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Deciphering seasonal shifts, genotype × environment interactions effect on antioxidant activity, bioactive compounds and antinutritional factors in a diverse panel of leafy amaranth (*Amaranthus* spp.) germplasm: genetic and multivariate insights for breeding

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Abstract

Background Leafy amaranths (*Amaranthus* spp.) are promising traditional food and nutritional sources in Asia and Africa. They hold excellent value as underutilized, climate-resilient vegetable crops with versatile potential for food and nutritional security. Majority of the vegetable amaranth germplasm cultivated by Indian farmers consists of local landraces, and has not been subjected to systematic characterization. Hence, comprehensive characterization of amaranth germplasm for bioactive, antioxidant, and antinutritional factors in contrasting environments is vital for accelerating breeding of vegetable amaranth to underscore its potential in fulfilling United Nations sustainable development goals (SDGs) of Zero Hunger (SDG2) and Climate Action (SDG13).

Results A diverse panel of 57 vegetable amaranth germplasm was investigated for antioxidant activity, bioactive compounds and antinutritional factors across winter (2024–2025) and summer (2025) seasons. Field experiment was laid out in randomized augmented block design with three commercial checks. Seasonal variations revealed

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the influence of G × E interactions on antioxidant activity, bioactive compounds and concentration of antinutrients. Clear temporal trends were recorded for antioxidant capacities (DPPH, CUPRAC, FRAP), bioactive traits and anti-nutrients (oxalates and nitrates). Genetic and multivariate analysis critically discriminated the germplasm and enabled the identification of potential parents for hybridization to exploit heterosis. Mahalanobis-D^2 cluster analysis suggested IC-0647405, IC-0647419, IC-0647408, IIHR-B-AM247 and IIHR-B-AM248 as a promising source of antioxidant phytochemicals, β-carotene, protein, vitamin C and phenolics with lower content of anti-nutrients. Pusa Kiran, IC-0647419 and IHR_B_AM248_3_2 exhibited lower concentration of oxalates, while IC-0647414, IC-0647411 and IIHR-B-AM237_5_14_3 depicted lowest accumulation of nitrates. High heritability and genetic advance estimates for studied traits indicated the scope of selective breeding in improvement of vegetable amaranth. Both synergistic and antagonistic trait associations were recorded between antioxidant and antinutrient traits.

Conclusion Our study revealed substantial seasonal and genetic variations for antioxidant, bioactive and antinutritional traits in leafy amaranth germplasm, which is crucial for escalating crop breeding programmes. Results of genotype × environment interactions demonstrated the need of multi-environment, multi-year stability analysis for robust selection of multi-trait genotypes. The multivariate insights paved the way to breed amaranth cultivars with high nutritional quality and wider adaptability.

Keywords Vegetable amaranth, G × E interaction, Multivariate analysis, Bioactive compounds, Anti-nutrients, Antioxidants, Biofortified breeding

Background

The challenges of food and nutritional security are becoming severe in face of climate catastrophe [1, 2]. Accelerating targeted breeding programmes, search for alternative food sources, exploration of genetic potential of underutilized crops, adopting advanced genomics tools, and systematic characterization of local germplasm, are need of the hour to tackle the issues of food and nutritional security in sustainable manner [1, 3–6]. In line with this, tapping of neglected and underutilized vegetable crops germplasm is a vital strategy to enhance the dietary diversity and diversifying traditional food systems [7, 8]. Vegetable crops play an important role in ensuring healthy diet, and are also referred as protective foods due to their rich phytochemistry [8–10]. Among vegetable species, leafy vegetables are rich blend of mineral nutrients, vitamins, antioxidants, bioactive compounds and other phytochemicals [10–13]. Realizing the nutritional value of leafy vegetables, Indian Council of Medical Research (ICMR) has recommended a daily uptake of 100 g of leafy vegetables [14]. Hence, exploration and characterization of underexploited vegetables with climate-resilient potential and nutraceutical value is pivotal to combat the challenges of worldwide hunger and malnutrition [11, 15–17]. Among the group of leafy vegetables, vegetable *Amaranthus* spp. offers an excellent opportunity to tackle the issues of food and nutritional security [11, 17, 18].

Amaranthus is a complex genus comprising nearly 70–80 species which are utilized as fodder, pseudocereal, leafy vegetables, weedy types, and ornamental types [11, 19, 20]. Amaranth is a suitable candidate for cropping systems due to short life cycle, and C_4 photosynthetic system makes it to supersedes staple crops like maize and wheat under poor soils [21, 22]. The leafy amaranth species have nutritional power comparable or better than lettuce and spinach [23, 24]. The commonly grown leafy-type *Amaranthus* species include *tricolor*, *viridis*, *dubius*, *lividus*, *blitum*, and *spinosus* [11]. Global geographical variations do exist in their utilization, like in Western African region, *blitum*, *hybridus*, *cruentus*, and *dubius* are most common species. While, *Amaranthus tricolor* is most prevalent species in India and China [11, 25]. Similarly, in South America, the most common species is *Amaranthus caudatus*, and in South East Asia, *Amaranthus hybridus* is becoming popular as both grain and vegetable type [11, 25]. Majority of the amaranth germplasm grown by Indian farmers as leafy vegetable belongs to *Amaranthus tricolor* [11]. This traditional germplasm mainly consists of local landraces exhibiting high phenotypic plasticity, where substantial variations are present in terms of colour of leaf, pigmentation of stem tissues, plant type, and inflorescence colour [26, 27]. The vegetable amaranth species are considered as abundant source of bioactive and nutraceutical compounds (betalains, carotenoids, ascorbic acids, phenolics, and anthocyanins) with high antioxidant activity [11, 23, 24, 28]. These compounds impart antioxidant, anti-inflammatory, anti-diabetic, anticancer, antimicrobial, hepatoprotective, and anti-cholesterolemic activities [24, 26, 29, 30]. The protection of human neuroblastoma cells against glycation end-products has been reported by using extracts of *Amaranthus* species, *tricolor* and *lividus* [31]. These pharmacological, therapeutic and medicinal properties trigger the true assessment of local germplasm of leafy amaranths to identify suitable parental lines for biofortification breeding and functional food industry. Additionally, wide range of variations have been recorded in leafy

amaranth species (*Amaranthus tricolor* and *Amaranthus lividus*) for various antioxidant and bioactive compounds [32]. The antioxidant and phytochemical content may also vary with leaf colour pigment, where in general, the genotypes with deep red colour exhibits high antioxidant potential [32, 33]. But these variations are also influenced by genotype, environmental factors, edaphic factors, and agronomic practices [12, 34–36].

Deciphering genetic variations and environmental influence on nutritional composition of bioactive and antioxidant compounds, variations in concentration of antinutrients across the seasons in a diverse panel of vegetable amaranth germplasm is pivotal in a fight with hunger and malnutrition. High phenotypic plasticity, tremendous diversity and climate change are main challenge for taxonomic characterization of amaranth germplasm [37]. Sufficient agro-morphological and biochemical diversity is present in the local germplasm of vegetable amaranth species grown in India. However, efforts on systematic characterization and exploring true genetic potential of these landraces are limited in India. The genetic and multivariate analysis is central in analysis of genetic variation and identification of parental lines for heterosis breeding [37]. Therefore, the present study was prudently undertaken for genetic and multivariate analysis of diverse panel of amaranths germplasm to underscore their antioxidant, bioactive and functional food potential to comply with United Nations sustainable development goals of zero hunger (SDG2) and climate action (SDG13). Season dependent shifts in antioxidant activity, bioactive compounds and antinutritional factors were investigated in the studied germplasm by analysing G × E interactions. Consequently, the study aimed to facilitate the selection of promising parents for escalating vegetable amaranth breeding to develop climate resilient cultivars with high nutritional quality.

Materials and methods

Study location

The field experiments of present study were conducted across winter (2024–2025) and summer (2025) seasons at the research farm of ICAR– CHES (Central Horticultural Experimental Station), Bhubaneswar, Odisha, India. The research farm is located at 20°15' N latitude and 85°40′ E longitude, which is at 35 m above mean sea level. The climate of study location is warm and humid with an average annual rainfall of about 1500 mm and 75–80% humidity [22]. Hot summers prevails during March to June followed by a long rainy season from mid-June to September. Winters are mild from November to January [22]. Soil of research farm is sandy loam soil with an acidic pH of 4.8 to 5.2 and a poor organic carbon content of 0.2% [22, 38]. The Agro-meteorological weather data during the cropping seasons is depicted in the supplementary file (Table S1 and Table S2).

Experimental design and plant materials

In the present study, a diverse panel of 57 leafy amaranth germplasm lines, developed through individual plant selection and selfing, including three check cultivars, Pusa Kiran, Arka Neelachal Bainishi and Arka Neelachal Ruchitha, were used to investigate the seasonal variations and season × genotype interactions influencing antioxidant, bioactive and antinutritional traits (Table S3). All the plant material used in the present study was provided by the research centre itself, that is Central Horticultural Experiment Station (ICAR-Indian Institute of Horticultural Research), Bhubaneswar, Odisha, India. The plant material is maintained and conserved at the centre for the past five years and belongs to two cultivated species, *Amaranthus tricolor* and *Amaranthus blitum.* The field trials were conducted by following randomized augment block design (RABD) with six blocks and 12 entries per block. The three standard check cultivars were replicated six times.

Estimation of antioxidant, biochemical and antinutritional traits

The studied traits were classified into biochemical, antioxidant, and anti-nutritional categories based on their functional roles in plant metabolism and human nutrition (Table 1).

Determination of total chlorophyll (SPAD value)

Chlorophyll content was assessed using a Soil Plant Analysis Development (SPAD) meter, which measures the ratio of transmitted radiation at two wavelengths: red light, which is largely absorbed by chlorophyll and near infrared light, which is not. In the present study we used atLEAF CHL BLUE Chlorophyll meter for recording the SPAD value. For data collection, five healthy leaves were selected randomly and tagged from each genotype, and the mean SPAD value was calculated.

Determination of antioxidant activity

To determine the antioxidant activity of studied leafy-type amaranth germplasm, CUPRAC assay (Cupric ion reducing antioxidant capacity), FRAP assay (Ferric reducing ability of plasma), and DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical-scavenging assays were performed.

CUPRAC assay

To determine the antioxidant capacity based on CUPRAC assay (μmol Trolox g^{-1}), the protocol of Apak et al. [39] was used with minor modifications. A sample of 5 g from each genotype was homogenized in 15 ml of 100% ethanol using a sterilized mortar pestle and this extract was

Table 1 Classification of measured biochemical, antioxidant, and anti-nutritional traits under study

Trait	Abbreviation	Category
Total chlorophyll	TC	Biochemical
Total carotenoids content	TCC	Biochemical
β-carotene	βC	Biochemical
Protein content	Protein	Biochemical
Total phenolic content	TPC	Biochemical
Vitamin C (total ascorbic acid content)	VITC	Biochemical
Total anthocyanin content	TAC	Biochemical
2,2-diphenyl-1-picrylhydrazyl (radical scavenging activity) assay	DPPH	Antioxidant
Ferric reducing antioxidant power or ferric reducing ability of plasma assay	FRAP	Antioxidant
Cupric Reducing Antioxidant Capacity assay	CUPRAC	Antioxidant
Oxalate content	Oxalate	Anti-nutritional
Nitrate content	Nitrate	Anti-nutritional

centrifuged at 10,000 rpm for a period of 15 min at 4 °C. The supernatant was stored at -20 °C. Thereafter, 100 µl of sample extract was blended with 4 ml of CUPRAC reagent. CUPRAC reagent contained 1 ml of neocuproine, 1 ml of ammonium acetate, 1 ml of $CuCl_2$ and 1 ml of distilled water with a pH 7.4. The absorbance was noted at a wavelength of 450 nm using a double beam Shimadzu UV-3600i plus spectrophotometer (Tokyo, Japan). The CUPRAC estimation was performed in triplicate for each of the leafy-type amaranth germplasm line.

FRAP assay

FRAP assay determines the ability to reduce ferric iron (Fe^{3+}) to the blue-colored ferrous (Fe^{2+}) form, that indicates the antioxidant power through a colour change. To determine the antioxidant potential of leafy-type amaranth germplasm under study based on FRAP assay (µmol Trolox g^{-1}), the procedure demonstrated by Benzie and Strain [40] was adopted with minor modifications. The FRAP reagent was prepared by mixing the acetate buffer (300 mM, pH 3.6), 10 mM TPTZ solution made in 40 mM HCl, and 20 mM FeCl3 at the ratio of 10:1:1 (*v*/*v*/*v*). Thereafter 100 µL of sample was thoroughly mixed with this FRAP reagent (3.0 ml). After 30 min, the absorbance was noted at 593 nm in double beam Shimadzu UV-3600i plus spectrophotometer (Tokyo, Japan). The FRAP assay for each of the sample was performed in triplicate.

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The antioxidant potential based on DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of amaranth leaves was determined as per Brand-William et al. [41] and Ghosh et al. [42] with minor modifications. For each of the leafy-type amaranth germplasm line, a sample of 2 g was mixed in 25 ml ethanol followed by overnight extraction in dark at room temperature. Thereafter, 1 ml of DPPH (80 µg/ml of ethanol) was added into a 0.2 ml of eluted extract. The blank sample was maintained by taking 0.2 ml of distilled water and 1 ml of DPPH solution. Both of these were kept for 30 min at room temperature. The absorbance was noted at 517 nm in double beam Shimadzu UV-3600i plus spectrophotometer (Tokyo, Japan) and initially spectrophotometer reading was set to zero using ethanol. The percentage of inhibition was calculated against blank as per following equation.

$$Percentage\ inhibition\ of\ DPPH = (Ablank - Asample)/Ablank \times 100,\ where\ Ablank \text{ is absorbance of blank, and } Asample \text{ is absorbance of sample.}$$

Estimation of total phenolic content (TPC)

To estimate the total phenolic content (TPC) (mg gallic acid 100 g^{-1} FW) in vegetable amaranth germplasm collections under study, the Folin– Ciocalteu method [43, 44] with minor modifications was employed. The extract preparation for estimation of TPC in leafy-typeamaranth, a sample of 5 g of leaves was homogenized in 15 ml of 80% ethanol using the mortar pestle. The centrifuging of aqueous extract was done at 10,000 rpm for 20 min at 4 °C and supernatant was stored at -20 °C. After that, 0.50 ml of extract was mixed with 2.5 ml of 1:10 diluted Folin–Ciocalteu reagent. The mixture was neutralized with 2 ml of 20% solution of sodium carbonate (Na_2CO_3). Then reaction mixture was incubated at room temperature for 30 min. The absorbance of the final blue coloured supernatant was noted at 650 nm using a double beam Shimadzu UV-3600i plus spectrophotometer (Tokyo, Japan). The gallic acid was used for calibration purpose.

$$TPC\ (mg\ gallic\ acid\ 100\ g^{-1}FW) = [(Absorbance\ 650\ nm + 0.022) \times\ volume \times\ dilution \times 10]/0.016 \times\ sample\ weight$$

Estimation of total ascorbic acid content

To determine the vitamin C (total ascorbic acid content) (mg 100 g^{-1}) in the leaves of leafy-type amaranth germplasm under study, the colorimetric method described by Ranganna [45] was employed by using a 2, 6-dichlorophenol indo-phenol solution (2,6-DCPIP) (dye) that is decolourized by vitamin C in the sample extracts. The standard of ascorbic acid was prepared by taking 100 mg of L-ascorbic acid and it was dissolved in 3% HPO_3 (metaphosphoric acid). The final volume was made up to 100 ml with 3% metaphosphoric acid and further it was diluted by taking 10 ml of 1 mg ml^{-1} ascorbic acid. To prepare the dye solution 50 mg of the sodium salt of 2,6-dichlorophenol indo-phenol dye was dissolved in 150 ml of hot glass distilled water containing 42 mg of $NaHCO_3$ (sodium bicarbonate). Then the final volume was made up to 200 ml and dye was stored in dark bottle under refrigerate conditions. The dye was further

standardized for each day. The dye factor was calculated as, Dye factor = 0.5/titer value. The 5 g of homogenized sample of vegetable amaranth leaves of each germplasm line was extracted with 4% oxalic acid. The volume was made up to 100 ml followed by centrifugation. Then 10 ml of 4% oxalic acid was added to 5 ml of supernatant that was pipette out. The titration was done against 2, 6-dichlorophenol indo-phenol dye and titre value was noted.

$$\text{Total ascorbic acid}\left(\frac{\text{mg}}{100\text{g}}\right) = \frac{titarte\ value \times dye\ factor \times sample\ volume\ made\ up \times 100}{titrate\ volume \times weight\ of\ sample}$$

Quantification of total carotenoid content (TCC)

The total carotenoid content of leafy-type amaranth germplasm was determined following the method of Panpraneechaeron et al. [46] with slight modifications. For estimation of TCC, 0.2 g of dry sample was taken for n-hexane extraction using an ultrasonic water bath (WUC-A03H, Witeg Labortechnik GmbH, Germany). The extracted solutions were filtered through Whatman No. 1 filter paper (#144150, Maidstone, England) and then the volume was made up to 25 ml with hexane. The absorbance of the resulting solution was recorded at 446 nm using a double beam Shimadzu UV-3600i plus spectrophotometer (Tokyo, Japan). The total carotene content (TCC) was calculated using the formula: TCC = 25 × 383× (As – Ab)/100 W.

where A_s is the absorbance of the sample, Ab is the absorbance of the blank, and W is the weight of the sample in grams. TCC was expressed as mg/kg of dry powder.

Determination of β-carotene content

The β-carotene analysis was performed as per the method described in AOAC [47]. To determine the β-carotene content in leafy-type amaranth germplasm, dispersed the 10 g of sample in 50 ml of water-saturated n-butanol to obtain a homogeneous suspension followed by gentle shaking, and allowed it to stand for 16 h at room temperature in the dark. Then it was mixed again and filtered the mixture through Whatman No. 1 filter paper. Then, filtrate volume was made up to 100 ml. The absorbance of the clear filtrate was noted at 440 nm using saturated n-butanol as the blank in a double beam Shimadzu UV-3600i plus spectrophotometer (Tokyo, Japan). The β-carotene content was determined using the formula: β-carotene content (ppm) = 0.0105 + 23.5366×A. The value was converted into μg $100ml^{-1}$.

Quantification of total anthocyanin content

To determine the anthocyanin content, pH-differential method using two buffer systems [potassium chloride buffer, pH 1 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M)] was used as per Lee et al. [48] and Ghosh et al. [42] with slight alterations. A double beam Shimadzu UV-3600i plus spectrophotometer (Tokyo, Japan) was used to record the absorbance at 510 nm and 700 nm. The anthocyanin content was expressed as cyanidin 3-glucoside (Cyd 3-glu) per kg FW, using an extinction coefficient (e) of 26,900 L $cm^{-1}mol^{-1}$ and a molecular weight of 449.2 g mol^{-1}.

Absorbance (A) = (A510 nm - A700 nm) pH1 - (A510 nm - A700 nm) pH4.5.

Monomeric anthocyanin content (mg L^{-1}) = A×MW×DF×1000/e×1.

Where, A = absorbance, MW = molecular weight (449.2), DF = dilution factor, e = molar absorptivity (26,900). The final value was converted into mg $100ml^{-1}$.

Protein (%) estimation

The proximate analysis system estimated protein content by measuring total nitrogen (N), as described by Sahrawat et al. [49], and multiplying it by a conversion factor. A standard factor of 6.25 is used to convert total nitrogen to total protein.

Determination of oxalates

To determine the anti-nutrient factor 'oxalate' in leafy-type amaranth, about 1 g of the ground sample was weighed into a conical flask, and 75 ml of 3 N H_2SO_4 was added to it. The mixture was allowed to stand for 30 min, after which it was filtered using Whatman No. 1 filter paper. A 25 ml aliquot of the filtrate was transferred into a beaker and 2 drops of methyl red indicator were added. The solution was then heated to boiling and titrated hot against 0.05 M $KMnO_4$ until a faint pink color persisted for at least 30 s. Oxalate content in the sample was calculated using the relation that 1 ml of 0.05 M $KMnO_4$ = 2.2 mg oxalate, and the oxalate content (mg $100\ g^{-1}$) was computed using:

$$\text{Oxalate content}\left(\text{mg } 100\text{g}^{-1}\right) = Titre\ value \times 2.2 \times\ DF \times\ 100/Weight,\ where:\ mg\ is\ mass\ equivalent\ of\ oxalate\ for\ 1\ ml\ of\ 0.05\ M\ KMnO4,\ DF\ is\ dilution\ factor,\ W\ is\ weight\ of\ sample\ in\ grams$$

Determination of nitrates

To determine the nitrate content (mg 100 g^{-1}) in leafy-type amaranth germplasm collections under study, the calorimetric method utilizing salicylic acid was employed [50]. For the nitrate estimation, a sample of about 500 mg was weighed and kept in a test tube for analysis. In the test tube carrying sample, 10 mL of hot (90–95 °C) distilled water was poured. Thereafter, the tubed were closed and placed in a hot water bath at 80 °C for a time period of 30 min with intermittent shaking. After hot water bath the samples were allowed to cool at room temperature and centrifugation was done at 4500 rpm. Then supernatant was taken out and weighed to estimate the total volume of extract. To remove the chlorophyll content in the leaf extract 0.5 g of $MgCO_3$ was added to the supernatant

and again centrifugation was done. Then supernatant consisting the nitrate extract was treated with 2 N NaOH followed by a combination of Salicylic acid and H_2SO_4 in a ratio of 1:20. Sodium nitrate was utilized in the preparations of standards. The absorbance was noted at 410 nm using a double beam Shimadzu UV-3600i plus spectrophotometer (Tokyo, Japan).

Statistical analysis

Data pertaining to antioxidant and bioactive compounds, antinutrients in the studied germplasm during both winter and summer seasons was subjected to statistical analysis for ANOVA (analysis of variance), mean performance, and determination of genetic components of variance using augmentedRCBD, a R statistical package as per Arvind et al. [51]. The three commercial checks were considered as fixed effects, while studied germplasm lines were considered as random effects [52]. Hence a mixed linear model was employed where seasons were treated as fixed effects, while G × E interactions were considered as random effects. The following mixed model was used:

$$Y_{ijk} = \mu + E_i + B_{j(i)} + G_k + C_k + GE_{(ik)} + \epsilon_{ijk},$$

where Y_{ijk} is observed response, μ is overall mean value, E_i is environment effect (fixed), $B_{j(i)}$ is block within environment (random effect), G_k is genotype effect (random), C_k is checks effect (fixed), $GE_{(ik)}$ is G × E interactions effect (random) and ϵ_{ijk} is residual error.

As in the augmented design, the test entries are unreplicated, the error variance is estimated based on the replicated check cultivars. The linear mixed models, where blocks are treated as random effects and checks as fixed effects, were used [52].

The GCV (genotypic coefficient of variation) and PCV (phenotypic coefficient of variation) estimates were categorized as low (0–10%), medium (10–20%) and high (> 20%) [53, 54].

$$\text{PCV}\,(\%) = \frac{\sqrt{\sigma^2 \text{p}}}{\bar{\text{x}}} \times 100 \text{ and } \text{GCV}\,(\%) = \frac{\sqrt{\sigma^2 \text{g}}}{\bar{\text{x}}} \times 100$$

Heritability was estimated based on variance components obtained using a mixed linear model, where genotypes were treated as random effects [52]. Low (0–30%), medium (30–60%) and high (> 60%) estimates of heritability were determined [55, 56].

Multivariate analysis

R studio, a statistical software carrying multiple packages was used (R studio 2020, https://rstudio.com) was employed for graphical representation of data. Mahalanobis distance (D^2) and Euclidian distance based statistical analysis was performed for discrimination of studied germplasm. Cluster analysis was employed utilizing silhouette technique and NbClust statistical package in R studio [57, 58]. Inter-and intra-cluster distances were determined using 'clv' package of R studio. The principal component analysis (PCA) was performed using R statistical packages 'FactoMineR and 'factoextra' [53, 56]. Major principal components contributing total variations were determined based on eigenvalues of more than 1 [37]. The trait associations based on Pearson correlation coefficient were computed using R statistical package 'corrplot' [59].

Results

Variance analysis (ANOVA) for antioxidant, bioactive, and anti-nutrient traits

The analysis of variance (ANOVA) for anti-nutrients, biochemical and antioxidant compounds during the winter season of 2024–2025 and summer season of 2025 in the leafy-type amaranth germplasm under study revealed that the treatment effects were significant for all the traits except for total anthocyanin content (TAC) during summer season of 2025 (Table 2, Supplementary Table S4, Table S5). The results indicated presence of substantial variations for all the studied biochemical, antioxidant and antinutrient traits in the studied leafy-type amaranth germplasm. The ANOVA analysis further indicated significant variations for all the biochemical and antioxidant traits in the check cultivars (Pusa Kiran, Arka Neelachal Bainishi and Arka Neelachal Ruchita) during both winter and summer seasons. The variance analysis further revealed that block effects were non-significant for majority of the traits except for vitamin C, DPPH, β-carotene content and oxalate content. The pooled ANOVA analysis indicated significant genotypic effects for all the biochemical and antioxidant compounds (Table 2). The season × genotype (G × E) interaction effects were also highly significant for majority of the biochemical traits except for vitamin C and nitrate content (Table 1).

Seasonal shifts in biochemicals, antioxidant compounds, and anti-nutrients

The phytochemistry of biochemical, antioxidants and anti-nutrients is complex in diverse collections of leafy-type amaranth. The profiling of leafy-type amaranth germplasm under study revealed significant variations for biochemical and antioxidant compounds (CUPRAC, FRAP, DPPH) during both winter season (2024–2025) and summer season of 2025 (Supplementary Table S6, Table S7, Fig. 1). Wide range of compositional and seasonal variations were also recorded for antinutrients, oxalates and nitrates (Table S6, Table S7, Fig. 1). The SPAD value for total chlorophyll (TC) varied from 38.89 (IIHR-B-AM285) to 74.30 (IC-0647421). The highest TC

Table 2 Pooled ANOVA depicting Season × Genotype (G × E) interaction for biochemical, anti-nutrients, and antioxidant compounds

Source	Season	Genotype	Season × Genotype (G × E)
Df	1	56	56
TC	27.209***	2358.342***	99.572***
CUPRAC	3341.79***	1135.99***	760.94***
FRAP	2491.23***	3980.54***	944.25***
DPPH	2463.9***	179473.9***	3219.3***
TPC	492.37***	5129.18***	140.77***
VITC	127.386***	534.428***	56.899
TCC	21,836,530***	636,903,707***	433,003,848***
βC	18,365***	6,661,082***	13,448***
TAC	47941.4***	8321.9***	8691.9***
Protein	21,928***	37,732***	10,500***
Oxalate	16,062***	5,372,597***	281,178***
Nitrate	0.010	1.9988e + 10***	0.001

TC Total chlorophyll (SPAD value), *CUPRAC* (µmol Trolox/g) Cupric Reducing Antioxidant Capacity, *FRAP* (µmol Trolox/g) ferric reducing ability of plasma, *DPPH* (%) 2,2-diphenyl-1-picrylhydrazyl, *TPC* (mg of gallic acid/100 g FW) Total phenolic content, *VITC* (mg/100 g) Vitamin C, *TCC* (mg/100 g) Total carotenoid content, βC (µg 100ml^{-1}), β-carotene content, *TAC* (mg/100 ml) Total anthocyanin content, *Protein* (%), Oxalate (mg/100 g), Nitrate (mg/100 g)

ns $p > 0.05$; *** $p <= 0.001$

value was recorded in IC-0647421, followed by IIHR-B-AM291 and IIHR-B-AM43. The cupric reducing antioxidant capacity (CUPRAC) (µmol Trolox g^{-1}) exhibited considerable variations, ranging from 17.38 (IIHR-B-AM296) to 28.89 (Pusa Kiran). The highest CUPRAC value was observed in the Pusa Kiran that is at par with IC-0647408 and IIHR-B-AM301. CUPRAC estimation suggested the potential role of these lines in enhancing antioxidant.

potential of amaranth. Marked variations were recorded for FRAP (Ferric Reducing Ability of Plasma) (µmol Trolox g^{-1}) based antioxidant power in the studied germplasm, ranging from 20.41 (IIHR-B-AM26) to 32.23 (IIHR-B-AM247). The highest FRAP activity was recorded in IIHR-B-AM247, followed by IIHR-B-AM245 and IC-0647405. The DPPH radical scavenging activity (%) exhibited a broad variation, ranging from 56.67 (IC-0647409) to 88.45 (IC-0647408).

The highest DPPH based antioxidant activity was depicted in IC-0647408, followed by IIHR-B-AM202 and IIHR-B-AM158. Substantial variations were found for total phenolic content (mg of gallic acid 100 g^{-1} fresh weight) ranging from 433 (IC-0647409) to 923.67 (Pusa Kiran). Pusa Kiran, followed by IIHR_B_AM231_13_2_15 and IIHR-B-AM230 exhibited high total phenolic content. Similarly, marked variations were also recorded for vitamin C content (mg 100 g^{-1}) that varied from 42 (IC-0647417 and IC-0647405) to 64 (IHR_B_AM248_3_2). The highest vitamin C content was recorded in IHR_B_AM248_3_2, followed by IC-0647419 and Pusa Kiran. Similarly wide range of variations were found for total carotenoids (mg 100 g^{-1}) among the tested germplasm ranging from 4.69 (IIHR-B-AM279) to 12 (IIHR-B-AM247). The genotype IIHR-B-AM247, followed by IIHR-B-AM239 and IIHR-B-AM202 exhibited maximum total carotenoid content. Likewise, substantial variations were recorded for β-carotene content (µg 100 ml^{-1}) ranging from 490 (IIHR-B-AM237_5_14_3) to 2845 (IC-0647405). The highest β-carotene content was recorded in IC-0647405, followed by Pusa Kiran and IIHR-B-AM253. Total anthocyanin content (mg 100 ml^{-1}) varied from 2.13 (Pusa Kiran) to 6.23 (IIHR-B-AM279). The genotype IIHR-B-AM279, followed by IIHR-B-AM230 and IIHR_B_AM231_13_2_15 depicted highest anthocyanin content. Remarkable variations were recorded from protein concentration (%) as well ranging from 14.05 (IC-0647420) to 29.57 (IIHR-B-AM301). The highest protein content was recorded in IIHR-B-AM301, followed by IC-0647407 and IIHR_B_AM237_5_14_3. Wide range of variations were exhibited in the studied leafy-type amaranth germplasm for anti-nutrients (mg 100 g^{-1}), where oxalate and nitrate contents varied from 467 (IC-0647419) to 1580 (IIHR-B-AM204) and 28.32 (IC-0647414) to 196.89 (IC-0647421), respectively. The lowest oxalate content was recorded in IC-0647419, followed by Pusa Kiran and IHR_B_AM248_3_2. The lowest nitrate concentration was recorded in IC-0647414, followed by IC-0647411 and IIHR-B-AM237_5_14_3. These germplasm collections can be utilized in breeding safe amaranth cultivars with low antinutritional factors.

Substantial variations were recorded for summer season as well in the studied leafy-type amaranth germplasm (supplementary Table S7; Fig. 1), where total chlorophyll (TC) content varied from 37.13 (IIHR-B-AM285) to 67.3 (IC-0647421) SPAD value. The antioxidant potential measured by CUPRAC and FRAP content (µmol Trolox g^{-1}) varied from 12.11 (IIHR-B-AM290) to 18.67 (IC-0647423), and 17.88 (Arka Neelachal Bainishi) to 28.15 (IC-0647408), respectively. While DPPH radical scavenging activity (%) ranged between 50.23 (IC-0647418) and 84.47 (IC-0647414). The total phenolic content (TPC) (mg of gallic acid 100 g^{-1} fresh weight) depicted wide range of variations, and varied from 280 (IC-0647409) to 859 (Pusa Kiran). The vitamin C content (mg 100 g^{-1}) varied from 24 (IC-0647405) to 60 (IHR_B_AM248_3_2). The carotenoid content (TCC) (mg 100 g^{-1}) varied from 2.554 (IIHR-B-AM279) to 5.362 (Pusa Kiran), whereas β-carotene content (µg 100ml^{-1}) varied from 379 (IIHR-B-AM237_5_14_3) to 2597 (IC-0647405). Tremendous variations were recorded for the total anthocyanin content (TAC) (mg 100ml^{-1}), and ranged from 0.303 (IIHR_B_AM231_13_2_15) to 1.132 (IIHR-B-AM285). Protein concentration (%) among the studied germplasm varied from 14.70 (IIHR-B-AM19) to 25.94 (IC-0647407). Regarding anti-nutritional factors, the oxalate content

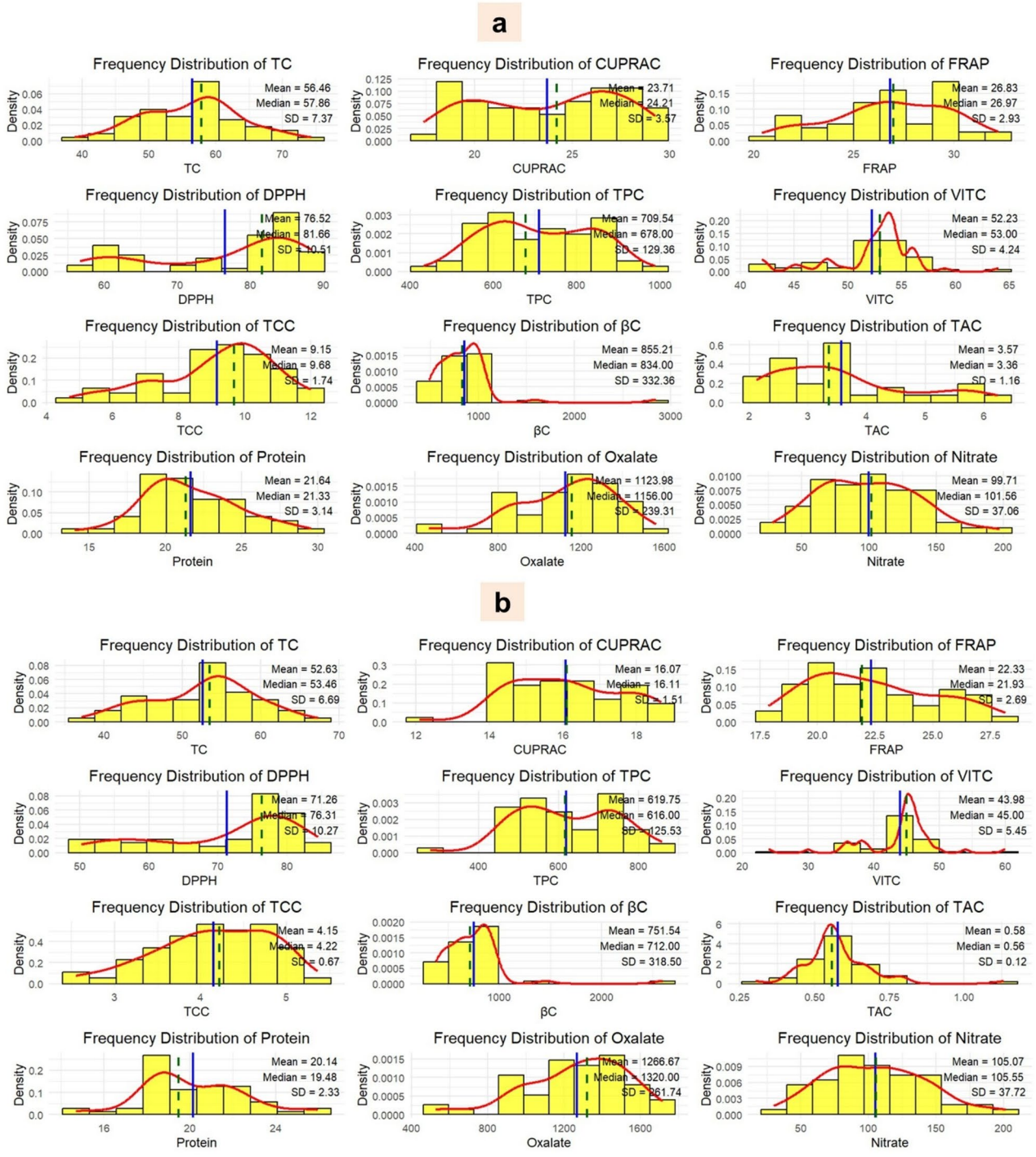


Fig. 1 **a** Frequency distribution of biochemical and antioxidant compounds, anti-nutrients, across the vegetable amaranth germplasm under study during winter season of 2024–2025 (**b**) Frequency distribution of biochemical and antioxidant compounds, anti-nutrients, across the vegetable amaranth germplasm under study during summer season of 2025. TC: total chlorophyll, TPC: total phenolic content, VITC: vitamin C, βC: β-carotene, TAC: total anthocyanin content, TCC: total carotenoid content

(mg 100 g^{-1}) varied from 528 (Pusa Kiran) to 1716 (IIHR-B-AM292), while nitrate content (mg 100 g^{-1}) ranged from 30.15 (IC-0647414) to 201.25 (IC-0647421). The germplasm line IC-0647421 exhibited highest total chlorophyll (TC) content followed by IIHR-B-AM43 and IC-0647423. The highest antioxidant activity based on CUPRAC content was recorded in IIHR-B-AM19 and IC-0647423 followed by IC-0647405 and IC-0647424.

Likewise, IC-0647408 depicted highest FRAP content followed by IC-0647405 and IIHR-B-AM268. Highest DPPH antioxidant activity (%) was recorded in IC-0647414 followed by Pusa Kiran and IC-0647408. Highest total phenol content (TPC) (mg of gallic acid 100 g^{-1} fresh weight) was reported in the check cultivar, Pusa Kiran followed by germplasm lines IIHR_B_AM231_13_2_15 and IIHR-B-AM230. The highest Vitamin C content (mg 100 g^{-1}) was found in IHR_B_AM248_3_2 followed by IC-0647419 and Pusa Kiran. Further, the check cultivar, Pusa Kiran exhibited highest content of total carotenoids (TCC) followed by IC-0647411 and IIHR-B-AM02. The Indigenous collection, IC-0647405 exhibited highest β-carotene (µg 100ml^{-1}) content followed by Pusa Kiran and IC-0647411. The highest total anthocyanin content (TAC) (mg 100ml^{-1}) was found in IIHR-B-AM285 followed by Pusa Kiran and IIHR-B-AM296. The highest protein percentage (%) was found in IC-0647407 followed by in IC-0647409 and IIHR-B-AM301. The check cultivar, Pusa Kiran exhibited lowest content of oxalates, besides germplasm lines IC-0647419 and IHR_B_AM248_3_2. Then, the leafy-type amaranth germplasm lines, IC-0647414, IC-0647411 and IIHR-B-AM237_5_14_3 depicted lowest accumulation of anti-nutrient, nitrates.

Genetic parameters of variability

The appraisal of genetic components of variance for biochemical, antioxidant compounds and anti-nutrients revealed significant variations among the studied leafy-type amaranth germplasm across the seasons (Table S8 and Table S9). The GCV and PCV analysis indicated that, the PCV estimates were slightly higher to GCV estimates for all the biochemical, antioxidant and anti-nutrient traits under study across the seasons. These findings indicated that genetic factors predominantly influenced the trait expression than environmental factors during respective seasons. In the winter season of 2024–2025, the medium estimates of GCV and PCV were recorded for majority of the traits except for vitamin C (low GCV, PCV), β-carotene, oxalate and nitrate content (high GCV, PCV) (Table S8). During the summer season, for majority of the traits, the medium estimates of GCV and PCV were recorded. For CUPRAC and total anthocyanin content (TAC) low estimates of GCV were observed, while for β-carotene and nitrate content, the high estimates of GCV were recorded (Table S9).

Additionally, the high (> 60%) estimates of broad sense heritability (h^2b) were documented for all the traits during both winter and summer cropping seasons except for low heritability of total anthocyanin content (TAC) (18.45%) during summer season. Similarly, for majority of the traits, the high (> 20%) estimates of genetic advance as percent of mean (GAM) were recorded during both the seasons, except for vitamin C (medium GAM, 11.18%) during winter season, and for CUPRAC (medium GAM, 18.87%) and TAC (low GAM, 7.83%) during summer season. Overall, the results of present investigation revealed the traits viz. CUPRAC, FRAP, DPPH, total chlorophyll, phenolic content, total carotenoids, β-carotene, protein, anthocyanins, oxalate and nitrate, showed very close difference between the PCV with corresponding GCV, indicating the least impact of the environmental factors in expression of the traits. Therefore, such traits with least difference between PCV and GCV should be selected strongly on the basis of phenotypic expression. Similarly, the traits viz. CUPRAC, FRAP, DPPH, total chlorophyll, phenolic content, total carotenoids, β-carotene, protein, oxalate and nitrate, exhibiting high broad sense heritability (> 60%) coupled high GAM may be selected directly towards development of superior variety in future crop improvement program in leafy-type amaranths.

Multivariate analysis

The multivariate analysis involving principal component analysis (PCA) and Mahalanobis D^2 cluster analysis depicted substantial variations for various biochemical and antioxidant compounds, and anti-nutrient traits, among the diverse collections of 57 leafy-type *Principal component analysis*.

The results pertaining to PCA analysis for biochemical and antioxidant compounds, and anti-nutrients indicated substantial genetic variations among the studied leafy-type amaranth germplasm (Tables 3 and 4; Figs. 2 and 3, Supplementary Table S10, Table S11). The PCA results based on winter season data analysis for biochemical and antioxidant compounds indicated that the first four (PC1 to PC4) principal components are effective for interpretation of PCA results based on eigen values of more than 1 viz. 3.442, 1.491, 1.322, and 1.172, respectively (Table 3). These four components explained over 61% of total variations. The percent variations explained by first four principal components in the first season was 28.684%, 12.421%, 11.015%, and 9.763%, respectively. The precent contribution of variables in each principal component is depicted in supplementary Table S10 and Fig. 2. The results indicated that in the first principal component (PC1), total carotenoids (19.33%) and anthocyanin content (14.89%) contributed maximum in total variations (Table S10 and Fig. 2), while in PC2, protein (31.73%) and nitrate content (19.56%) contributed maximum in total variations. The correlation analysis of variables with respective principal components indicated that in the PC1, total chlorophyll, CUPRAC, FRAP, DPPH and total carotenoids had positive influence, while anthocyanin content had strong negative influence (Fig. 2d). In the PC2, protein had positive influence, while anti-nutrients (oxalate and nitrate) had negative influence.

Table 3 Eigen values of principal components based on anti-nutrients, biochemical and antioxidant compounds based on data analysis for winter season 2024–2025

Prin_comp	eigenvalue	percentage of variance	cumulative percentage of variance
PC1	3.442	28.684	28.684
PC2	1.491	12.421	41.105
PC3	1.322	11.015	52.12
PC4	1.172	9.763	61.884
PC5	0.937	7.805	69.689
PC6	0.818	6.817	76.506
PC7	0.661	5.51	82.016
PC8	0.575	4.792	86.808
PC9	0.549	4.571	91.379
PC10	0.455	3.791	95.17
PC11	0.324	2.697	97.867
PC12	0.256	2.133	100

Table 4 Eigen values of principal components based on anti-nutrients, biochemical and antioxidant compounds based on data analysis for summer season 2025

Prin_comp	eigenvalue	percentage of variance	cumulative percentage of variance
PC1	1.95	16.248	16.248
PC2	1.591	13.261	29.509
PC3	1.51	12.587	42.096
PC4	1.249	10.408	52.504
PC5	1.056	8.798	61.302
PC6	1.043	8.688	69.989
PC7	0.916	7.633	77.622
PC8	0.699	5.821	83.443
PC9	0.638	5.319	88.762
PC10	0.52	4.337	93.099
PC11	0.448	3.736	96.835
PC12	0.38	3.165	100

The PCA results for biochemical and antioxidant compounds revealed that first six principal components (PC1 to PC6) were effective for interpretation of results as they had eigen value more than 1, viz. 1.95, 1.591, 1.51, 1.249, 1.056, and 1.043, respectively (Table 4). These six components (PC1 to PC6) explained over 69% of total variations. The percent variation explained by first six principal components was, 16.248%, 13.261%, 12.587%, 10.408%, 8.798%, and 8.688%, respectively (Table 4; Fig. 3). The percent contribution of variables in total variations in respective principal components is presented in supplementary Table S11 and Fig. 3. The PCA results indicated that, in the first principal component (PC1), TAC contributed maximum in total variations (20.379%) followed by DPPH (19.818%) (Table S11). Likewise, in PC2, vitamin C contributed maximum in total variations (24.438%) followed by β-carotene (23.905%). The association analysis of variables with respective principal components indicated that in the first component (PC1), total chlorophyll (TC), DPPH and oxalate had positive influence, while total anthocyanin content (TAC) had strong negative influence (Fig. 3d). In PC2, β-carotene and total carotenoid content (TCC) along with FRAP had positive influence, while vitamin C had strong negative influence (Fig. 3d).

Mahalanobis D^2–Cluster analysis based on biochemical and antioxidant traits

The Mahalanobis D^2–based hierarchical clustering based on data analysis for biochemical and antioxidant traits *via* Ward's method using Euclidean distance revealed the substantial variations among the studied leafy-type amaranth germplasm for biochemical and antioxidant traits during both winter (2024–2025) and summer (2025) cropping seasons. The cluster analysis grouped the diverse collections of leafy-type amaranth germplasm into five distinct clusters (C1 to C5), suggesting the presence of sufficient genetic variations among the leafy-type amaranth germplasm for anti-nutrients, biochemical and antioxidant compounds across the seasons (Tables 5 and 6, Table S12, Table S13, Fig. 4). The cluster analysis indicated that during both winter and summer seasons, indigenous collection 'IC-0647405' remained in separate cluster solely in Cluster-II and Cluster-III, respectively. The cluster mean analysis suggested that this indigenous collection can be used as a source for high antioxidant activity (CUPRAC, FRAP), total phenolic content, total carotenoids, β-carotene content, high protein and low nitrate content (Tables 5 and 6). The maximum number of genotypes were included in Cluster-III and Cluster-II during winter and summer seasons, respectively (Fig. 4a and b). During both the seasons, IC-0647419 and IHR_B_AM248_3_2 remained together in single cluster viz. Cluster-V in winter season and Cluster-I including check cultivar Pusa Kiran during summer season (Fig. 4). These genotypes belonging to Cluster-V and Cluster-1 during winter and summer seasons, respectively, can be utilized as a genetic resource for high vitamin C, high anthocyanin and low oxalate content (Tables 5 and 6). The cluster mean analysis based on winter season data indicated that the Cluster-IV was represented by the genotypes rich in total chlorophyll, CUPRAC and good source of total carotenoids. Likewise, the Cluster-I in winter season was represented by the genotypes rich in total phenol, anthocyanins and protein content.

The cluster analysis based on summer season data revealed that genotype IIHR-B-AM285 remained separate in a single Cluster-IV and is a good source of high anthocyanins, low antinutrients and high DPPH based antioxidant activity (Fig. 4; Table 6). While in the winter season, this genotype was part of largest Cluster-III

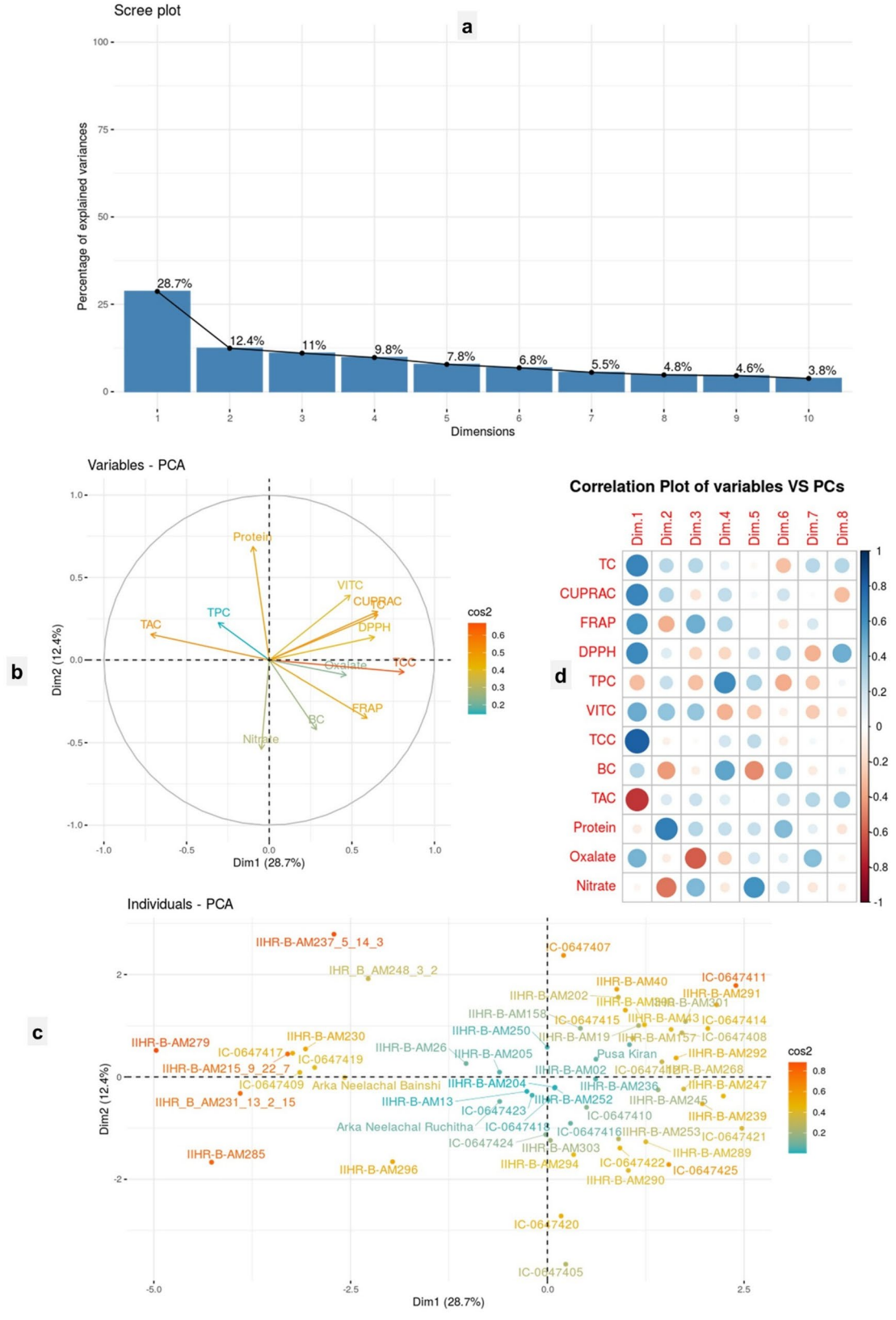


Fig. 2 (See legend on next page.)

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Fig. 2 PCA analysis based on biochemical and antioxidant compounds demonstrating genetic variations among the studied leafy-type amaranth germplasm during winter season. **a** Scree-plot depicting the contribution of first 10 principal components in total variations. The first two principal components 28.7% and 12.4% of total variation (**b**) PCA biplot depicting the contribution of individual variable in total variations across first two dimensions. The intense orange colour indicates higher contribution of variables in total variations (**c**) PCA biplot depicting the variations among studied leafy-type amaranth germplasm and plotting them across first two dimensions (**d**) Correlation between variables and principal components. In each dimension, the intense blue colour depicted strong positive association of respective variables with their principal component and intensity towards red colour indicated negative influence of variables in respective principal components

(Fig. 4). The cluster mean analysis suggested that the largest Cluster-III during the winter season is represented by the genotypes with highest DPPH activity, good source of vitamin C, total carotenoids and low nitrate content. The genetic variation represented by cluster analysis during both winter and summer cropping seasons in the studied leafy-type amaranth germplasm also supported the effect of season × genotype (G × E) interaction in influencing the compositional variations for biochemical and antioxidant compounds. The inter-cluster distance matrix (Table S12) based on biochemical and antioxidant traits for the winter season evaluation of studied leafy-type amaranth germplasm indicated highest inter-cluster distance between Cluster-II and Cluster-IV (6.18), followed closely by Cluster-II and Cluster-V (6.13), and Cluster-I and Cluster-II (5.69). these results suggested that Cluster-II was highly diverse from Cluster-I, Cluster-IV and Cluster-V. The lowest inter-cluster distance was recorded between Cluster-I and Cluster-III (2.66), indicating close similarity in their biochemical and antioxidant trait profiles. Thus, the crosses between genotypes belonging to highly divergent clusters (especially Cluster-II × Cluster-IV or Cluster-II × Cluster-V) are expected to generate greater heterosis and wider transgressive segregation for biochemical and antioxidant traits in leafy-type amaranth. The inter-cluster distance matrix based on Mahalanobis D^2 statistics for the summer season data analysis (Table S13) indicated that the highest inter-cluster distance was recorded between Cluster III and Cluster IV (D^2 = 6.16), followed by Cluster I and Cluster IV (D^2 = 5.45) and Cluster I and Cluster III (D^2 = 5.10). These results indicated that the genotypes of these respective clusters were highly diverse for biochemical and.

antioxidant compounds, and crosses between these genotypes from distinct clusters might give high heterosis and genetic gains for biochemical and antioxidant traits in leafy-type amaranth. The lowest inter-cluster distance was found between Cluster II and Cluster V (D^2 = 3.15). This indicates a close genetic relationship between these clusters, and crosses among genotypes within these groups may result in limited genetic gain.

Trait association among biochemical, anti-nutrient and antioxidant compounds

The interaction analysis among biochemical and antioxidant compounds analysed in a large set of leafy-type amaranth germplasm under study, indicated significant and non-significant positive or negative associations between different traits across the seasons (Supplementary Table S14, Table S15, Fig. 5). The correlation matrix indicated that total chlorophyll (TC) exhibited significant positive correlations with CUPRAC ($r = 0.414^{**}$), FRAP (0.433^{***}), DPPH (0.305^{*}), vitamin C (0.397^{**}), and total carotenoid content (0.467^{***}). CUPRAC correlated positively with DPPH (0.369^{**}) and total carotenoid (0.462^{***}). FRAP also exhibited significant positive association with total carotenoid (0.549^{***}) and β-carotene content (0.324^{*}). Concurrently, DPPH exhibited significant positive interaction with total carotenoid (0.463^{***}) and vitamin C (0.283^{*}). These findings indicated that total carotenoids have strong antioxidant activity based on their significant interaction with chlorophyll, CUPRAC, FRAP and DPPH, suggesting their role in free radical scavenging. Anthocyanins exhibited significant negative association with anti-nutrient, oxalate (-0.377^{**}). Protein content did not show any significant association with most biochemical traits under study indicating independence from antioxidant-related traits. The correlation matrix analysis based on summer season data for biochemical and antioxidant traits also indicated significant positive or negative interactions between studied traits (Table S27). The correlation matrix analysis indicated significant negative association of total chlorophyll (TC) with total anthocyanin content (TAC) ($r = -0.293^{**}$). Total chlorophyll had weak non-significant correlation with other antioxidant traits. The significant positive interaction was reported for FRAP content with β-carotene (βC) ($r = 0.268^{*}$) and nitrate ($r = 0.276^{*}$). Similarly, DPPH exhibited significant positive association with vitamin C ($r = 0.31^{*}$) and total carotenoid content ($r = 0.307^{*}$). Further, a strong significant negative correlation was found between total anthocyanin content (TAC) and anti-nutrient, oxalate content ($r = -0.377^{**}$).

Discussion

Climate change is posing a serious threat to food and nutritional security of billions [1]. Revitalizing the orphan crops, systematic characterization of local germplasm, delineation of genotype, environment and G × E interaction effects, promoting genomics in germplasm conservation efforts, identification of elite lines with climate resilient nature and nutri-dense potential, are the key strategies to upscale targeted breeding in a fight with hunger and malnutrition [1, 5, 11, 22, 60–63]. Tapping of

local germplasm of vegetable amaranth for antioxidant, bioactive and antinutritional traits holds promise in this context owing to its phenomenal nutraceutical, therapeutic, antioxidant and climate resilient potential [11, 37, 60–63]. Season, genotype, soil types, environmental factors, fertilizer applications, and agronomic practices have been reported to alter phenotypic, biochemical, antioxidative and physiological parameters of amaranth leaves [12, 34, 64, 65]. The pooled ANOVA results of present investigation for antioxidant, bioactive and antinutritional traits across the seasons depicted significant treatment effects for majority of the traits, suggesting the presence of substantial genetic variations among a diverse panel of 57 leafy amaranth germplasm accessions under study. The data analysis indicated significant effects of season, genotype and season × genotype (G × E) interaction for all the antioxidant and bioactive traits under study, except for nitrate and vitamin C content. Results highlighted clear temporal trends in the studied traits across the seasons. The findings of the present investigation are in agreement with the previous reports on significant influence of genotypes and genotype × environment interactions (G × E) in shaping nutritional profile of crop plants [12, 34, 64, 65]. Notably, a small number of germplasm lines under study demonstrated constant performance for antioxidant and bioactive compounds across the seasons, underpinning the response of selection in choosing parental lines with marvellous nutritional quality and adaptation to marginal conditions [12, 22, 34]. Thes results suggested strong scope of further multi-location and multi-year trials to identify the genotypes with wider adaptability and stability for nutrient rich safe cultivars. The excess of antinutrients like oxalates and nitrates in daily diet significantly alters availability of essential nutrients. Oxalates react with Ca and other minerals to from insoluble crystals which are responsible for hyperoxaluria, a risk factor for kidney stones [66]. Hence, it is imperative to understand the genetics and environmental influence on accumulation of these antinutrients in leafy vegetables. In the present study, significant G × E interaction effects were recorded for anti-nutrients (oxalate and nitrate) indicated the role of season and genotypes in accumulation of these antinutrients. These results are in line with previous reports on oxalates concentration in spinach, where nitrogen forms, seasonal changes and genetic effects have been reported to alter oxalate accumulation [66, 67]. Similarly, Kaminishi and Kita [68] reported strong seasonal variations for oxalates and nitrates concentration in spinach leaves. Moreover, the excessive root uptake of nitrates facilitates high accumulation of oxalates in leafy vegetables [69]. Additionally, the block effects were non-significant across the seasons indicating role of genetic factors in observed variation for antioxidant, bioactive and antinutritional traits. For the success of targeted crop breeding programmes related to biofortification and climate crisis, the existence of substantial genetic variation is pivotal [38, 63].

Seasonal variation analysis highlighted wide range of variations for biochemical, anti-nutrients, and antioxidant compounds among the studied leafy-type amaranth germplasm. The germplasm lines IC-0647421, IIHR-B-AM43 and IC-0647423 were found promising for total chlorophyll content. The highest antioxidant activity based on CUPRAC content was recorded in IIHR-B-AM19, IC-0647423 and IC-0647405, while IC-0647408 depicted highest FRAP content. Similarly, DPPH based radical scavenging activity was found highest in IC-0647414 and check cultivar, Pusa Kiran. The data analysis identified IHR_B_AM248_3_2, IC-0647419 and Pusa Kiran as promising parents for high vitamin C content. Further, the check cultivar, Pusa Kiran along with IC-0647411 and IIHR-B-AM02 were identified as suitable parents for high total carotenoid content. The indigenous collection, IC-0647405 exhibited highest β-carotene content along with check cultivar, Pusa Kiran. The highest total anthocyanin content (TAC) was found in IIHR-B-AM285 and Pusa Kiran. The indigenous collections, IC-0647407, IC-0647409 and IIHR-B-AM301 were found superior for protein concentration and can be effectively utilized in breeding for high protein content in amaranth leaves. The genotypic differences in nitrogen uptake, and assimilation pathways might be responsible for substantial variation among the studied germplasm for protein content. The check cultivar, Pusa Kiran exhibited lowest content of oxalates, besides germplasm lines IC-0647419 and IHR_B_AM248_3_2. Then, the leafy-type amaranth germplasm lines viz. IC-0647414, IC-0647411 and IIHR-B-AM237_5_14_3 accumulated lowest nitrates. Hence, these lines can be used as parents for breeding leafy-type amaranth cultivars with low content of anti-nutrients like nitrates. The results are in conformity with the previous findings of Sarker and Oba [32], Sarker and Oba [28], Khanam and Oba [70], and Jimenez-Aguilar and Grusak [71], who also reported wide genotypic and species variations for the bioactive and antioxidant compounds in red and green colour amaranth accessions. The species-specific variation has also been reported for TPC, FRAP, and ORAC content in amaranth leaves, where high total antioxidant activity of these biochemical was reported in the leaves of *A. hypochondriacus* as compared to *A. caudatus* [72]. The wide range of variations recorded among the studied germplasm lines for different antioxidant, bioactive and antinutritional traits could be attributed to differences in underlying metabolic pathways and their regulation [70–72]. Higher DPPH scavenging activity in selected genotypes is likely correlated with enhanced level of non-enzymatic antioxidants such as phenolics, ascorbic acid, and carotenoids, which collectively contribute

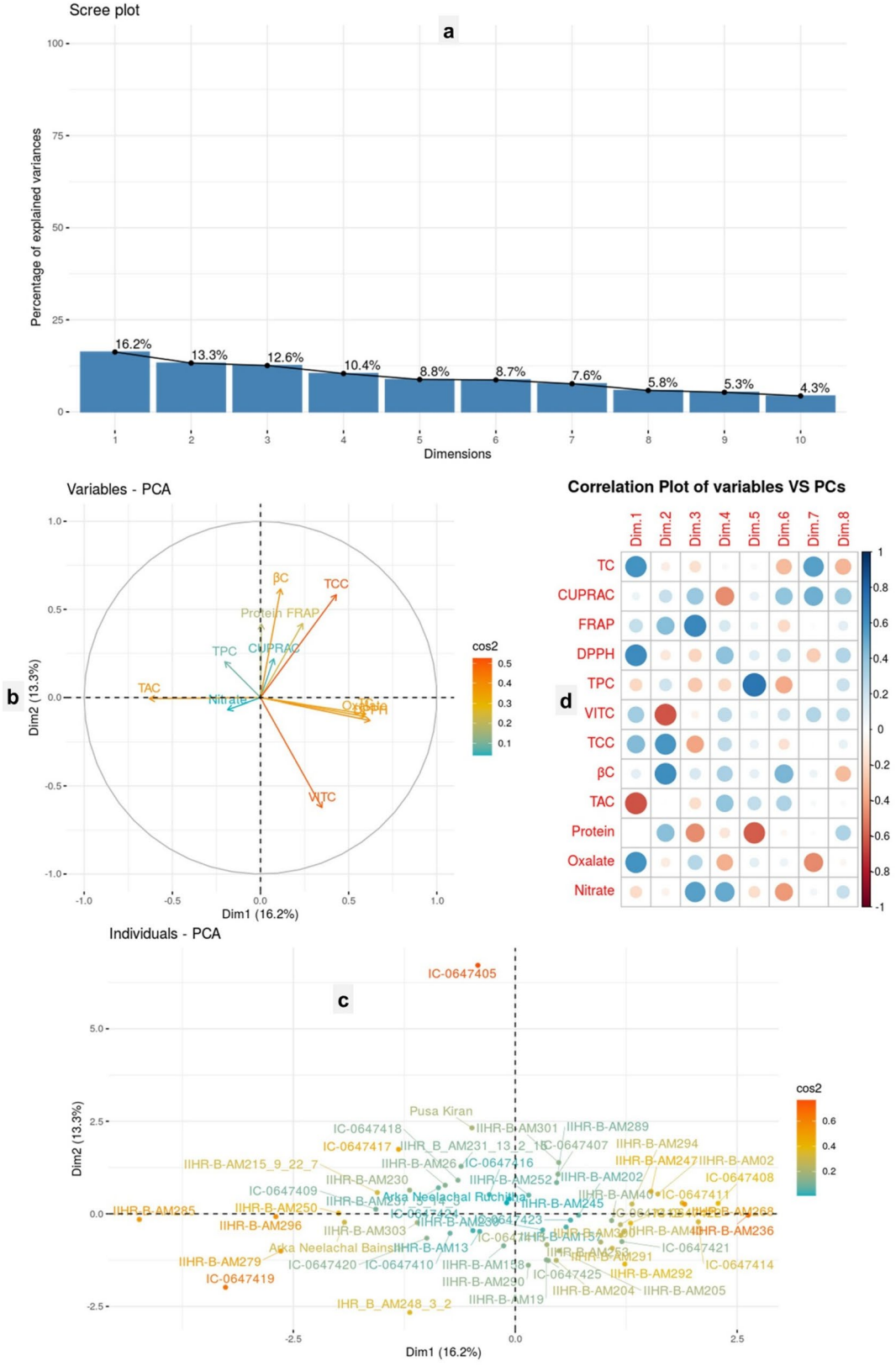


Fig. 3 (See legend on next page.)

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Fig. 3 PCA analysis based on biochemical and antioxidant compounds demonstrating genetic variations among the studied leafy-type amaranth germplasm during summer season. **a** Scree-plot depicting the contribution of first 10 principal components in total variations. The first two principal components (PC1 and PC2) explained 16.2% and 13.3% of the total variation, respectively (**b**) PCA biplot depicting the contribution of individual variable in total variations across first two dimensions. The intense orange colour indicates higher contribution of variables in total variations (**c**) PCA biplot depicting the variation among studied leafy-type amaranth germplasm and plotting them across first two dimensions (**d**) Correlation between variables and principal components. In each dimension, the intense blue colour depicted strong positive association of respective variables with their principal component and intensity towards red colour indicated negative influence of variables in respective principal components

to enhanced ROS scavenging capacity [73]. Likewise, substantial variation in total phenolic contents could be attributed to alterations in activities of phenylpropanoid pathway [74]. Genotypes with high phenolic content are generally considered to have high antioxidant potential [74]. Further, the variations in ascorbate biosynthesis and recycling pathways might be responsible for substantial differences in vitamin C accumulation in studied germplasm lines [75]. Similarly, the alterations in variation in isoprenoid biosynthesis pathways, particularly the methylerythritol phosphate (MEP) pathway might be responsible for substantial variations in carotenoids content [76].

The results of genetic components of variance, suggested moderate to high estimates of GCV and PCV for antioxidant and bioactive compounds along with antinutritional factors. These findings suggested the scope of simple selective breeding in improvement of nutritional quality [43]. Moreover, the high estimates of heritability (h^2b) coupled with genetic advance (GAM) also supported the scope of simple selection procedures in improving nutritional quality of leafy amaranths [77]. In a previous study, high heritability of about 100% was reported for β-Cyanins, β-xanthins, and total polyphenols in vegetable amaranth genotypes, indicating role of additive gene action in expression of these traits [78]. The quality traits are governed by mono or oligogenes, and hence less influence by environmental factors [78]. Thus, high estimates of heritability were recorded for these quality traits [79]. Additionally, the overestimates of heritability could be attributed to design error of randomized augmented block design because environmental variance is often underestimated, owing to the absence of replication for test entries and dependence on replicated check varieties. Therefore, heritability estimates should be interpreted with caution [80]. Nevertheless, the estimates of genetic components of variance provided preliminary information for screening of germplasm for high quality and wider adaptability.

The multivariate techniques of PCA and Mahalanobis D^2 cluster analysis also discriminated the studied germplasm based on antioxidant activity, bioactive compounds and antinutrients concentration, depicting substantial variation for section of ideal parents. The PCA is an important multivariate approach to identify and determine the degree of association between desirable traits and principal components (PCs) [22, 37]. The role of individual variables contributing in total variation was also determined using PCA approach [37, 81]. PCA results indicated that first six components explained over 69% of total variations based on biochemical and antioxidant traits. The total carotenoid content (TCC), vitamin C, oxalates, nitrates and β-carotene contributed maximum in total variations in the studied leafy-type amaranth germplasm. Similar trends were recorded by Abrar et al. [37], who reported first five major principal components (PCs) based on physiological traits and mineral nutrients. Likewise, in another study comprising 39 leafy-type amaranth germplasm lines, 68% of the total variation for different phenotypic and nutritional quality traits was explained by first four principal components [82]. Similarly, in a previous study on vegetable amaranth, the PCA analysis based on yield, physiological and biochemical traits in vegetable amaranth genotypes depicted that first five PCs explained 86.2% of total variation [78]. Further, the significant correlation between individual principal components, especially PC1 and PC2, with biochemical and antioxidant compounds, can be utilized for identification of ideal indigenous or exotic collections of leafy-type amaranth species [37, 81].

In the present study, Mahalanobis D^2 analysis for biochemical and antioxidant compounds along with antinutritional factors, grouped the 57 diverse leafy amaranth germplasm collections into five different clusters. The cluster mean analysis and cluster distance matrix facilitated the selection of diverse parents for heterosis breeding in improving quality of leafy amaranths. The broad genetic spectrum of studied germplasm was depicted by cluster analysis [37]. These results are in line with previous reports on amaranth germplasm for nutritional quality and phenotypic traits by Abrar et al. [37], Shukla et al. [82], and Jahan et al. [78]. The hybridization programme between the genotypes from divergent clusters like cluster-II × cluster-IV and cluster-II × cluster-V, would be instrument to get high heterotic response for biochemical and antioxidant compounds. These cross combinations will enable generation of high genetic diversity and recombination for useful nutritional quality traits [37, 82]. Overall, the cluster analysis results suggested that strategic hybridization between highly divergent clusters, combined with selection within nutritionally superior clusters, can accelerate the development of high-yielding, antioxidant-rich, and nutritionally balanced amaranth varieties. Additionally, the correlation analysis in the present study, demonstrated both synergistic and antagonistic trait

Table 5 Cluster Means from Mahalanobis D^2 Analysis based on biochemical and antioxidant traits for winter season 2024-25

Cluster	TC	CUPRAC	FRAP	DPPH	TPC	VITC	TCC	βC	TAC	Protein	Oxalate	Nitrate
I	57.34	20.16	27.71	71.63	736.90	48.40	8.37	645.40	4.33	22.24	929.60	114.76
II	50.97	24.39	31.28	69.86	750.00	42.00	9.90	2845.00	4.23	18.27	1098.00	61.45
III	55.86	24.61	26.09	81.82	707.34	52.71	9.38	838.51	3.31	21.90	1186.31	92.75
IV	59.52	25.23	28.79	63.78	690.11	53.67	9.75	929.00	3.17	20.56	1226.11	120.87
V	51.43	18.63	24.37	68.89	678.50	61.50	5.99	869.50	5.63	20.77	558.50	70.12

Table 6 Cluster Means from Mahalanobis D^2 Analysis biochemical and antioxidant traits for summer season 2025

Cluster	TC	CUPRAC	FRAP	DPPH	TPC	VITC	TCC	βC	TAC	Protein	Oxalate	Nitrate
I	51.52	15.81	20.85	70.11	682.33	54.67	3.61	1005.00	0.75	20.62	616.00	85.55
II	53.22	15.89	22.13	74.49	619.30	43.64	4.19	679.36	0.55	20.19	1298.23	103.93
III	45.97	18.56	27.15	64.45	690.00	24.00	4.99	2597.00	0.57	21.79	1187.00	65.87
IV	37.13	16.11	19.99	71.60	541.00	37.00	3.94	698.00	1.13	17.57	935.00	140.22
V	52.58	16.86	23.70	54.68	599.88	45.25	4.05	829.50	0.58	19.79	1388.50	119.12

TC Total chlorophyll (SPAD value), *CUPRAC* (µmol Trolox/g) Cupric Reducing Antioxidant Capacity, *FRAP* (µmol Trolox/g) ferric reducing ability of plasma, *DPPH* (%) 2,2-diphenyl-1-picrylhydrazyl, *TPC* (mg of gallic acid/100 g FW) Total phenolic content, *VITC* (mg/100 g) Vitamin C, *TCC* (mg/100 g) Total carotenoid content, βC (µg/100 ml) β-carotene content, *TAC* (mg/100 ml) Total anthocyanin content, Protein (%), Oxalate (mg/100 g), Nitrate (mg/100 g)

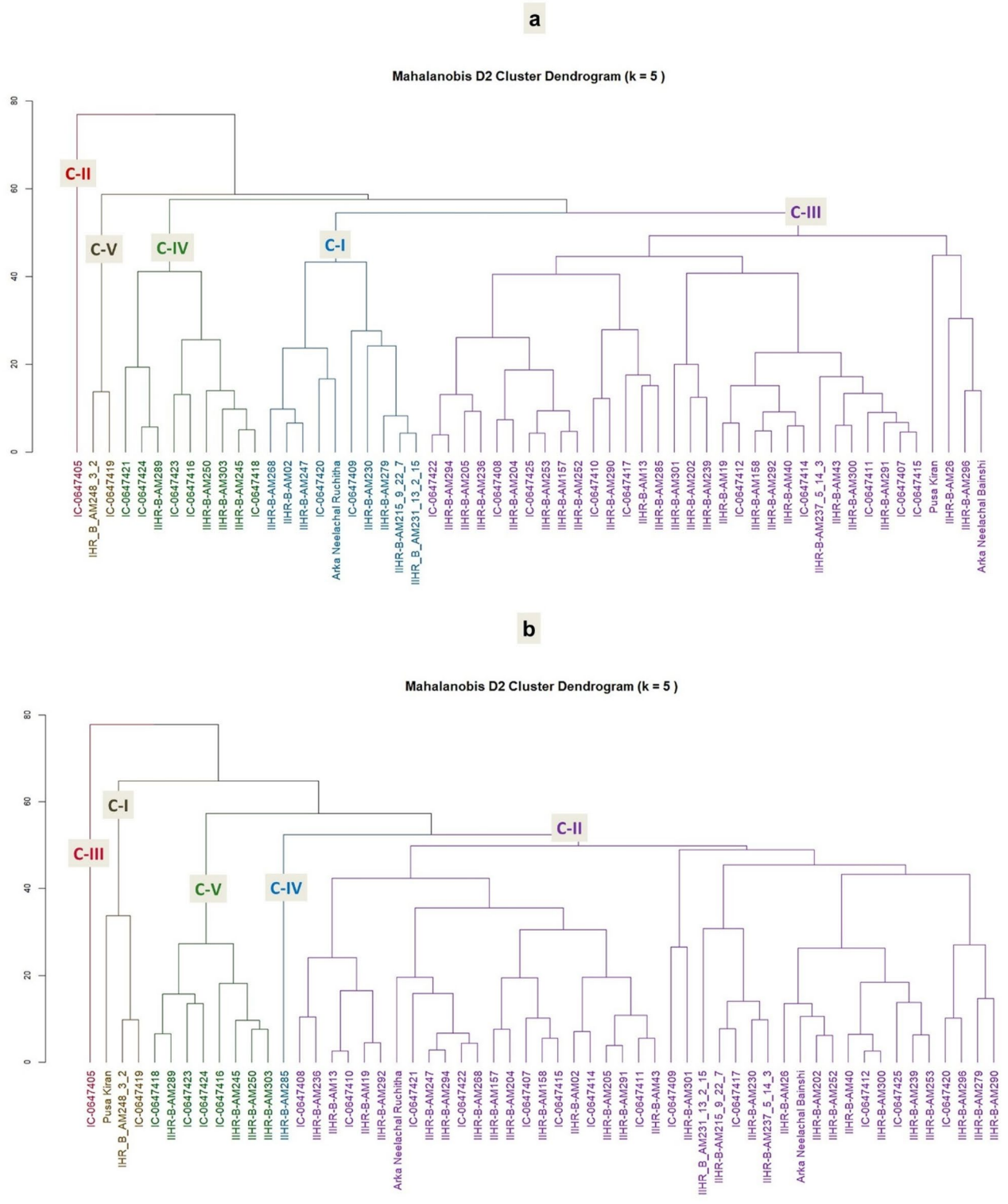


Fig. 4 **a** Mahalanobis D^2 cluster dendrogram based on data analysis for biochemical and antioxidant compounds for the first season (winter season of 2024–2025) enabling the grouping of studied leafy-type amaranth germplasm into five different clusters (**b**) Mahalanobis D^2 cluster dendrogram based on data analysis for biochemical and antioxidant compounds for the second season (summer 2025) enabling the grouping of studied amaranth germplasm into five different clusters

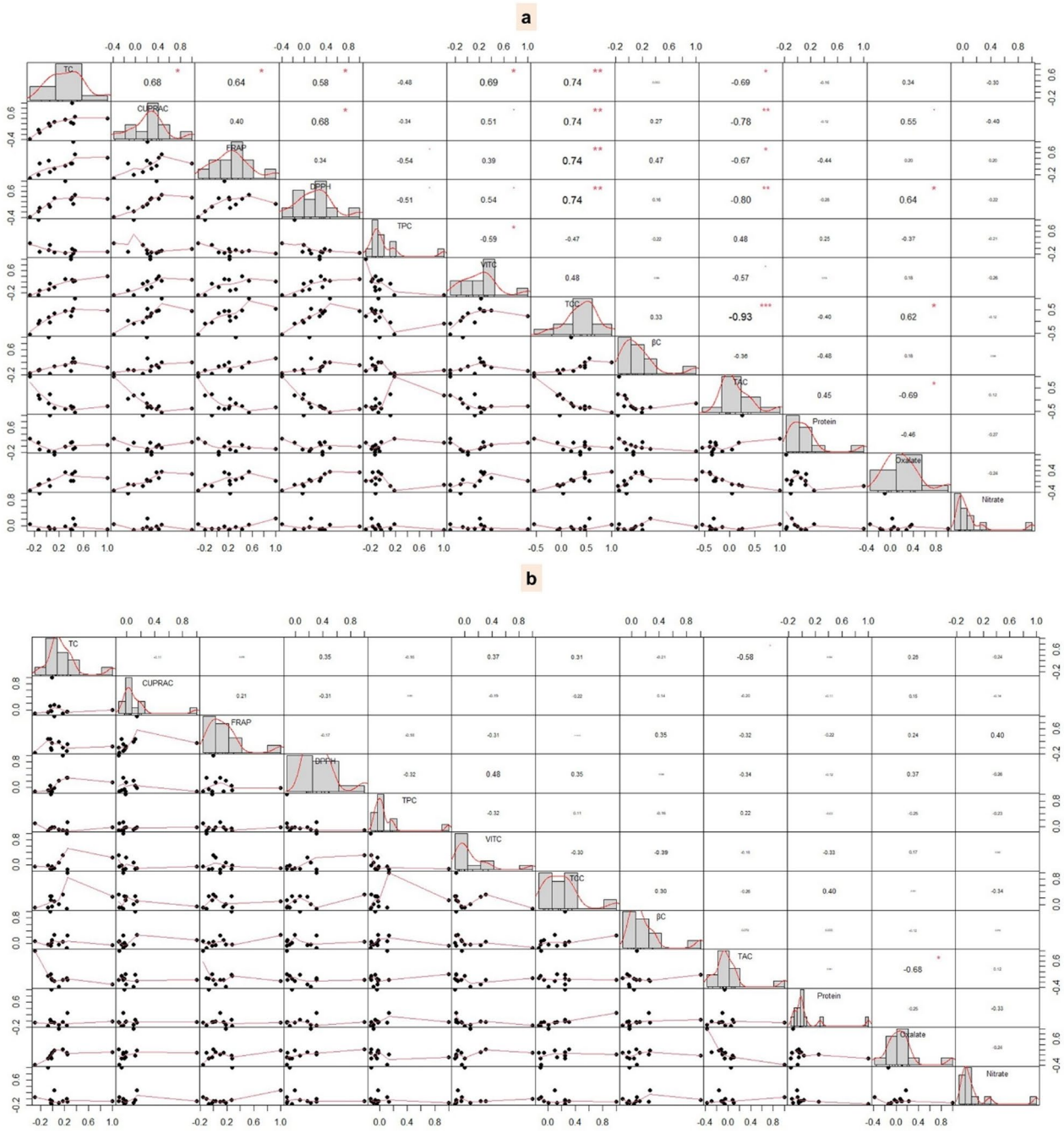


Fig. 5 Correlation matrix showing distributions (diagonal), scatter plots with trends (lower triangle), and Pearson correlation coefficients (upper triangle) (**a**) biochemical and antioxidant traits for the winter season of 2024–2025 (**b**) biochemical and antioxidant traits for the summer season of 2025

associations among antioxidant, bioactive and antinutritional traits. The correlation matrix analysis indicated significant negative correlation of total chlorophyll (TC) with total anthocyanin content, which indicated a metabolic and functional divergence between these pigment groups. Chlorophyll is primarily associated with photosynthetic activity, whereas anthocyanins are stress-responsive secondary metabolites involved in photoprotection and antioxidative defense [83, 84]. While, significant positive association was recorded for FRAP content with β-carotene (βC) and nitrate content. Similarly, DPPH exhibited significant positive interaction with vitamin C and total carotenoid content. These findings corroborate with the results of Jimenez-Aguilar and Grusak [71], Sarker and Oba [32] and Jahan et al. [78] for the correlation analysis of biochemical and antioxidant traits. From a breeding perspective, this indicates that carotenoid-rich genotypes can be targeted as key donors for improving

antioxidant properties, enabling simultaneous enhancement of nutritional quality and health-promoting traits. Overall, the interaction analysis results indicated a coordinated network for nutritional potential of leafy-type amaranth germplasm, where different classes of bioactive and antioxidant compounds interact either synergistically or antagonistically. These findings indicated that simultaneous improvement of antioxidant potential and nutritional value is feasible, although trade-offs between some negatively associated antioxidants, photosynthetic pigments, and anti-nutrients needs to be considered carefully during biofortification related breeding programmes [85].

Conclusion

The results of present study demonstrated a comprehensive understanding of seasonal dynamics, G × E interactions, and their integrated response on antioxidant activity, bioactive compounds and antinutrients in a diverse panel of vegetable amaranth germplasm. The substantial genetic variations were recorded for antioxidants and phytochemicals (TPC, vitamin C, total anthocyanin content, protein, CUPRAC, FRAP, DPPH), which is a prerequisite for effective selection. The strong influence of G × E interactions, emphasized the need for further multi-location, multi-season evaluation of leafy-type amaranth germplasm for identification of genotypes with stability for nutritional quality and low concentration of antinutritional factors. Study highlighted pronounced seasonal effects, hence G × E interactions need to account in selection strategies. The integration of genetic parameters of variability with multivariate analysis could enable the identification of superior lines with high antioxidant activity, bioactive compounds, and reduced burden of antinutrients [86]. The cluster analysis facilitated the selection of genotypes from diverse clusters for heterosis breeding. The strong positive relationships of carotenoids with multiple antioxidant parameters (DPPH, FRAP, CUPRAC) suggest that selection for carotenoid content can serve as an effective indirect selection approach to enhance overall antioxidant capacity. The findings of present investigation underscore the potential of leafy amaranth germplasm in accomplishing SDG2 and SDG13 of United Nations. The study paved a robust framework for targeted breeding to develop cultivars with high nutritional quality and wider adaptability. The findings also highlighted the importance of robust systematic characterization of locally adapted germplasm for sustainable food and nutritional security.

Supplementary Information

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Supplementary Material 1.

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Authors' contributions
Srija Priyadarsini: Writing original draft, Methodology, Data recording; Gobinda Chandra Acharya: Supervision, Resources, Conceptualization, Review and Editing; Pradyumna Tripathy: Review and editing; Sidharth Kumar Palai: Review and Editing; Manasi Dash: Formal analysis; Ashok Mishra: Formal data analysis **; A.V.V. Koundinya: Review of manuscript; Nityamanjari Mishra: Data curation; Saurabh Singh: Writing – review & editing, Writing – original draft, Data curation, Data analysis, Software; Pushpajeet Choudhari: Formal data analysis, Review and editing; Chetna Nimje: Data recording, Formal analysis; Alok Nandi: Review and editing; Umasankar Pal : Data analysis; Jyotirmayee Samantaray: Data analysis; Sipra Mohapatra: Data analysis; Meenu Kumari : Review and editing.

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Data availability
All the data generated in presented in the manuscript and supplementary files.

Declarations

Ethics approval and consent to participate
The authors declare that the research work described in this manuscript was conducted in accordance with the ethical standards of academic research and integrity. The study did not involve human participants or experimental animals.

Consent for publication
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Competing interests
The authors declare no competing interests.

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