 25 ml of double distilled water. Standard stock solution for vitamin B5 (calcium salt of pantothenic acid) was prepared by dissolving 21.4 mg of calcium d-pantothenate in 25 ml of double distilled water. Standard stock solution for vitamin B6 (pyridoxine HCl) was prepared by dissolving 20.8 mg of pyridoxine hydrochloride in 25 ml of double distilled water.

The working standards were prepared as follows: i) For B1, 1.068 mg/ml and 562 μ l was taken in 10 ml volumetric flask, ii) B2, 0.069 mg/ml and 5.1 ml was taken in 10 ml volumetric flask, iii) B3, 1.66 mg/ml and 60.5 μ l was taken in 10 ml volumetric flask, iv) B5, 0.856 mg/ml and 292 μ l was taken in 10 ml volumetric flask, v) and for B6, standard 0.832 mg/ml and 72.2 μ l was taken in 10 ml volumetric flask.

Preparation of buffer, solutions and samples

For buffer preparation, 1.08 g of hexane sulphonic acid sodium salt and 1.36 g of potassium dihydrogen phosphate were dissolved in 940 ml of HPLC water and 5 ml of triethylamine was added to it and the pH was adjusted to 3.0 with orthophosphoric acid.

To prepare the mobile phase, buffer and methanol were mixed with a ratio of 96:4 and filtered through 0.45 μ membrane filter and degassed by using helium gas.

Extraction solution was made by mixing 50 ml of acetonitrile with 10 ml of glacial acetic acid and the volume was finally made up to 1000 ml with double distilled water.

10 g of each sample was homogenized, weighed and transferred into conical flasks and 25 ml of extraction solution was added, kept on shaking water bath at 70°C for 40 min. Thereafter, the sample was cooled down, filtered and finally the volume was made up to 50 ml with extraction solution.

High performance liquid chromatography (HPLC)

Calibration curve was made by using mix standards in mobile phase with five point calibrations, analyzed independently by HPLC and a standard curve was plotted between concentration and peak area. The injected quantities showed good linearity. The data of peak area vs. used standard vitamin concentration were treated by linear least-square regression and the regression equation thus

obtained from standard curve, was used to estimate water-soluble vitamins in different samples.

For HPLC analysis, a waters symmetry C18 column (4.6 x 250 mm 5 μ m) was used with a linear gradient of Buffer: methanol (96:4) at a constant flow rate of 1 ml/min with 2300 pressure by using waters pump (1515 isocratic) and a UV (2487) detector was employed for the detection of peaks, using two channels simultaneously at a wavelength of 210 nm, a bandwidth of 5 nm and another wavelength of 280 nm. All the analyses were performed with 3 replicates.

The accuracy of the present method was checked in the sample by recovery study. Pre-analyzed samples were spiked with extra 50, 100 and 150% of the standard vitamins; the mixtures were reanalyzed by adapting the proposed method. The experiment was repeated 3 times, average recovered vitamin content was quantified using regression equation, and the % recovery was calculated accordingly.

The precision of the used method was obtained by determining inter and intra-day variation in the three replicates of B-group vitamins at two concentration levels (200-400 ng per spot) and the Percent Standard Relative Deviation (RSD) were calculated.

Samples of *in vitro* and field sown (*ex-vitro*) seedlings, 60 μ l each were used in triplicates. The vitamin yield was quantified using regression equation of calibration curve of each standard.

RESULTS AND DISCUSSION

The solvent system developed produced a sharp and compact peak of the B-group vitamins. Densitometric analysis of water-soluble vitamins were carried out at 210 and 280 nm absorbance mode. The regression analysis data showed a good linear relationship (Table 1). The method used for vitamin extraction and subsequent estimation afforded recovery of 99.18 - 100.88%. The inter- and intra-variation of vitamins at two different concentration levels showed a low relative standard deviation (0.95-1.57%). The content of B-group vitamins in different samples was analyzed from the regression equation using value of area obtained from win-cat software.

In the present study, the quantitative HPLC analysis of water soluble vitamins were carried out in *in vitro* and *ex vitro* germinated seedlings. The mean values of the various B-group vitamins analyzed from *ex vitro* and *in vitro* germinated chickpea seedlings are presented in Table 2. The vitamins production was age and culture conditions (*ex vitro* and *in vitro*) dependent. The vitamin B1 (thymine-HCL) and B2 (riboflavin) contents were

Table 2. Water soluble vitamins contents in *ex vitro* and *in vitro* germinated chickpea seedlings.

Water soluble vitamins	Water soluble vitamins ($\mu\text{g/g}$)									
	3 Days		5 Days		7 Days		9 Days		11 Days	
	<i>In vitro</i>	<i>Ex vitro</i>	<i>In vitro</i>	<i>Ex vitro</i>	<i>In vitro</i>	<i>Ex vitro</i>	<i>In vitro</i>	<i>Ex vitro</i>	<i>In vitro</i>	<i>Ex vitro</i>
B1	0.05b	0.07b	0.08c	0.11bc	0.11c	0.14c	0.09e	0.15c	0.04e	0.10c
B2	0.02b	0.05b	0.04c	0.15b	0.15c	0.30a	0.17c	0.27b	0.09d	0.52a
B3	0.00c	0.00	0.00d	0.00d	0.00d	0.02d	0.12d	0.25b	0.10c	0.00d
B5	0.80a	0.62a	0.67a	0.48a	0.55b	0.16b	0.48b	0.13c	0.21b	0.13bc
B6	0.00c	0.0c	0.22b	0.08c	0.75a	0.12bc	1.20a	0.35a	0.67a	0.15b

Values are mean of three replicates. Means with common letters within a column are not significantly different ($p \geq 0.05$) according to Duncan's Multiple Range Test (DMRT).

maximum in *ex vitro* grown seedlings, whereas a linear increase was found up to 9 days old seedlings. Thereafter, their amount started to decrease. In both the conditions, vitamin B3 was detected after 5 days onwards, which was maximum in 9 days old *ex-vitro* grown seedlings and later it, however, declined. A significant linear increase in vitamin B5 content was noticed up to 9 days old seedlings. *In vitro* culture condition proved to be highly effective compared to *ex-vitro* in terms of B5 production, although a sudden decrease in its amount was found in 11 days old seedlings. In both the conditions, vitamin B6 was not detected up to 3 days old seedlings; however, seedlings developed in *in vitro* conditions showed a significant linear increase up to 9 days.

Vitamin B-group contents in *in vitro* and *ex vitro* germinated chickpea seedlings have been analyzed. It could be noted from the results that vitamin B-group contents were directly influenced by the seedlings age and germinating conditions. Vitamin B1, B2, and B3 production was high in seedlings raised under natural environmental condition, however, the yield of B5 and B6 increased considerably in *in vitro* grown seedlings. These variations in vitamin content might be due to the light intensity, which regulates the metabolic pathways of particular type of the B-group vitamin. The results of water-soluble vitamins *in vitro* and *ex vitro* agreed with those of medicinal plants whereas (Pande et al., 2002; Yohe, 2002; Paul and Shaha, 2004; Bhat et al., 2008). The present study revealed that water-soluble vitamin production was age and environmental conditions dependent. These results corroborate the previous finding in some other plant system (Elliot and McPherson, 1971; Kundu and Das, 1986; Datta and Srivastava, 1997; Bhat et al., 2008). It has also been established in other plants that the metabolic pathway was not only restricted to certain tissues, but it was also modulated by different developmental and environmental conditions (Pasquali et al., 1992; Adebisi et al., 2005).

In summary, *in vitro* and *ex vitro* germinated chickpea seedlings showed a considerable variation in water-soluble vitamins yield. A linear increase in yield was

found in the entire vitamin B group. B1, B2 and B3 were high in *ex vitro*-germinated seedlings; however, B5 and B6 were maximum in *ex vitro* conditions. The present study determined the suitable age and the conditions for the production of water-soluble vitamins in chickpea. Based on these results, it could be concluded that germinated chickpea could be used for human consumption in order to supply vitamin B group in the diet of vegetarians.

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