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Characterization and identification of annual wild *Cicer* species for seed protein and mineral concentrations for chickpea improvement

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Abstract

Developing nutrient-rich crop cultivars is the most economic strategy to combat malnutrition resulting from protein and mineral deficiencies. Chickpea (*Cicer arietinum* L.) is an important staple grain legume source of good quality dietary protein around the world, particularly in southern Asia, northern Africa, and the Middle East. In the present investigation, the genetic variability for protein and mineral concentrations was studied in 41 accessions of cultivated chickpea and eight annual wild *Cicer* species of primary, secondary, and tertiary gene pool. Large variability was observed between and within *Cicer* species for seed protein, Fe, Zn, Cu, Mn, Ca, and Mg concentration with high heritability. *C. chorassanicum* (Bunge) Popov was found to be the most promising species for high seed protein and Ca; *C. judaicum* Boiss. for high seed Fe, Cu. and Mg; *C. yamashiatae* Kitam. for high seed Zn and Fe; and *C. pinnatifidum* Jaub. & Spach for high seed Mn concentrations. All the wild *Cicer* accessions except ICC20190 (*C. echinospermum* P. H. Davis) had high concentration of at least one or more seed nutrients. Wild *Cicer* accessions such as ICC17141 (*C. chorassanicum*), ICC17269 and ICC17303 (both *C. pinnatifidum*), ICC17261 and ICC17262 (*C. reticulatum*), ICC20236 (*C. chorassanicum*), and ICC17117 and ICC17281 (*C. yamashitae*) were found promising for multiple seed nutrients. As *C. reticulatum* Ladiz. and *C. echinospremum* accessions are crossable with cultivated chickpea, promising *C. reticulatum* accessions identified in the present study can be used in crossing program for developing new nutrient-rich chickpea cultivars.

1 | INTRODUCTION

One of the United Nation's sustainable development goals is to "end hunger, achieve food security and improved nutrition, and promote sustainable agriculture." The ever-increasing world population necessitates doubling the food grain production by developing new high-yielding and nutrient-rich cultivars to address the global issue of hunger and malnutrition. Stagnant yields of most crop varieties coupled with poor nutritional quality are the major limitations to achieve this goal. At the global level, protein and mineral (micro- and macro-nutrient) deficiencies have been identified as serious health problems (Frossard, Bucher, Machler, Mozafar, & Hurrell, 2000; Grusak & Cakmak, 2005; Rude & Gruber,

2004; Thacher, Fischer, Strand, & Pettifor, 2006; Welch & Graham, 2005) especially among people having vegetarian diets. Among the micronutrients, iron (Fe) and zinc (Zn) deficiencies are the major health problems prevalent in Asia, Africa, and Latin America affecting about 3 billion people (Darnton-Hill, Bloem, & Chopra, 2006; Welch & Graham, 2004). The Fe and Zn are the cofactors of important proteins such as hemoglobin, and cytochrome and transcription factors. Inadequate intake of these minerals in the diet of about 50% of the world population may affect their proper growth and development (Brown, Peerson, Rivera, & Allen, 2002; Welch, 2002). The calcium (Ca) deficiency causes rickets in children and osteomalacia, a generalized demineralization of bones, in adults and may also contribute to osteoporosis (Hays & Swenson, 1985; Malhotra, 1998; Murray, Granner, Mayes, & Rodwell, 2000). The magnesium (Mg) deficiency increases neuromuscular hyperirritability, which may lead to irregular heartbeat and tremors (Murray et al., 2000). Similarly, copper (Cu) deficiency may also lead to impaired cardiovascular system development, neurological and immunological abnormalities, and alterations in cholesterol metabolism (Gambling & McArdle, 2004; Georgieff, 2007; Klevay et al., 1984; Reiser, Yang, & Canary, 1987). Manganese (Mn) is an essential micronutrient for metabolic regulation, bone mineralization, and formation of glycosaminoglycans (Wedler, 1994).

To combat nutritional deficiencies, the conventional efforts such as micronutrient supplementation and food fortification, are not affordable in developing countries. Hence, one of the most important and economic approaches is to biofortify common staple crops with macro- and micronutrients by breeding for nutritionally dense, high-yielding crop cultivars (Bouis, 2003). Staple cereals alone do not meet the daily requirement of essential micronutrients (Shi et al., 2008). In contrast, grain legumes or pulses, having high protein, vitamin, and mineral contents, are essential for alleviating micronutrient deficiencies and malnutrition and thus complement the conventional cereal-based diets (Broughton et al., 2003; Ufaz & Galili, 2008).

Among the grain legumes, chickpea (*Cicer arietinum* L.) is the second most important food legume crop and is cultivated in about 60 countries across the Indian subcontinent, the Middle East, southern Europe and northern Africa on ~17.8 million ha area with 17.2 Tg production (http://www.fao.org/faostat/en/#data/QC). India ranks first in chickpea production across the globe with an annual production of 11.4 Tg (http://www.fao.org/faostat/en/#data/QC). Chickpea is an important source of dietary proteins, micronutrients, vitamins, and carbohydrates (Jukanti, Gaur, Gowda, & Chibbar, 2012). The quality of chickpea protein is considered better than other pulses, such as pigeonpea (*Cajanus cajan L. Huth*), green gram [*Vigna radiata* (L.) R. Wilczek var. *radiata*], and black gram (*Vigna mungo* Hepper var. *mungo*) (Kaur, Singh, & Sodhi, 2005), and contains 20–22% protein (Gaur, Sami-

neni, Sobhan, & Chibbar, 2015). Chickpea is a potential staple food crop in ~60 countries, and improving its nutritional quality will help in reducing protein and mineral deficiencies for people in these countries. To achieve this objective, new and diverse sources of variations are needed to further improve its nutritional quality.

Sufficient variability is present in genus *Cicer*, which contains 43 wild *Cicer* species for chickpea improvement. Wild *Cicer* species possess many useful traits and can be exploited as important sources of variability for improving nutritional quality including protein and mineral content of cultivated chickpea. Limited information is available regarding variability for seed nutrient concentration in the wild *Cicer* species compared with the cultivated chickpea. The present investigation was carried out to study the genetic variability for seed protein and mineral concentrations among different accessions—originating from or collected from different countries and belonging to eight annual wild *Cicer* species and cultivated chickpea—and to identify promising wild *Cicer* accessions having high seed protein and mineral concentration for use in chickpea improvement programs.

2 | MATERIALS AND METHODS

2.1 | Plant material

Thirty-five accessions of eight annual wild *Cicer* species belonging to primary gene pool (*C. reticulatum* Ladiz.), secondary gene pool (*C. echinospermum* P.H. Davis), and tertiary gene pool (*C. bijugum* Rech. f., *C. chorassanicum* (Bunge) Popov, *C. cuneatum* Hochst. ex A. Rich., *C. judaicum* Boiss., *C. pinnatifidum* Jaub. & Spach, *C. yamashitae* Kitam.) along with six popular chickpea varieties were used in this study (Table 1). The wild *Cicer* accessions have origin in seven countries (Table 1). Six chickpea varieties, belonging to desi (ICCV96029, JG 11, ICCV10, and G130) and kabuli (KAK 2 and L 550) types, are extensively cultivated in India and were used as checks (Table 1). ICCV96029 (Kumar & Rao, 2001), JG 11 (Dattatri, Mahadeva, Sudhakar, Dhanalakshmi, & Sudhakar, 2010), ICCV10 (Gowda et al., 1995), and G130 (Singh, 1987) are super early-, early-, medium-, and late-flowering

TABLE 1 List of annual wild and cultivated *Cicer* species and their accessions used in this study

Species	No. of accessions	Accession identity	Country of origin
C. arietinum	6	ICCV96029	India
		JG 11	India
		ICCV10	India
		KAK 2	India
		L550	India
		G130	India
C. bijugum	3	ICC17156	Turkey
		ICC17187	Syria
		ICC17289	Turkey
C. chorassanicum	2	ICC17141	Afghanistan
		ICC20236	Afghanistan
C. cuneatum	4	ICC17162	Ethiopia
		ICC20175	Ethiopia
		ICC20176	Ethiopia
		ICC20215	Ethiopia
C. echinospermum	5	ICC20190	Turkey
		ICC20192	Turkey
		ICC20218	Turkey
		ICC20244	Turkey
		ICC20257	Turkey
C. judaicum	7	ICC17148	Lebanon
		ICC17149	Israel
		ICC17188	Syria
		ICC17204	India
		ICC17271	Lebanon
		ICC17274	Syria
		ICC17316	Ethiopia
C. pinnatifidum	5	ICC17126	Turkey
		ICC17200	Syria
		ICC17269	Turkey
		ICC17276	Syria
		ICC17303	Turkey
C. reticulatum	7	ICC17123	Turkey
		ICC17124	Turkey
		ICC17163	Turkey
		ICC17164	Turkey
		ICC17261	Turkey
		ICC17262	Turkey
		ICC17326	Turkey
C. yamashitae	2	ICC17117	Afghanistan
		ICC17281	Afghanistan

desi-type varieties. KAK 2 (Zope, Wanjari, Kumar, Van, & Rao, 2002) is a medium-flowering, kabuli-type variety, and L 550 (Dua, Chaturvedi, & Sewak, 2001) is late-flowering, kabuli-type variety.

2.2 | Methodology

The study was carried out under the controlled greenhouse conditions maintained at 22 °C air temperature in 2014, 2015, and 2016 at ICRISAT, Patancheru, India. The hard seed coat of wild *Cicer* accessions was scarified to initiate germination. Before sowing, the seeds were treated with a combination of fungicides (2 g thiram plus 1 g carbendazim kg^{-1} seed) for prevention from seed and soil-borne fungal diseases. Non-scarified seeds of chickpea varieties and scarified seeds of wild *Cicer* accessions were kept for germination in wet Petri plates at room temperature for 3 d. The germinated seedlings were transplanted (one seedling per pot) in pots containing a mixture of sterilized black soil, sand, and farmyard manure in 2:1:1 proportion. Plants were maintained in three replications containing two pots per replication. After 1 mo of germination, the seedlings were grown under 18-h extended daylength till maturity. At maturity, pods from individual plants were harvested and the healthy matured seeds free from dust and metal particles were used for analysis. About 20 g of seeds of each accession wase analyzed at Charles Renard Analytical Laboratory, ICRISAT, Patancheru to estimate seed protein and mineral (Fe, Zn, Cu, Mn, Ca and Mg) concentrations.

Total N was estimated colorimetrically on continuous-flow auto-analyzer by using sulfuric acid and selenium digestion (Sahrawat, Kumar, & Murthy, 2002). Protein concentration was calculated by using conversion factor 6.25 (total N × 6.25). Concentration of micronutrients (Fe, Zn, Cu, Mn) and macronurtients (Ca and Mg) were estimated on inductively coupled plasma–optical emission spectrometry method (Wheal et al., 2011).

The seeds were washed with distilled water followed by drying in the oven at 60 °C for 48 h before grinding. About 20 g of dried seed sample from each accession was powdered in a stainless steel grinder and the powered seed samples were kept overnight in the oven at 55 °C. The samples and the standards were simultaneously digested with appropriate blanks in triplicate (three independent analyses). About 0.3 g of powered sample was taken in a 50-ml polypropylene tube, and 2 ml of concentrated nitric acid and 0.5 ml hydrogen peroxide was added. The contents were left overnight for cold digestion in a digestion chamber. Initially, the samples were digested at 80 °C for 0.5 h and afterwards at 125 °C for ~2 hr to get colorless and clear digest. Upon cooling of the digests, the contents were dissolved in distilled water and the final volume made up to 25 ml followed by mixing on vortexer. Digested samples were filtered and analyzed for seed Fe, Zn, Cu, Mn,

Ca, and Mg concentrations by inductively coupled plasma–optical emission spectrometry (Teledyne Leeman Labs). The concentration of micro- and macronutrients were expressed in mg kg^{-1} and g kg^{-1} seed, respectively. Protein concentration was expressed as a percentage.

2.3 | Statistical analysis

The ANOVA was carried out using data recorded on five traits (seed protein, Fe, Zn, Cu, and Mn concentration) in three replications during 3 yr (2014, 2015, and 2016) and on two additional traits (seed Ca and Mg concentration) in 2016. and The replicate-wise values of protein and mineral concentrations recorded in 41 accessions from nine *Cicer* species were used for year-wise statistical analysis following general ANOVA for various factors and their interactions using GenStat (15th ed.) (http://www.genstat.co.uk). Bartlett's homogeneity of variance test (Bartlett, 1937) revealed that the error variances between 3 yr were homogeneous for most traits, and therefore, pooled analysis was performed. For pooled ANOVA, data on five traits (seed protein, Fe, Zn, Cu, and Mn concentration) in 41 accessions belonging to nine *Cicer* species was used. For analysis, the accessions were nested within species (species/accessions) and the significance of differences were tested within and between species, species/accessions, and interaction means by using respective least significant differences.

Broad-sense heritability (h^2) was calculated using pooled data for seed protein, Fe, Zn, Cu, and Mn and using 2016 data for seed Ca and Mg. Broad-sense heritability was categorized as low (<0.30), moderate (0.30–0.60), and high (> 0.60). Pearson correlation coefficients were estimated to identify the useful associations between different seed nutrients.

Pooled data on five seed nutrient traits—protein, Fe, Zn, Cu and Mn concentration—was used to create a phenotypic distance matrix for each trait by calculating the differences between each pair of entries. The diversity index was calculated for each trait by averaging the differences in the phenotypic values divided by the respective range (Johns et al., 1997). The mean, minimum, and maximum diversity were calculated, and the pair of species and accessions showing the maximum and minimum diversity were identified. Cultivated and wild *Cicer* species and accessions were clustered using Euclidean phenotypic distance matrix following hierarchical clustering (Ward, 1963) in R package 'cluster.'

The performance of wild *Cicer* accessions over years for seed nutrient concentration was compared with the best cultivated chickpea, used as checks. The promising accessions with higher seed Fe, Zn, Cu, Mn, and protein concentrations were identified based on the pooled analysis. For seed Ca and Mg concentration, the promising accessions were identified based on the performance of wild accessions compared with cultivated chickpea in 2016.

3 | RESULTS

The year-wise ANOVA showed significant differences among species and species/accessions for all the traits ($P \leq .001$). Pooled analysis showed significant differences between years, species × years, and species/accessions × year (Table 2). Partitioning of the total sum of squares revealed the greater importance of species/accessions followed by species toward total variability in seed protein concentration in individual years (30–39% variations attributed to species/accessions and 28–34% attributed to species) and equal contribution of species/accessions (16%) and species (17%) in pooled analysis. Variability in all seed mineral concentrations was mainly due to species in year-wise and pooled analyses (Table 2).

3.1 | Between-species variability

Seed protein concentration in eight wild *Cicer* species and cultivated chickpea varied from 21.8 (*C. yamashitae*) to 24.9% (*C. chorassanicum*), wherein only *C. chorassanicum* had significantly higher seed protein concentration (24.9%) compared with chickpea (23.5%). Six wild *Cicer* species—*C. reticulatum*, *C. echinospermum*, *C. bijugum*, *C. pinnatifidum*, *C. chorassanicum*, and *C. yamashitae*—had significantly higher seed Fe (48.6–166 mg kg^{-1}) and Zn (35.3–47 mg kg^{-1}) concentration compared with chickpea (~42 and 28 mg kg^{-1} for Fe and Zn, respectively). Further, amongst eight wild *Cicer* species, *C. judaicum* had the highest seed Fe (199 mg kg^{-1}) but the lowest seed Zn concentration (~28 mg kg^{-1}) whereas *C. cuneatum* had the highest seed Zn concentration (~31 mg kg^{-1}) but the lowest seed Fe concentration (~40 mg kg^{-1}). Except for *C. echinospermum* (3.1 mg kg^{-1}) and *C. bijugum* (3.6 mg kg^{-1}), the remaining wild species had significantly higher seed Cu concentration, which ranged from 4.3 mg kg^{-1} in *C. reticulatum* to 7.7 mg kg^{-1} in *C. judaicum* compared with 3.4 mg kg^{-1} in *C. arietinum*. Similarly, seed Mn concentration was also significantly higher in most of the wild species (ranging from 57.9 mg kg^{-1} in *C. chorassanicum* to 162 mg kg^{-1} in *C. pinnatifidum*) compared with chickpea (37.1 mg kg^{-1}) (Table 3). An almost similar pattern was observed for seed protein and mineral concentrations in individual year-wise analysis (data not given). In 2016, seed Ca concentration was significantly higher in all wild *Cicer* species (3.02 g kg^{-1} in *C. echinospermum* to 6.09 g kg^{-1} in *C. chorassanicum*) than chickpea (2.22 g kg^{-1}), whereas five wild *Cicer* species—*C. bijugum*, *C. chorassanicum*, *C. judaicum*, *C. pinnatifidum*, and *C. reticulatum*—had significantly higher seed Mg concentration (1.65–1.83 g kg^{-1}) than chickpea (1.41 g kg^{-1}) (Table 3).

TABLE 2 Analysis of variance (ANOVA) for protein and mineral concentration in annual wild and cultivated *Cicer* accessions evaluated over three seasons (2014–2016) at ICRISAT, Patancheru, India

Source of variations	Pooled					2016	
	Protein	Fe	Zn	Cu	Mn	Ca	Mg
	%	———————————mg kg⁻¹———————————				———g kg⁻¹———	
Replication	6.92	1.06	5.75	0.60	39.19	0.19	0.01
Season	51.71**	1,421.15**	401.53**	22.89**	580.39**	–	–
Species	27.33**	153,391.92**	1,039.53**	124.41**	83,510.09**	21.54**	0.50**
Season × species	8.64**	211.93**	27.00**	3.53**	54.22**	–	–
Residual	3.24	34.16	2.63	0.60	13.09	0.07	0.01
Species/accession	6.53**	1,100.98**	127.17**	6.23**	955.92**	0.72**	0.05**
Season × species/accession	3.26**	94.73**	6.22**	0.93**	33.79**	–	–
Residual	1.24	26.44	3.15	0.27	14.70	0.08	0.00

**Significant at $P < .001$.
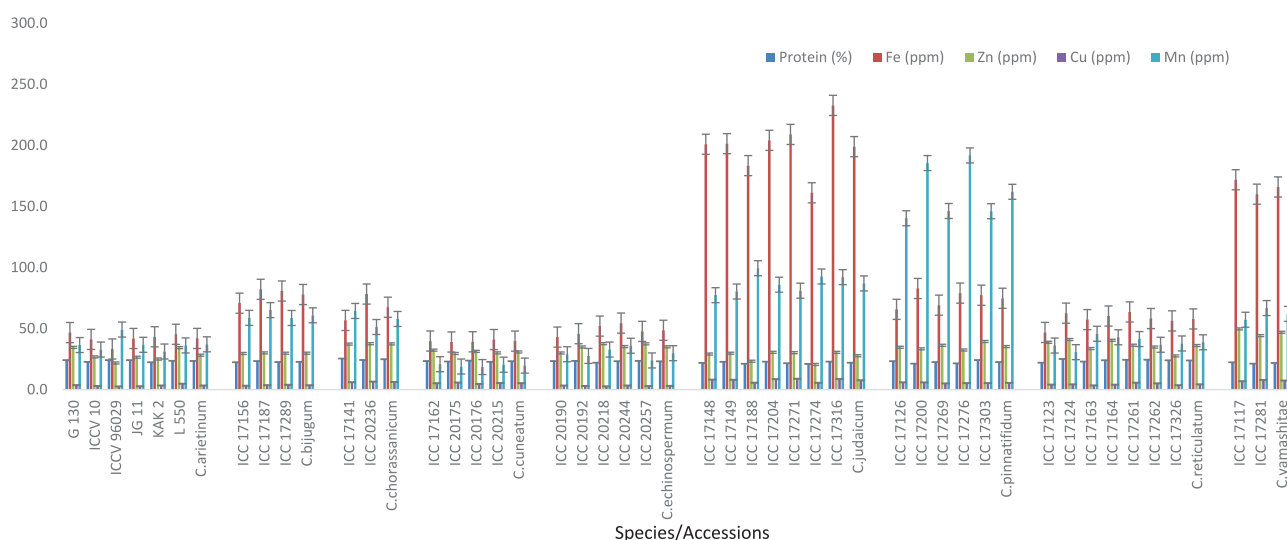
FIGURE 1 Variations for seed protein and mineral concentration in eight annual wild and cultivated *Cicer* species evaluated over 3 yr at ICRISAT, Patancheru, India

Overall, *C. chorassanicum* was superior for all the seven seed nutrients (protein, Fe, Zn, Cu, Mn, Ca, and Mg) and *C. pinnatifidum* for six seed nutrients (Fe, Zn, Cu, Mn, Ca, and Mg).

3.2 | Within-species variability

Based on the narrow range, it is evident that low variability was present within cultivated and different wild *Cicer* species for seed protein concentration (Table 3; Figure 1). When the accessions were compared with the respective species means, only one accession, ICC17303 from *C. pinnatifidum* showed significantly higher seed protein concentration (24.2%). For seed Fe concentration, the highest variability was observed within *C. judaicum* (161.2–232.7 mg kg⁻¹), wherein three accessions, ICC17204, ICC17271, and ICC17316, showed significantly higher seed Fe concentration (204–233 mg kg⁻¹) when compared with species mean (199 mg kg⁻¹). Though there were only two accessions, ICC17141 and ICC20236, of *C. chorassanicum*, a large variation was observed between them for seed Fe and Mn concentrations (57 mg kg⁻¹ Fe and 64 mg kg⁻¹ Mn concentration in ICC17141 and 78 mg kg⁻¹ Fe and 51 mg kg⁻¹ Mn concentration in ICC20236). Similar pattern was observed between two accessions of *C. yamashitae* wherein seed Fe and Zn concentration was significantly higher in ICC17117 (~172 mg kg⁻¹ Fe and 50 mg kg⁻¹ Zn) and seed Mn concentration in ICC17281 (67 mg kg⁻¹). Variability for seed Fe concentration was also observed within *C. reticulatum* and *C.*

TABLE 3 Variability for protein and mineral concentration in annual wild and cultivated *Cicer* accessions evaluated over three seasons (2014–2016) at ICRISAT, Patancheru, India

Species[a]	Mean (range within species)						
	Pooled					2016	
	Protein	Fe	Zn	Cu	Mn	Ca	Mg
	%	mg kg⁻¹				g kg⁻¹	
C. arietinum (6)	23.5 (22.4–24.1)	41.9 (33.3–46.7)	28.2 (21.8–34.6)	3.4 (2.6–4.9)	37.1 (31.2–49.2)	2.22 (1.62–3.16)	1.41 (1.21–1.59)
C. bijugum (3)	22.8 (22.4–23.4)	77.9 (70.8–82.1)	29.8 (29.6–30.1)	3.6 (3.2–4.0)	60.9 (58.7–65.1)	4.24 (3.54–4.63)	1.77 (1.55–1.88)
C. chorassanicum (2)	24.9 (24.3–25.5)	67.5 (56.7–78.3)	37.4 (37.3–37.6)	6.3 (6.1–6.6)	57.9 (51.3–64.4)	6.09 (6.06–6.12)	1.65 (1.60–1.71)
C. cuneatum (4)	23.3 (22.8–23.8)	39.7 (39–41)	30.8 (29.6–32.3)	5.3 (4.7–5.8)	19.7 (18.5–20.8)	3.42 (3.19–3.58)	1.44 (1.41–1.48)
C. echinospermum (5)	23.2 (22.1–23.6)	48.6 (43–54.5)	35.1 (30–37.6)	3.1 (2.7–3.4)	29.8 (23.9–35.9)	3.02 (2.37–3.58)	1.40 (1.31–1.43)
C. judaicum (7)	22.1 (21–23)	199.0 (161.2–232.7)	27.7 (20.6–30.6)	7.7 (5.6–8.9)	87.0 (77.3–99.4)	5.49 (4.52–5.96)	1.83 (1.76–1.87)
C. pinnatifidum (5)	22.6 (21.3–24.2)	74.8 (65.8–82.8)	35.3 (32.4–39.4)	5.5 (5.1–6.0)	162.0 (140.3–191.8)	3.98 (3.39–4.48)	1.68 (1.43–1.97)
C. reticulatum (7)	23.9 (22.1–25.1)	57.9 (46.8–63.6)	36.1 (27.6–41.0)	4.3 (3.5–5.7)	38.8 (30.7–45.8)	2.98 (2.56–3.38)	1.70 (1.56–2.01)
C. yamashitae (2)	21.8 (21.3–22.4)	165.9 (160–171.8)	47.0 (44.2–49.7)	7.4 (6.9–8.0)	62.0 (57.2–66.8)	5.03 (4.95–5.11)	1.25 (1.22–1.28)
Mean	23.1	85.9	34.2	5.2	61.7	4.05	1.57
Range (between species)	21.8–24.9	39.7–199	27.7–47	3.1–7.7	19.7–162	2.22–6.09	1.25–1.83
SEM (between species)	0.326	1.057	0.294	0.141	0.655	0.085	0.024
SEM (accessions within species)	0.456	1.792	0.576	0.204	1.256	0.160	0.039
Broad-sense heritability	68.8	93.2	95.3	88.6	96.8	95.0	96.1

[a]value in parenthesis indicates the number of accessions in each species.
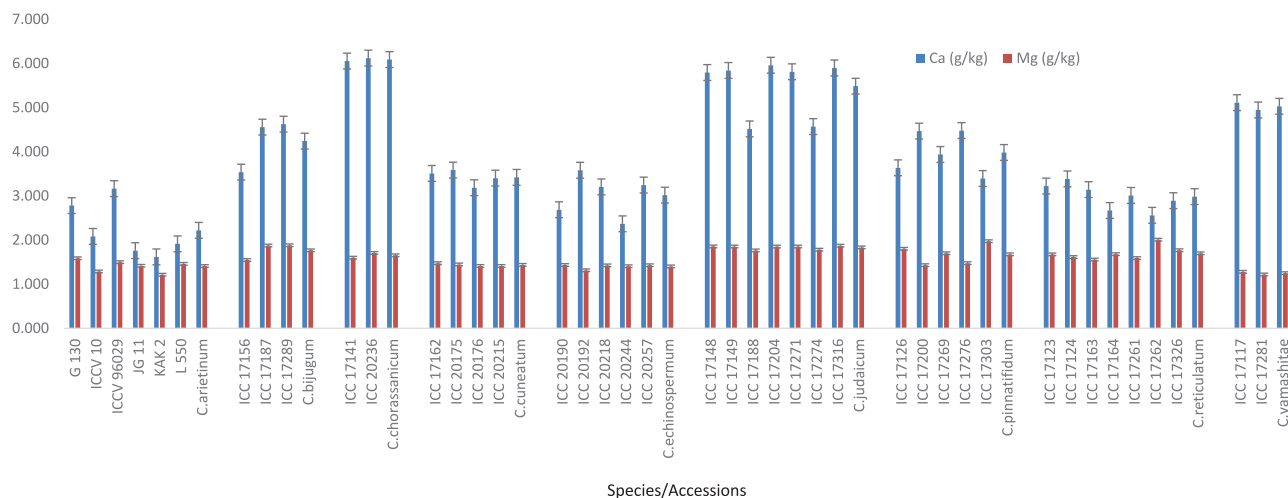
FIGURE 2 Variations for seed calcium (Ca) and magnesium (Mg) concentration in eight annual wild and cultivated *Cicer* species evaluated in 2016 at ICRISAT, Patancheru, India

echinospermum accessions. One accession of *C. reticulatum*, ICC17261, and one accession of *C. echinospermum*, ICC20244, showed significantly higher seed Fe concentration when compared with the respective species means. For seed Zn concentration, accessions of *C. reticulatum* showed high variability (Table 3), and three accessions, ICC17123, ICC17124, and ICC17164, showed significantly higher seed Zn concentration (39–41 mg kg^{-1}) than the species mean (Figure 1). The highest variability for seed Cu and Mn concentration was observed within accessions of *C. judaicum* (Table 3). Within *C. judaicum* accessions, three accessions, ICC17204, ICC17271, and ICC17316, had high seed Cu concentration (8.6–8.9 mg kg^{-1}) and ICC17188, ICC17274, and ICC17316 had high seed Mn concentration (92–99 mg kg^{-1}). High variability for seed Ca and Mg concentration was observed within both cultivated and wild species (Table 3). Within *C. arietinum*, ICGV 96029 showed high seed Ca, and G130 showed high seed Ca and Mg concentration (Figure 1). Amongst the wild accessions, *C. echinospermum* accession ICC20192 (3.58 g kg^{-1}), *C. judaicum* accession ICC17204 (5.96 g kg^{-1}), and two *C. pinnatifidum* accessions, ICC17200 (4.47 g kg^{-1}) and ICC17276 (4.48 g kg^{-1}), had significantly higher seed Ca concentration than the respective species means (Figure 2). Similarly, two *C. pinnatifidum* accessions, ICC17126 (1.80 g kg^{-1}) and ICC17303 (1.97 g kg^{-1}), and one *C. reticulatum* accession, ICC17262 (2.01 g kg^{-1}), had high seed Mg concentration (Figure 2).

3.3 | Correlation between seed nutrients

Based on accession means, the significant positive correlation was observed between seed Fe and Cu ($r = .816$), seed

Fe and Mn ($r = .377$), and seed Mn and Cu ($r = .401$) concentration (Table 4), whereas seed protein concentration had significantly negative association with seed Fe ($r = -.492$) and Mn ($r = -.419$) concentration. At the species level, protein concentration was negatively associated with seed Fe ($r = -.684$) concentration. Significantly positive association was observed between seed Fe and Cu ($r = .800$) concentration (Table 4). Further, significantly positive correlation of seed Ca was observed with seed Fe and Cu concentration, both at species and accessions levels. Positive but nonsignificant correlation was observed between seed Ca and Mg concentration based on accession means ($r = .426$) and species mean (.330) in 2016 (data not given).

3.4 | Phenotypic diversity, cluster analysis, and identification of promising accessions

At the species level, the mean phenotypic diversity index was 0.220. The maximum diversity was found between *C. yamashitae* and chickpea (0.534) and the least diversity was between *C. reticulatum* and *C. echinospermum* (0.029). For accessions, the mean phenotypic diversity index was 0.135. The maximum diversity was found between chickpea genotype ICCV96029 and *C. judaicum* accession ICC17271 (0.444) and minimum diversity was between *C. cuneatum* accession ICC20215 and *C. cuneatum* ICC20175 (0.001). The five most similar and diverse pairs of species accessions were identified (Table 5).

Hierarchical cluster analysis based on five seed nutrients over 3 yr was performed to separate the cultivated and wild species and accessions into distinct groups. The cluster analysis following Ward's method grouped nine cultivated and wild *Cicer* species into five clusters (Figure 3). Cluster 1

TABLE 4 Correlation between protein and mineral concentrations in annual wild and cultivated *Cicer* accessions evaluated over years (2014–2016) at ICRISAT, Patancheru, India

Variable	Level	Protein	Fe	Zn	Cu
Fe	Species	−0.684*	–	–	–
	Accession	−0.492*	–	–	–
Zn	Species	−0.101	0.216	–	–
	Accession	0.216	−0.057	–	–
Cu	Species	−0.360	0.800*	0.372	–
	Accession	−0.288	0.816*	0.140	–
Mn	Species	−0.389	0.344	0.076	0.377
	Accession	−0.419*	0.377*	−0.002	0.401*

*Significant at $P < .05$.

TABLE 5 Phenotypic diversity in annual wild and cultivated *Cicer* species and accessions evaluated over years (2014–2016) at ICRISAT, Patancheru, India

Most similar pair of species or accessions	Similarity	Most diverse pair of species or accessions	Diversity
Species			
C. reticulatum–C. echinospermum	0.971	*C. yamashitae–C. arietinum*	0.534
C. bijugum–C. arietinum	0.970	*C. judaicum–C. echinospermum*	0.465
C. echinospermum–C. arietinum	0.970	*C. judaicum–C. arietinum*	0.437
C. echinospermum–C. bijugum	0.963	*C. yamashitae–C. echinospermum*	0.415
C. reticulatum–C. cuneatum	0.962	*C. reticulatum–C. judaicum*	0.393
Accessions			
ICC20215– ICC20175	0.999	ICCV96029– ICC17271	0.444
ICC20257– ICC20192	0.998	ICCV96029–ICC17316	0.433
ICC20244– ICC17163	0.998	ICCV96029– ICC17281	0.427
ICC17204–ICC17149	0.998	JG11–ICC17271	0.414
ICC17262–ICC17261	0.998	ICCV96029–ICC17117	0.406

is comprised of chickpea, *C. reticulatum*, *C. echinospermum*, *C. bijugum*, and *C. cuneatum*. The remaining four wild *Cicer* species were clustered separately with each species in individual cluster (Figure 3). Based on accession means, 41 cultivated and wild *Cicer* accessions were grouped into five clusters (Figure 4). Cluster 1 was the largest cluster, consisting of 21 accessions that included all cultivated chickpea genotypes, all the accessions of *C. bijugum* (3 accessions), *C. echinospermum* (5 accessions), *C. cuneatum* (4 accessions), and three accessions of *C. reticulatum*. The remaining four accessions of *C. reticulatum* and both the accessions of *C. chorassanicum* were clustered into Cluster 2. All the accessions of *C. judaicum* (7 accessions), *C. pinnatifidum* (5 accessions), and *C. yamashitae* (2 accessions) were clustered into Cluster 3, Cluster 4, and Cluster 5, respectively. Cluster 2 had the highest mean for seed protein concentration followed by Cluster 1. Cluster 3 had the highest mean seed Fe concentration and Cluster 4 had the highest seed Mn concentration.

The performance of wild *Cicer* accessions was compared with the cultivated chickpea genotypes (used as checks). Among the cultivated chickpea, G130 had significantly higher seed protein and mineral concentration and hence this genotype was selected as the best check for comparing the wild *Cicer* accessions. For seed protein concentration, only one accession, ICC17141 (*C. chorassanicum*, 25.5%), was significantly better than G130 (24.1%). A total of 27 wild *Cicer* accessions for seed Fe concentration (52–233 mg kg^{-1}), 12 accessions for seed Zn concentration (36–50 mg kg^{-1}), 23 accessions for seed Cu concentration (4.3–8.9 mg kg^{-1}), 22 accessions for seed Mn concentration (41.4–191.8 mg kg^{-1}), 25 accessions for seed Ca concentration (3.245–6.122 g kg^{-1}) and 15 accessions for seed Mg concentration (1.699–2.007 g kg^{-1}), were significantly better than the best check, G130 (46.7 mg kg^{-1} seed Fe, 34.6 mg kg^{-1} seed Zn, 3.7 mg kg^{-1} seed Cu, 36.5 mg kg^{-1} seed Mn, 2.78 g kg^{-1} seed Ca, and 1.59 g kg^{-1} seed Mg) (Table 6). The top
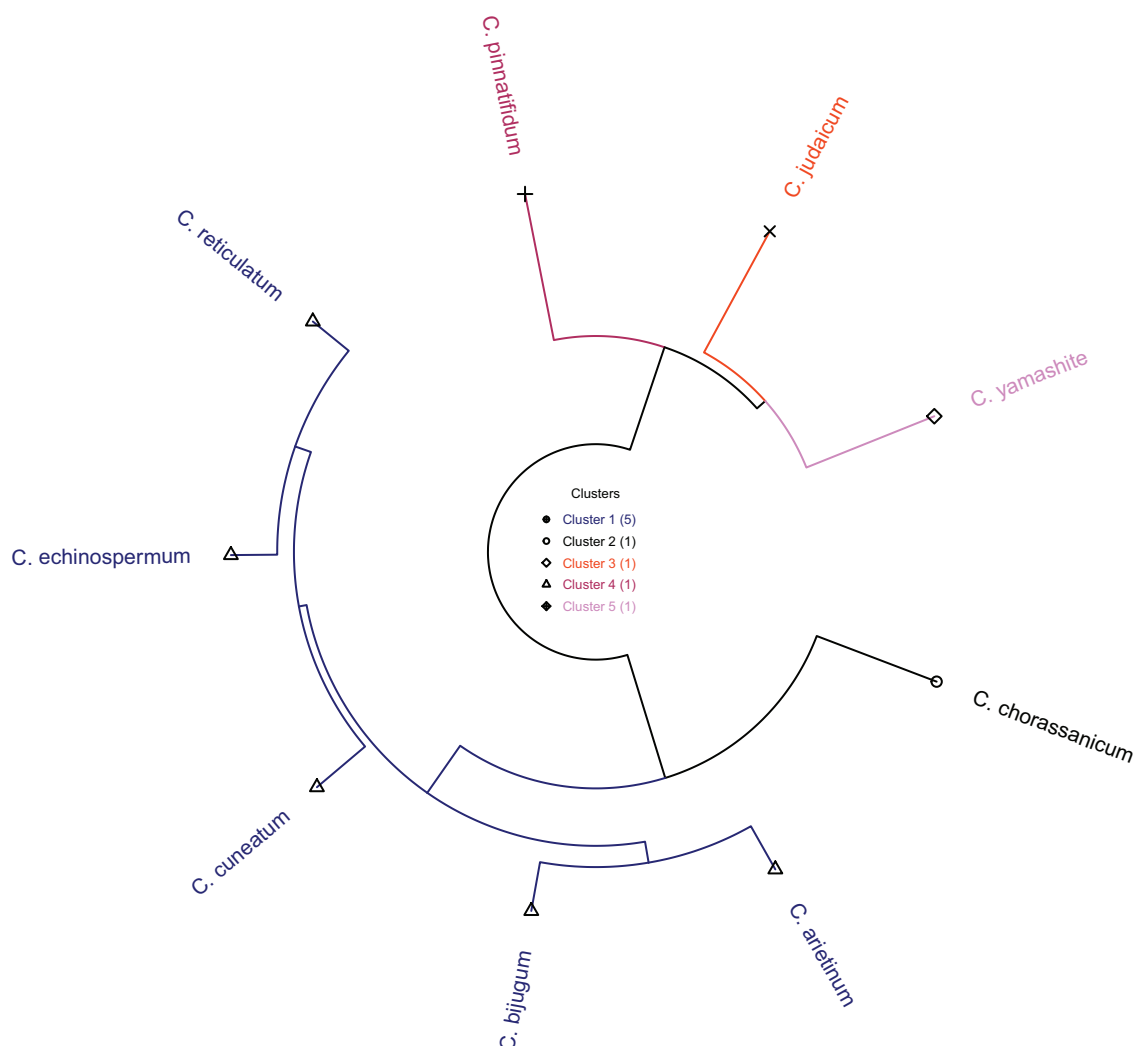
FIGURE 3 Cluster diagram depicting different clusters formed using nine annual wild and cultivated *Cicer* species following Ward's method based on five seed nutrient concentrations. Cluster 1 consisted of cultivated chickpea, progenitor species *C. reticulatum*, cross-compatible secondary gene pool species *C. echicospermum*, and cross incompatible tertiary gene pool species, *C. bijugum*, and *C. cuneatum*. Remaining cross-incompatible tertiary gene pool species were clustered separately in different clusters

five accessions for each seed nutrient are given in Table 7. Overall, on pooled analysis, only one accession, ICC17141 (*C. chorassanicum*), was found promising for all five seed nutrients (protein, Fe, Zn, Cu, and Mn) followed by two accessions, ICC17269 and ICC17303 of *C. pinnatifidum*, ICC17261 of *C. reticulatum*, ICC20236 of *C. chorassanicum*, and two accessions, ICC17117 and ICC17281, of *C. yamashitae* were found promising for all four minerals (Table 6).

4 | DISCUSSION

Identification and exploitation of wild species harboring new and diverse genetic variability for seed protein and mineral concentration is the most economic strategy to alleviate pro-

tein and nutrient deficiency. Enormous variability for seed protein and nutrients was observed between and within eight annual *Cicer* species used in this study. Thus, nutrient-rich accessions can be selected for use in chickpea breeding programs. The previous attempts to study the genetic variability for grain nutrient concentration in chickpea were mainly based on cultivated germplasm (Aliu et al., 2016; Diapari et al., 2014; Upadhyaya et al., 2016; Vandemark et al., 2018) or on a few accessions belonging mostly to the primary, secondary, or a few tertiary gene pool species (Kaur, Grewal, Gill, & Singh, 2019; Wettberg et al., 2018) mostly limited to estimation of protein concentration (Ocampo, Robertson, & Singh, 1998). The present study provides a comprehensive analysis of variability for protein and mineral concentrations in eight annual wild and cultivated *Cicer* species originating or collected from seven countries. Significant variations
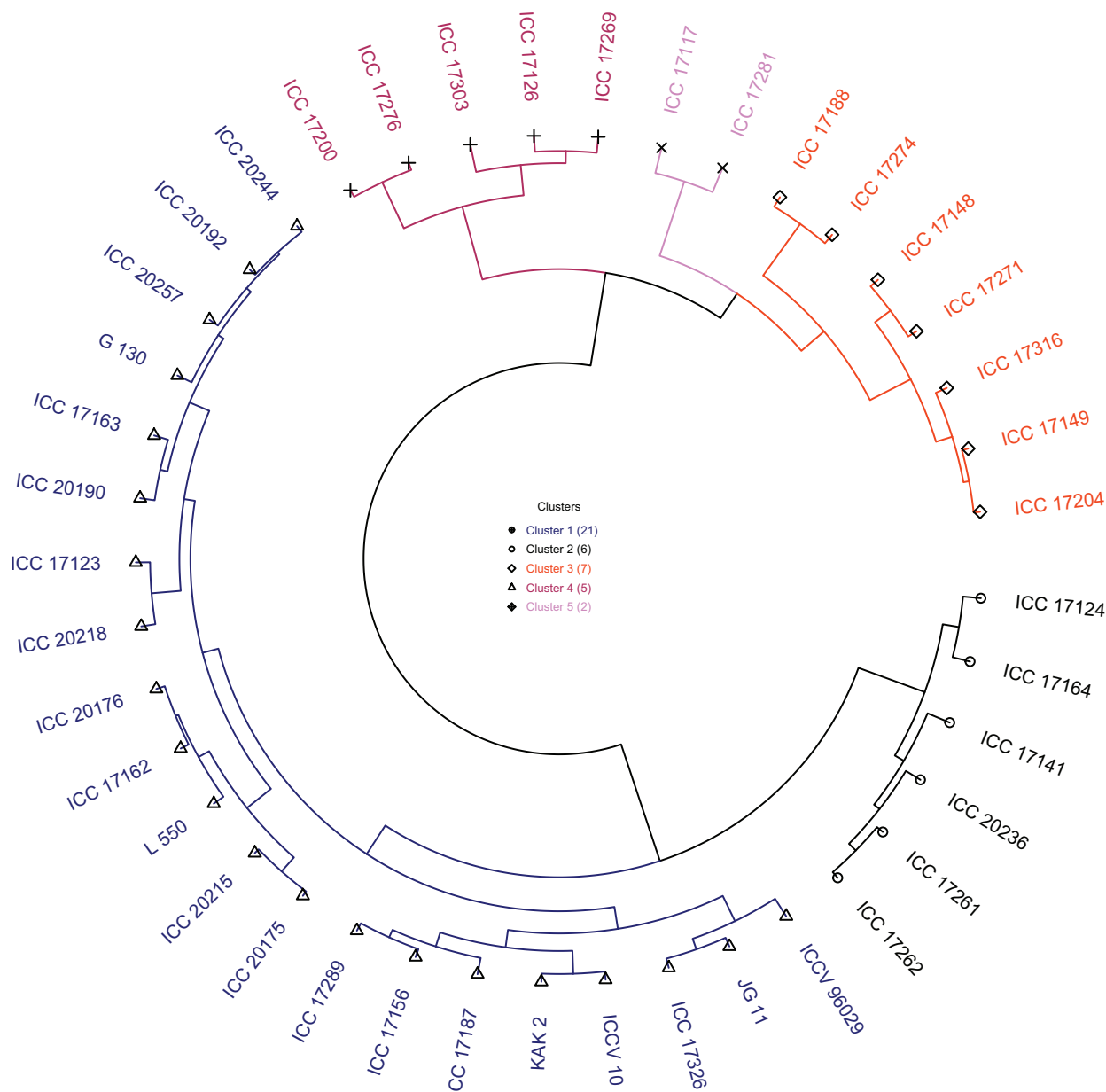
FIGURE 4 Cluster diagram depicting different clusters formed using 41 annual wild and cultivated *Cicer* accessions following Ward's method based on five seed nutrient concentrations. Cluster 1 consisted of all accessions of cultivated chickpea, *C. echinospermum*, *C. bijugum*, *C. cuneatum*, and three out of seven accessions of *C. reticulatum*

for seed protein concentration in annual wild *Cicer* species has been reported, which ranged from 16.8% in *C. cuneatum* to 26.8% in *C. pinnatifidum* amongst eight wild *Cicer* species (Ocampo et al., 1998) and 24.3% in *C. judaicum* to 25.3% in *C. pinnatifidum* amongst three wild *Cicer* species: *C. judaicum*, *C. pinnatifidum*, and *C. echinospermum* (Kaur et al., 2019). In the present study, the highest seed protein concentration was found in *C. chorassanicum* (24.9%). Seed protein concentration in *C. cuneatum* accessions was found higher (22.8–23.8%) in the present study than the previous report (Ocampo et al., 1998). In contrast, the seed protein concentration in *C. pinnatifidum* accessions was lower (21.3–

24.2%) in the present study than the previous reports (Kaur et al., 2019; Ocampo et al., 1998). Differences were observed for most of the seed nutrient concentrations when compared with the previous report (Kaur et al., 2019). For example, the range of seed Fe and Zn concentrations in *C. pinnatifidum* was almost similar but large differences were observed in *C. echinospermum* (35.1 mg kg^{-1} Zn and 48.6 mg kg^{-1} Fe in the present study) when compared with the previous report (15.6 and 92.4 mg kg^{-1} Zn and Fe, respectively) (Kaur et al., 2019). Similarly, average seed Fe concentration in *C. judaicum* was reported to be 83.2 mg kg^{-1} (Kaur et al., 2019) compared with 199 mg kg^{-1} in the present study. These

TABLE 6 Identification of promising wild *Cicer* accessions for high protein and mineral concentrations

Species	Accessions	Protein	Fe	Zn	Cu	Mn	Ca	Mg
		%	————mg kg⁻¹————				——g kg⁻¹——	
C. bijugum	ICC17156	22.4	70.8*	29.6	3.2	58.8*	3.538*	1.545
C. bijugum	ICC17187	23.4	82.1*	30.1	3.6	65.1*	4.558*	1.874*
C. bijugum	ICC17289	22.6	80.8*	29.8	4	58.7*	4.625*	1.88*
C. chorassanicum	ICC17141	25.5*	56.7*	37.3*	6.1*	64.4*	6.055*	1.6
C. chorassanicum	ICC20236	24.3	78.3*	37.6*	6.6*	51.3*	6.122*	1.707*
C. cuneatum	ICC17162	23.4	39.8	32.3	5.2*	20.8	3.509*	1.475
C. cuneatum	ICC20175	23	39	29.6	5.8*	18.9	3.584*	1.446
C. cuneatum	ICC20176	23.8	39.2	31.3	4.7*	18.5	3.185	1.412
C. cuneatum	ICC20215	22.8	41	30.1	5.4*	20.4	3.402*	1.412
C. echinospermum	ICC20190	23.4	43	30	3.3	29	2.686	1.434
C. echinospermum	ICC20192	23.5	45.8	34.9	3	27.6	3.581*	1.314
C. echinospermum	ICC20218	22.1	52*	37.6*	2.7	32.8	3.203	1.425
C. echinospermum	ICC20244	23.3	54.5*	35.3	3.4	35.9	2.367	1.406
C. echinospermum	ICC20257	23.6	47.7	37.5*	2.9	23.9	3.245*	1.428
C. judaicum	ICC17148	21.9	200.9*	29.1	8.2*	77.3*	5.795*	1.854*
C. judaicum	ICC17149	22.8	201.4*	29.8	8.1*	80.3*	5.842*	1.853*
C. judaicum	ICC17188	21.2	183.4*	23.3	5.6*	99.4*	4.517*	1.764*
C. judaicum	ICC17204	22.9	204.1*	30.6	8.6*	85.9*	5.96*	1.852*
C. judaicum	ICC17271	21.6	209*	30.3	8.9*	81*	5.812*	1.853*
C. judaicum	ICC17274	21	161.2*	20.6	5.6*	92.7*	4.57*	1.781*
C. judaicum	ICC17316	23	232.7*	30.5	8.7*	92.1*	5.899*	1.871*
C. pinnatifidum	ICC17126	23.3	65.8*	34.7	6*	140.3*	3.636*	1.801*
C. pinnatifidum	ICC17200	21.3	82.8*	33.4	5.9*	185.5*	4.467*	1.43
C. pinnatifidum	ICC17269	22.5	69.2*	36.3*	5.1*	146.3*	3.938*	1.699*
C. pinnatifidum	ICC17276	21.5	79*	32.4	5.3*	191.8*	4.48*	1.476
C. pinnatifidum	ICC17303	24.2	77.3*	39.4*	5.3*	146.1*	3.393*	1.973*
C. reticulatum	ICC17123	22.1	46.8	38.8*	4.1	36.1	3.222	1.674
C. reticulatum	ICC17124	25.1	62.6*	41*	4.3*	30.7	3.384*	1.614
C. reticulatum	ICC17163	23	57.3*	33.7	3.5	45.8*	3.142	1.557
C. reticulatum	ICC17164	24	60.3*	40.4*	4.1	42.9*	2.668	1.68
C. reticulatum	ICC17261	24.5	63.6*	36.4*	5.7*	41.4*	3.01	1.59
C. reticulatum	ICC17262	24.5	58.2*	34.9	5.1*	36.8	2.559	2.007*
C. reticulatum	ICC17326	24	56.3*	27.6	3.6	37.8	2.891	1.768*
C. yamashitae	ICC17117	22.4	171.8*	49.7*	6.9*	57.2	5.112*	1.279
C. yamashitae	ICC17281	21.3	160*	44.2*	8*	66.8*	4.946*	1.219
C. arietinum (check)	G130	24.1	46.7	34.6	3.7	36.5	2.78	1.586
SEM (accessions across species)		0.372	1.714	0.592	0.173	1.278	0.1619	0.0365

*Significantly better than the best check, G130 at *P* < .05.

differences could be due to the different accessions used in these studies. Specifically, *C. chorassanicum* was found promising for high seed protein and Ca concentration; *C. judaicum* for high Fe, Cu, and Mg; *C. yamashitae* for high Zn and Fe; and *C. pinnatifidum* for high seed Mn concentration; these sources can be used for improving seed nutritional quality of cultivated chickpea varieties.

One accession, ICC17141 of *C. chorassanicum* for seed protein, 27 wild *Cicer* accessions for seed Fe, 12 accessions for seed Zn, 23 accessions for seed Cu, 22 accessions for seed Mn, 25 accessions for seed Ca, and 15 accessions for seed Mg concentration were identified as the promising sources of nutritional traits. Amongst these accessions, ICC17141 (*C. chorassanicum*), ICC17269 and ICC17303 (both

TABLE 7 Top five promising *Cicer* accessions identified for each grain nutrient at ICRISAT, Patancheru, India

Trait	G130 (check)	Promising accessions
Protein (%)	24.1	ICC17141 (*C. chorassanicum*), ICC17124 (*C. reticulatum*), ICC17262 (*C. reticulatum*), ICC17261 (*C. reticulatum*), and ICC20236 (*C. chorassanicum*) (25.5–24.3%)
Fe (mg kg^{-1})	46.7	ICC17316 (*C. judaicum*), ICC17271 (*C. judaicum*), ICC17204 (*C. judaicum*), ICC17149 (*C. judaicum*), and ICC17148 (*C. judaicum*) (232.7–200.9 mg kg^{-1})
Zn (mg kg^{-1})	34.6	ICC17117 (*C. yamashitae*), ICC17281 (*C. yamashitae*), ICC17124 (*C. reticulatum*), ICC17164 (*C. reticulatum*), and ICC17303 (*C. pinnatifidum*) (49.7–39.4 mg kg^{-1})
Cu (mg kg^{-1})	3.7	ICC17271, ICC17316, ICC17204, ICC17148, and ICC17149 (all *C. judaicum*; 8.9–8.1 mg kg^{-1})
Mn (mg kg^{-1})	36.5	ICC17276, ICC17200, ICC17269, ICC17303, and ICC17126 (all *C. pinnatifidum*; 191.8–140.3 mg kg^{-1})
Ca (g kg^{-1})	2.78	ICC20236 (*C. chorassanicum*), ICC17141 (*C. chorassanicum*), ICC17204 (*C. judaicum*), ICC17316 (*C. judaicum*), and ICC17149 (*C. judaicum*) (6.12–5.84 g kg^{-1})
Mg (g kg^{-1})	1.59	ICC17262 (*C. reticulatum*), ICC17303 (*C. pinnatifidum*), ICC17289 (*C. bijugum*), ICC17187 (*C. bijugum*), and ICC17316 (*C. judaicum*) (2.01–1.87 g kg^{-1})

C. pinnatifidum), ICC17261 and ICC17262 (both *C. reticulatum*), ICC20236 (*C. chorassanicum*), and ICC17117 and ICC17281 (both *C. yamashitae*) were found to be promising for multiple seed nutrients. The highest seed Fe concentration was found in the accessions of *C. judaicum*. These accessions were originated in different countries; for example, ICC17316, having 233 mg kg^{-1} seed Fe concentration, is from Ethiopia; ICC17271 (209 mg kg^{-1}) is from Lebanon; ICC17204 (204 mg kg^{-1}) is from India; ICC17149 (201 mg kg^{-1}) is from Israel; ICC17148 (201 mg kg^{-1}) is from Israel; ICC17188 (183 mg kg^{-1}) is from Syria; and ICC17274 (161 mg kg^{-1}) is from Syria. These accessions also have high seed Cu, Mn, Ca and Mg concentration compared with other wild *Cicer* accessions.

Of these selected accessions, ICC17261 and ICC17262 (both from Turkey) hold a great potential for improving nutritional quality of chickpea as these accessions belong to the primary gene pool species *C. reticulatum*. *C. reticulatum* is easily crossable with cultivated chickpea (Singh & Ocampo, 1997), and the two promising accessions identified in this study can play an important role in improving seed Fe, Zn, Cu, and Mn concentration of cultivated chickpea. Another crossable species is *C. echinospermum* (Singh & Ocampo, 1997), and the two accessions, ICC20218 and ICC20244, of this species (both from Turkey) were found to have high seed Fe concentration. Another potent *C. echinospermum* accession having high seed Zn and Ca concentration was ICC20257. These accessions can be used in chickpea improvement programs for improving seed protein, Fe, Zn, and Ca concentration of cultivars. The promising *C. reticulatum* and *C.*

echinospermum accessions can also be used to develop multiparent populations following complex cross approach such as three-way and four-way crosses with a view to combine genes and alleles from different wild species into a common cultivated genetic background (Sharma, 2017). The complex cross approach may lead to the generation of desirable transgressive segregants as a result of unexpected epistatic effects as has already been reported in *Cicer* (Ocampo et al., 1998).

Use of promising accessions belonging to tertiary gene pool species *C. chorassanicum*, *C. pinnatifidum*, *C. judaicum*, *C. bijugum*, *C. cuneatum*, and *C. yamashiatae* is hindered because of cross-incompatibility barriers. Several efforts were made in the past to use tertiary gene pool species for chickpea improvement (Ahmad & Slinkard, 2004; Ahmad, Slinkard, & Scoles, 1988; Badami, Mallikarjuna, & Moss, 1997; Clarke et al., 2006; Clarke, Kumari, Khan, & Siddique, 2011; Croser, Ahmad, Clarke, & Siddique, 2003; Mallikarjuna, 1999; Mallikarjuna & Muehlbauer, 2011; Mallikarjuna, Jadhav, Nagamani, Amudhavalli, & Hoisington, 2007). In all these studies, efforts were made to generate interspecific hybrids between cultivated chickpea and different cross-incompatible tertiary gene pool species following embryo rescue techniques. However, no success was obtained in these studies except in *C. arietinum* × *C. bijugum* (Mallikarjuna et al., 2007). Hence, there is a need to develop novel techniques and strategies for accessing genes and alleles from these important and unexploited sources. One of the strategies could be to study the crossability relationships of primary and secondary gene pool species, *C. reticulatum* and *C. echinospermum*, respectively, with

six tertiary gene pool species. Based on these studies, the potential of *C. reticulatum* and *C. echinospermum* as 'bridge species' can be explored to access variability from the cross-incompatible tertiary gene pool species.

Highly positive association observed between seed Fe and Cu as well as seed Fe and Mn concentration provides an opportunity for simultaneous improvement of these seed nutrient traits. Vandemark et al. (2018) also found significant positive correlation between Fe and Cu content in chickpea and lentil and between Fe and Mn in chickpea. However, negative correlation between seed protein and Fe concentration will hinder the progress in improving both seed protein and Fe concentration simultaneously in chickpea cultivars. Non-significant association of seed protein with other nutrients such as Zn, Cu, Ca, and Mg indicates the possibility of combining higher seed Zn and other nutrient concentrations with higher seed protein.

This study led to the identification of wild *Cicer* accessions for multiple seed nutrient traits as well as the most diverse and similar pair of accessions. The five most diverse pairs of accessions or species indicated that the cultivated chickpea is highly diverse from *C. judaicum* and *C. yamashitae*, whereas it was found to be closely related to *C. echinospermum* and *C. bijugum*. In contrast, *C. cuneatum* accessions ICC 20215 and ICC 20175 (both from Ethiopia), *C. echinospermum* accessions ICC 20257 and ICC 20192 (both from Turkey), and *C. reticulatum* accessions ICC17262 and ICC17261 (both from Turkey) were found to be the most similar accessions. These results indicate the presence of duplicate accessions of these species in the gene bank.

Further, the cluster analysis grouped 41 cultivated and wild accessions into five clusters, wherein similar accessions were placed in the same cluster. Interestingly, all cultivated chickpea genotypes along with all accessions of *C. echinospermum*, *C. bijugum*, *C. cuneatum*, and three of the seven *C. retictulaum* accessions were clustered together in one cluster, whereas remaining four accessions of *C. reticulatum* were grouped in a separate cluster. This helps in selecting the most diverse accessions within cross-compatible gene pool for immediate use in chickpea improvement program. It is interesting to note that the accessions of *C. choarssanicum*, *C. judaicum*, *C. pinnatifidum*, and *C. yamashitae* were clustered in the species-specific groups and clusters. This shows that the variability between species is higher than the within species variability for seed nutrient concentrations. Systematic hybridization between promising accessions for seed protein and mineral concentrations chosen from different clusters will help to create new genetic variability for improving the seed nutrient concentrations of cultivated chickpea. Further, the contrasting pair of accessions within or between cross-compatible species such as *C. reticulatum* accession ICC17261, having significantly higher concentrations of seed Fe, Zn, Cu, and Mn, and *C. echinospermum* accession ICC 20190, having the lowest concentration of all the seed nutri-

ents, can be used to develop mapping populations for the mapping of genes and quantitative trait loci for multiple seed nutrients. Overall, the selected wild *Cicer* accessions with high seed nutrient concentrations hold promise in improving the nutritional content of high-yielding and well-adapted chickpea cultivars.

5 | SUMMARY AND CONCLUSION

Developing nutrient-rich chickpea cultivars is the most economic strategy to combat malnutrition resulting from protein and mineral deficiencies. In this study, efforts were made to identify new and diverse sources of variations for seed protein and mineral (Fe, Zn, Cu, Mn, Ca, and Mg) concentrations among 41 accessions of cultivated chickpea and eight annual wild *Cicer* species belonging to primary, secondary, and tertiary gene pools. Overall, large variability was observed both between and within species for seed protein, Fe, Zn, Cu, Mn, Ca, and Mg concentration with high heritability. Most of the wild *Cicer* accessions were found to have high concentration of at least one or more seed nutrients. Two *C. reticulatum* accessions, ICC17261 and ICC17262; two *C. chorassanicum* accessions, ICC17141 and ICC20236; two *C. pinnatifidum* accessions, ICC17269 and ICC17303; and two *C. yamashitae* accessions, ICC17117 and ICC17281, were found promising for multiple seed nutrients. Though promising *C. reticulatum* accessions can be used in crossing program for improving cultivated chickpea, the use of tertiary gene pool species for chickpea improvement needs concerted efforts in standardizing protocols and strategies to overcome cross-incompatibility barriers.

AUTHOR CONTRIBUTIONS
SHS planned the study; SHS and SAL raised the material under green house; CN analyzed the seed samples for seed nutrient concentrations in quality lab; SS analyzed the data; SHS and SAL prepared the manuscript; SAL, CN and SS provided their inputs. All the authors reviewed and approved the final manuscript.

CONFLICT OF INTEREST

Authors have no conflict of interest.

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