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 ICC17262 (*C. pinnatifidum* Jaub. & Spach) 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,
Cicer species possess many useful traits and can be exploited (Bunge). Plant material C. chorassanicum species accessions having high seed protein and mineral C. yamashitae. Most diverse pair of accessions identified for use in crossing program for chickpea improvement.

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. chrorassianicum (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.

Core Idea

- Large variability present within and between cultivated and wild Cicer species for grain nutrients
- Promising wild accessions having high concentration of protein and mineral contents identified
- Most diverse pair of accessions identified for use in crossing program for chickpea improvement.
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 were 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 macronutrients (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 powdered 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. 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*, *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 chickpea (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\(^{-1}\)). 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 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 | 2016 | Protein % | Fe mg kg⁻¹ | Zn mg kg⁻¹ | Cu mg kg⁻¹ | Mn mg kg⁻¹ | Ca g kg⁻¹ | Mg g kg⁻¹ |
|--------------------------------------|-------------|------|-----------|-------------|------------|------------|------------|-----------|-----------|
| Replication                          | 6.92        | 0.19 | 1.06      | 5.75        | 0.60       | 39.19      |            |           |           |
| Season                               | 1.421      | 0.19 | 1.421     | 401.53      | 22.89      | 580.39     |            |           |           |
| Species                              | 153,391    | 0.72 | 153,391   | 83,510      | 21.54      | 0.50       |            |           |           |
| Season × species                      | 94.73      | 0.08 | 94.73     | 127.17      | 6.22       | 33.79      |            |           |           |
| Residual                             | 26.44      | 0.00 | 26.44     | 3.15        | 0.27       | 14.70      |            |           |           |
| Species/accession                    | 1,100.98   | 0.01 | 1,100.98  | 127.17      | 6.23       | 55.92      |            |           |           |
| Season × species/accession           | 94.73      | 0.05 | 94.73     | 6.22        | 0.93       | 33.79      |            |           |           |
| Residual                             | 1.24       | 0.00 | 1.24      | 3.15        | 0.27       | 14.70      |            |           |           |

**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.
| Species* | Mean (range within species) | 2016 |
|----------|-----------------------------|------|
|          | Pooled                      |      |
|          | Protein  %                  | mg kg⁻¹ | Ca | Mg |
| 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.*
**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...
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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. echinospermum*          | 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
Clusters
- Cluster 1 (5)
- Cluster 2 (1)
- Cluster 3 (1)
- Cluster 4 (1)
- Cluster 5 (1)

**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. echinospermum*, 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.

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 protein 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
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 (mg kg⁻¹) | Zn (mg kg⁻¹) | Cu (mg kg⁻¹) | Mn (mg kg⁻¹) | Ca (mg kg⁻¹) | Mg (mg 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. pinnaatifidum        | ICC17126   | 23.3        | 65.8*       | 34.7         | 6*           | 140.3*       | 3.636*       | 1.801*       |
| C. pinnaatifidum        | ICC17200   | 21.3        | 82.8*       | 33.4         | 5.9*         | 185.5*       | 4.467*       | 1.43         |
| C. pinnaatifidum        | ICC17269   | 22.5        | 69.2*       | 36.3*        | 5.1*         | 146.3*       | 3.938*       | 1.699*       |
| C. pinnaatifidum        | 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. pinnaatifidum 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
C. yamashitae, ICC20236 (C. reticulatum), ICC17262 (C. reticulatum), ICC17261 (C. reticulatum), and ICC20236 (C. chorassanicum) (25.5–24.3%)

Zn (mg kg\(^{-1}\))

34.6

ICC17117 (C. yamashitae), ICC17281 (C. yamashitae), ICC17214 (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\(^{-1}\))

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 triparent 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. yamashitae 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, & Scloes, 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. reticulatum* 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 nutrients, 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. pin- natifidum* 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.

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CONFLICT OF INTEREST
Authors have no conflict of interest.

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