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A multidisciplinary characterization of Apulian chickpea local varieties by integrating SSR markers, drought-related gene expression and biochemical traits

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Abstract: The ongoing climate changes are increasing the frequency and severity of drought events, thereby threatening the productivity of many crops in the Mediterranean basin. Chickpea (*Cicer arietinum* L.), a nutritionally valuable grain legume of global importance, is widely cultivated in Southern Italy and exhibits considerable local biodiversity. In this study, nine chickpea landraces were comprehensively characterized by an integrated approach combining microsatellite-based genetic analysis, seed biochemical profiling, and gene expression analysis under simulated water deficit. Genetic variability assessed by SSR markers resolved the landraces into three clusters that largely corresponded to seed coat colour (white, red, black). Seed quality was evaluated by quantifying key biochemical parameters, including protein content, total phenolic compounds and carotenoids; red and black seeds contained significantly higher levels of phenolics and carotenoids than white seeds. Drought tolerance potential was investigated by imposing polyethylene glycol (PEG)-induced osmotic stress and quantifying the transcript abundance of two Kunitz-type trypsin inhibitor genes by quantitative PCR. The *CaTPI-1* transcript exhibited a marked up-regulation in roots of red and black landraces under stress, whereas white-coated landraces showed reduced root accumulation of *CaTPI-1*. Collectively, these results indicate that red and black landraces combine enhanced antioxidant-related seed chemistry with a stress-responsive transcriptional profile potentially associated with improved tolerance to water deficit. These locally adapted chickpea genotypes constitute valuable genetic resources for conservation and breeding programmes aimed at enhancing chickpea resilience and nutritional quality in semi-arid Mediterranean agroecosystems.

Keywords: *Cicer arietinum* L.; genetic diversity; drought stress; microsatellite markers; seed composition

1. Introduction

The cultivation and consumption of legumes dates to the origins of agriculture. For millennia, they have played a central role in human nutrition and food systems thanks to a high nutritional value, especially in terms of proteins, complex carbohydrates, vitamins, minerals, and bioactive compounds, which have health-promoting properties (Duranti 2006; Gupta et al. 2021). In addition to their dietary significance, legumes contribute significantly to the sustainability of the agroecosystems through nitrogen fixation, which reduces the use of synthetic fertilizers, and enhancing the soil fertility (Graham and Vance 2003). Among legumes, chickpea (*Cicer arietinum* L.) is the third most widely produced legume worldwide, after common bean and pea, with over 16 million tonnes produced annually, nearly half of which produced in India (FAOSTAT 2024). Based on seed morphology, pigmentation and geographical distribution, two phenotypes, named *desi* and *kabuli*, are recognized within the species, although the underlying genetic differentiation is relatively low (Swamy 2023). *Desi* morphotype (Indian origin) are small, angular, and variegated in colour (brown, black or green), while *kabuli* morphotype (Mediterranean and Middle Eastern origin) are large, round, and white to cream coloured (Singh and Diwakar 1995).

The Apulian region (Southern Italy) is rich in agro-biodiversity, particularly regarding grain legumes such as faba bean, pea, and chickpea, which are traditional components of crop rotations (wheat-legume-forage crops) (Piergiovanni and Margiotto 2021). Local varieties are well adapted to the edaphic and climatic conditions of the Mediterranean microenvironments and have sustained small-scale agriculture and local food cultures over centuries (Casañas et al. 2017). Recent investigations have explored the genetic diversity and agronomic potential of Apulian chickpea landraces to facilitate their utilization in breeding programs aimed at improving stress resilience and seed nutritional quality (De Giovanni et al. 2017; Pavan et al. 2017).

Understanding and valorising this existing diversity is imperative in the face of climate change and shifting consumer demands towards foods with enhanced health properties. This is particularly critical for Apulia, which faces high desertification risk; chickpea cultivation represents a sustainable alternative given the crop capacity to thrive in semiarid

conditions (Gambella et al. 2021; Carvalho et al. 2022). The characterization of landraces provides the foundation for informed *on-farm* management, germplasm conservation strategies, and targeted breeding efforts (Smýkal et al. 2015; Salgotra and Chauhan 2023). Specifically, molecular fingerprinting using microsatellite (SSR) markers allows the accurate assessment of unique profiling patterns and genetic diversity, population structure, and relationships among plant material (Babay et al. 2020; Duhan and Kaundal 2021; Maurya et al. 2023; Sabetta et al. 2021; Savoia et al. 2023). At the same time, the evaluation of physiological responses to water deficit and the expression of key stress-responsive genes can help to identify genotypes with inherent tolerance to drought, a major constraint to chickpea production in semi-arid Mediterranean environments (Blum 2011; Maphosa et al. 2020). Protease inhibitors (PIs) are among the molecules involved in plant adaptive responses. They play a fine regulation of proteolysis during germination modulating endogenous proteinases (Pawar et al. 2024) and have a crucial role in the plant response to biotic stresses (Moloi and Ngara 2023; Paiva-Silva et al. 2025). Several legume Kunitz type trypsin inhibitors genes have been described in *Cicer*, in particular the *CaTPI-1* (*Cicer arietinum*, Trypsin Protein Inhibitor) is a Kunitz-type trypsin inhibitor characterized by serine protease domains and stabilized by four conserved cysteine residues forming two disulphide bridges. The CaTPI-1, a 24 kDa single-chain polypeptide, is encoded by the *CaTPI-1* gene, represents a specific isoform of the Kunitz family. It is predominantly expressed in vegetative tissues of seedling, where it contributes to plant defence system by contrasting or mitigating biotic and abiotic stresses. Another Kunitz trypsin inhibitor protein, designated as *CaKTI* is codified by a diverse gene described either within the Kunitz family, is seed-related, and primarily involved in the plant defence against insects. Together, these two proteinase inhibitors illustrate divergent functional specializations within the Kunitz family: CaTPI-1 is mainly involved in endogenous physiological processes and developmental regulation, whereas CaKTI is specialized in direct defence against herbivores. Thus, they represent two complementary evolutionary strategies by which Kunitz inhibitors contribute to plant protection and adaptation. The exploitation of the genetic variation present in Apulian chickpea local varieties, provides opportunities to identify alleles and mechanisms associated with stress resilience and yield stability. Advances in genomic and phenomics technologies have facilitated the detection of genomic regions linked to drought tolerance and other agronomical important traits, such as root architecture, osmotic adjustment, and antioxidant metabolism (Ali et al. 2022; Karkee et al. 2026; Singh et al. 2023 and 2026).

In this study, we aimed to characterize some Apulian chickpea local varieties at molecular and physiological levels, evaluating their responses to controlled drought stress. Moreover, the evaluation of seed composition enabled comparison of Apulian local varieties with two commercial chickpea varieties, providing valuable insights into their potential as legumes with enhanced nutritional value.

2. Materials and Methods

2.1 Plant material

In 2024, collecting missions were conducted in the framework of the project *SavegrainPugliaLeg* to acquire local varieties of chickpea (*Cicer arietinum* L.) still grown in central and southern Apulia region. Eleven local varieties were collected and characterised by using a multidisciplinary approach. About 300 g of dry seeds were obtained by the local farmers who grow them from more than 15 years. Collection sites and seed traits, recorded following the official descriptors (IBPGR 1993) are show in Table 1. The Spanish variety “*Blanco lechoso*” (BL), cultivated in Andalusia and Extremadura (Spain), and an Italian, black-seeded commercial variety “*Cece nero rugoso*” (black chickpea, BC), were included as references.

Table 1. List of studied local varieties, their collection sites (Apulian provinces: BA = Bari, LE = Lecce, TA = Taranto), and seed morphological traits.

Work code	Collection site	Local name	Seed shape	Coat texture	Coat colour	Morphotype
<i>Local varieties</i>						
L172	Ruvo di Puglia (BA)	chickpea	pea shaped	smooth	beige	<i>kabuli</i>
L179	Nardò (LE)	chickpea from Nardò	pea shaped	smooth	beige	<i>kabuli</i>
L301	Nardò (LE)	chickpea from Nardò	pea shaped	smooth	beige	<i>kabuli</i>
L316	Tricase (LE)	chickpea	pea shaped	smooth	beige	<i>kabuli</i>
L79	Alberobello (BA)	chickpea	angular	tuberculated	brown	<i>desi</i>
L171	Ruvo di Puglia (BA)	chickpea	angular	tuberculated	brown	<i>desi</i>

L303	Cassano delle Murge (BA)	red chickpea from Cassano	pea shaped	smooth	reddish brown	<i>desi</i>
L304	Laterza (TA)	chickpea	angular	tuberculated	brown	<i>desi</i>
L302	Cassano delle Murge (BA)	black chickpea from Cassano	owl's head	rough	black	<i>desi</i>
L315	Alberobello (BA)	chickpea	angular	tuberculated	black	<i>desi</i>
L317	Tricase (LE)	black chickpea from Muro Leccese	angular	tuberculated	black	<i>desi</i>
<i>Commercial varieties</i>						
BL	Andalusia, Spain	Blanco lechoso	pea shaped	smooth	ivory white	<i>kabuli</i>
BC	Apulia, Italy	Black chickpea	angular	tuberculated	black	<i>desi</i>

2.2 DNA Extraction

Seeds were pre-chilled for three days at 4 °C in Petri dishes on moistened Whatman filter paper, and subsequently transferred to a growth chamber and incubated in darkness at 25 °C. After germination, seedlings were transplanted into peat moss and grown for four weeks under controlled conditions with a 16 h/8 h light/dark photoperiod.

Total genomic DNA was extracted from young leaves (50-100 mg) using Plant DNAzol Reagent (Invitrogen, Carlsbad, CA, USA) and the cells were disrupted and homogenized in presence of 3.2 mm diameter stainless steel beads with a Bullet Blender storm (Next Advance, 2113 NY-7, Troy, NY 12180, USA) operated at maximum speed for 3 min. DNA concentration was measured using a NanoDrop™ ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and DNA quality was assessed by electrophoresis on a 0.8% Certified Molecular Biology Agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) prepared in 0.5× TBE buffer (1 M Trizma, 1 M boric acid, 20 mM EDTA, pH 8.3) and stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA). All DNA samples were diluted with ultrapure water (Gibco, Invitrogen, USA) to a standard working concentration of 20 ng µL⁻¹ and used for subsequent molecular analyses.

2.3 SNPs Analysis

Fragments covering the coding regions of the PIs *CaKTI*, *CaTPI-1* and *CaSIL* were used for SNP analysis. Primers were synthesized according to the literature (Srinivasan et al. 2005; Jimenez et al. 2007) (Table S1). The length of the amplified fragments ranged from 500 to 800 nt. DNA extracted as described above was diluted to 20 ng/µL and used for amplification of the genes in the respective local varieties. PCR reactions were carried out in a 25 µL mixture containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 100 µM each of dATP, dCTP, dGTP and dTTP, 1.5 mM MgCl₂, 1.25 units of Taq polymerase, and 500 nM of each primer. Amplification conditions were optimized by adjusting annealing temperature and extension time. For all assays, Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) was used. Following electrophoresis, target amplicons were excised from agarose gels and purified using the QIAEX II Gel Extraction Kit (Qiagen, The Netherlands), after polyadenylation reaction were cloned using the TA Cloning™ Kit with the pCR™ 2.1 vector (Invitrogen, Carlsbad, CA, USA). Recombinant clones were sequenced in both directions by an external sequencing service. Sequence analysis was performed using BioEdit (Hall 1999). Nucleotide sequences were aligned with ClustalW (Thompson et al. 1994) and compared with the *CaTPI-1* (NM_001279070.1), *CaSIL* (XM_004511089.3) and *CaKTI* (NM_001279121.1) sequences retrieved from the NCBI database.

2.4 SSR Analysis

A set of 17 nuclear SSR was used to assess the genetic variability of the chickpea collection. For each SSR, information on locus type, repeat motif, annealing temperature, and linkage group is listed in Table S2. PCR amplification were carried out in a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a final volume of 20 µL containing 1 X Dream Taq Buffer, 1.5 mM MgCl₂, 0.2 mM dNTP mix, 70 nM M13-tailed forward primer, 200 nM reverse primer, 200 nM M13 primer, 0.5 U/µL Dream Taq and 40 ng of genomic DNA. All reagents were supplied by Thermo Fisher Scientific® (Waltham, MA, USA). The M13 primer was fluorescently labelled with the dyes FAM, HEX and TAMRA (Eurofins Genomics, Milan, Italy). In many cases, a touchdown PCR protocol was applied, consisting of an initial denaturation at 94 °C for 30 s; 24 cycles of 94 °C for 30 s, annealing at locus-specific high temperature for 30 s, and 72 °C for 30 s; followed by 11 cycles at 94 °C for 30 s, annealing at locus-specific lower temperature for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. PCR products were separated by capillary electrophoresis by an external service and genotyped using GeneMapper software v.5.0 (Applied Biosystems, Foster City, CA, USA).

2.5 Genetic Diversity

For each SSR marker, the main genetic indexes were calculated using GenALEx software v. 6.51b2 (<http://biology-assets.anu.edu.au/GenALEx>) (Peakall and Smouse 2006): number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I) (Shannon & Weaver 1948), observed (Ho) and expected (He) heterozygosity, and fixation index (F) (Wright 1951). Moreover, principal coordinates analysis (PCoA) (Gower 2015) was performed on the similarity/dissimilarity matrix. Cervus v. 2.0 was used to calculate the polymorphic information content (PIC).

Genetic distance between the chickpea genotypes was estimated using the simple matching dissimilarity index. A weighted Neighbour-Joining tree (Saitou and Nei 1987) was constructed with the Dissimilarity Analysis and Representation for Windows (Darwin6) (<http://darwin.cirad.fr>) software version 6.0.21. The robustness of branches was assessed by 1000 bootstrap replicates (Felsenstein 1985).

2.6 Drought stress imposition

Water deficit stress was induced by treatment with 10% (w/v) polyethylene glycol (PEG-6000). A control treatment without PEG was included for each local variety. Drought stress was imposed on four-week-old chickpea plants for 24 h. Plants were carefully unrooted, rinsed under tap water, and transferred to the PEG solution. At the end of the treatment period, root and leaf samples were immediately collected, frozen in liquid nitrogen, and stored at -80°C for RNA extraction and subsequent analyses.

2.7 RNA isolation and RT-qPCR

The expression levels of *CaTPI-1* and *CaKTI*, genes associated with drought stress tolerance, were evaluated in roots and leaf tissue following drought imposition. Total RNA was extracted from approximately 100 mg of leaf or root tissue from each variety using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA samples were treated with DNase using the TURBO DNA-free™ Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol to eliminate genomic DNA contamination. RNA integrity was verified by electrophoresis on a 1.2% agarose gel, and RNA purity and concentration were determined using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For cDNA synthesis, 3 μg of total RNA was reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The resulting cDNA was used as a template for quantitative real-time PCR (RT-qPCR). Primer pairs were designed with the support of the online open software Primer3Plus (<https://www.primer3plus.com/>) (Table S1). RT-qPCR reactions were performed in a 96-well StepOnePlus RealTime System (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA USA) using 2X PowerUp SYBR Green PCR Master Mix (Thermo Fisher Scientific), with 400 nM forward and reverse primers, and approx. 20 ng of reverse-transcribed RNA, in a total volume of 12.5 μL . Relative gene expression levels were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen 2001), normalized to the expression of a reference gene (Acc. N. DQ321719; Shukla et al. 2006).

2.8 Seed biochemical characterization

For each local variety, approximately 30g of dry seeds randomly selected were milled in a laboratory mill Cyclotec 1093 (Tecator, Sweden) to obtain a fine meal that was used for the determination of the biochemical traits. Hydration index (HI), swelling index (SI) and coat percentage were measured as previously described (Piergiovanni et al. 2000). Moisture and ash contents were determined according to AOAC official methods (930.15 and 923.03, respectively). Trypsin inhibitor (TI) amounts were measured spectro-photometrically on aqueous extracts prepared according to (Della Gatta et al. 1988) and expressed as trypsin inhibitor units for milligrams of meal (TIU mg^{-1}). Total phosphorus (P_{tot}) was determined by the colorimetric method of Fiske and Subbarow (1925), inorganic phosphorus (P_i) was quantified according to Campion et al. (2009). Total phenolic compounds (TPC) and carotenoids were quantified according to Doria et al. (2012) and Ndolo and Beta (2013), respectively.

2.9 Statistical Analysis

All experiments were performed using three independent biological replicates, each assessed in triplicate (technical replicates). Data are expressed as mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA), applying a homoscedastic Student's *t*-test for gene expression data and one-way analysis of variance (ANOVA) for seed biochemical parameters.

3. Results

3.1. Genetic diversity

A total of 17 SSR loci were evaluated to assess the genetic variability of the chickpea local varieties under study. The number of alleles per locus (N_a) ranged from 2 (H1F14 and H4H01) to 11 (TR29), with a mean of 7 alleles per locus, indicating a moderately high level of allelic richness across the SSR panel. The effective number of alleles (N_e) was lower than N_a , with values ranging from 1 (CESSRDB47) to 10 (TR29) and a mean of 5, reflecting unequal allele frequency distributions at most loci. Shannon's information index (I) ranged from 0.32 (CESSRDB47) to 2.33 (TR29), with a mean of 1.61, further supporting moderate-to-high levels of genetic diversity within the collection. Expected heterozygosity (H_e), a widely used measure of gene diversity, ranged from 0.14 (CESSRDB47) to 0.90 (TR29), with a mean of 0.72, indicating overall substantial genetic variation among the accessions. In contrast, observed heterozygosity (H_o) was markedly lower across most loci, with a mean of 0.10 and values of zero or around zero recorded at ten loci, suggesting a predominance of homozygosity consistent with the predominantly self-pollinating nature of chickpea. The fixation index (F) was positive and high at most loci (mean 0.83), indicating a significant excess of homozygotes relative to Hardy-Weinberg expectations, with several loci (NCPGR21, H1F14, H4H01, ICCM0076, NCPGR127, STSM11) exhibiting $F=1.00$, indicative of complete fixation. A notable exception was CESSRDB47, which displayed a negative F value (-0.06), suggesting a slight excess of heterozygotes at this locus, though its overall diversity indices were the lowest among all markers evaluated.

The polymorphism information content (PIC) values ranged from 0.14 (CESSRDB47) to 0.89 (TR29), with a mean of 0.69. Thirteen out of 17 loci (76.4%) exhibited PIC values > 0.50 , classifying them as highly informative markers, while loci H1F14 (PIC=0.37), H4H01 (PIC = 0.30) and STSM11 (PIC=0.40) were moderately informative. These results demonstrate that the majority of the SSR loci employed in this study are highly suitable for diversity analysis in chickpea germplasm.

Table 2. Genetic parameters calculated by SSR analysis for the studied chickpeas. Locus name, allele size range (bp), number of detected (N_a) and effective (N_e) alleles, Shannon's index (I), observed (H_o) and expected (H_e) heterozygosity, fixation index (F), and polymorphism information content (PIC) are reported.

SSR locus	Allele size	N_a	N_e	I	H_o	H_e	F	PIC
H3C06	188-212	6	5	1,637	0,077	0,778	0,901	0,747
NCPGR21	162-184	9	7	2,098	0,000	0,864	1,000	0,850
H4D08	202-232	8	6	1,905	0,077	0,825	0,907	0,805
H1F14	217-223	2	2	0,679	0,000	0,486	1,000	0,368
H3F08	214-272	9	8	2,142	0,167	0,875	0,810	0,862
ICCM0069	220-258	9	8	2,098	0,182	0,868	0,790	0,853
TR29	194-246	11	10	2,332	0,077	0,896	0,914	0,887
H4H01	282-296	2	2	0,562	0,000	0,375	1,000	0,305
H5E11	217-273	8	4	1,746	0,077	0,772	0,900	0,745
GM37999	252-294	9	7	2,079	0,250	0,859	0,709	0,845
H6C07	220-242	7	5	1,791	0,167	0,813	0,795	0,788
ICCM0068	220-260	9	7	2,037	0,333	0,851	0,608	0,834
ICCM0076	270-310	7	6	1,906	0,000	0,844	1,000	0,825
CESSRDB47	256-268	3	1	0,325	0,154	0,145	-0,061	0,140
ICCM0249	143-167	9	6	1,944	0,154	0,822	0,813	0,802
NCPGR127	198-206	4	4	1,332	0,000	0,722	1,000	0,673
STMS11	149-153	3	2	0,790	0,000	0,462	1,000	0,404
Mean	-	7	5	1,612	0,101	0,721	0,829	0,690

3.2 Molecular characterization and genetic relationships

The genetic diversity and relationships among the Apulian chickpea local and the commercial varieties (BC and BL) were visualized by a Weighted Neighbour-Joining (WNJ) tree (Figure 1) and a Principal Coordinates Analysis (PCoA; Figure 2). Despite the geographically restricted collection area, predominantly within the provinces of Bari, Lecce, and Taranto, the analyses revealed a moderate but appreciable level of genetic variability, suggesting that traditional cultivation practices across even a limited territory have maintained meaningful differentiation among local varieties. Both the WNJ tree and the PCoA showed a broadly concordant pattern, although varieties did not group strictly according to their morphological type. Two main clusters are clearly resolved: the first encompassing most of the varieties (L79,

L303, L171, L179, L172, L316, L315, L304, BC and L317), and the second including the remaining ones (L302, L301, and BL). Within the first cluster, a well distinguished sub-cluster comprising the two black-seeded varieties BC and L317 was observed, whereas the other black-seeded varieties (L302 and L315) were distributed across separate branches of the WNJ tree. The brown/reddish-seeded genotypes (L79, L171, L303) formed a distinct and well-supported group, except for L304, which appeared more divergent. Three of four beige-seeded genotypes (L172, L179, L316) clustered together in the WNJ tree and occupied overlapping positions in the PCoA ordination space, whereas L301 was markedly more distant. Notably, the two varieties L179 and L301, both collected in fields near Nardò (LE) and named by local farmers as “Cece di Nardò”, were positioned distinctly in both the WNJ tree and the PCoA plot, with L301 distant from the main, beige-seeded local germplasm group. This result demonstrates that genetically distinct materials are currently classified under the same local farmer designation “Cece di Nardò”, a variety whose cultivation in that area of the Apulia region dates to the end of the XIX century. Although both samples exhibit identical seed morphology (Table 1), further investigation is required to determine the precise origin of each sample and, consequently, to identify which material should be classified as foreign to the local variety. The commercial variety BL consistently appeared as an outlier in both analyses, separated from all Apulian varieties along the first coordinate of the PCoA. Although BL shares the beige/white seed coat with several local varieties, its genetic divergence is consistent with its distinct geographic origin and breeding history.

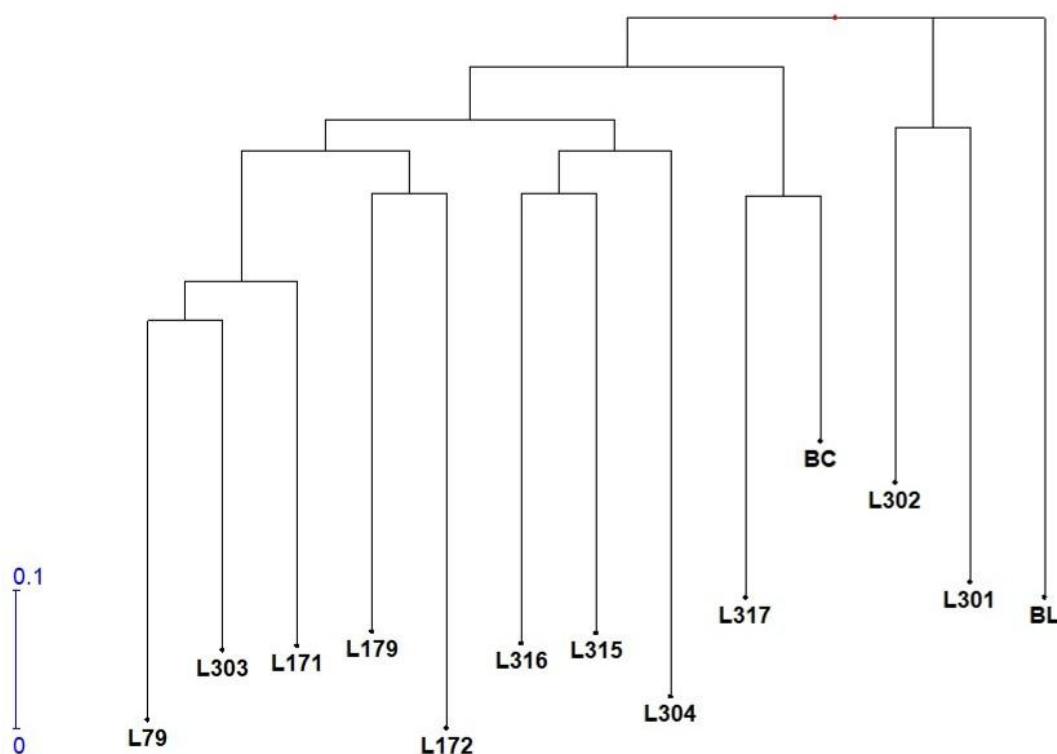


Fig. 1 Neighbour-Joining dendrogram of the chickpea varieties based on Nei's genetic distance estimated from 17 SSR markers. Bootstrap support was based on 1000 replicates. The Spanish commercial variety *Blanco lechoso* (BL) was used as outgroup

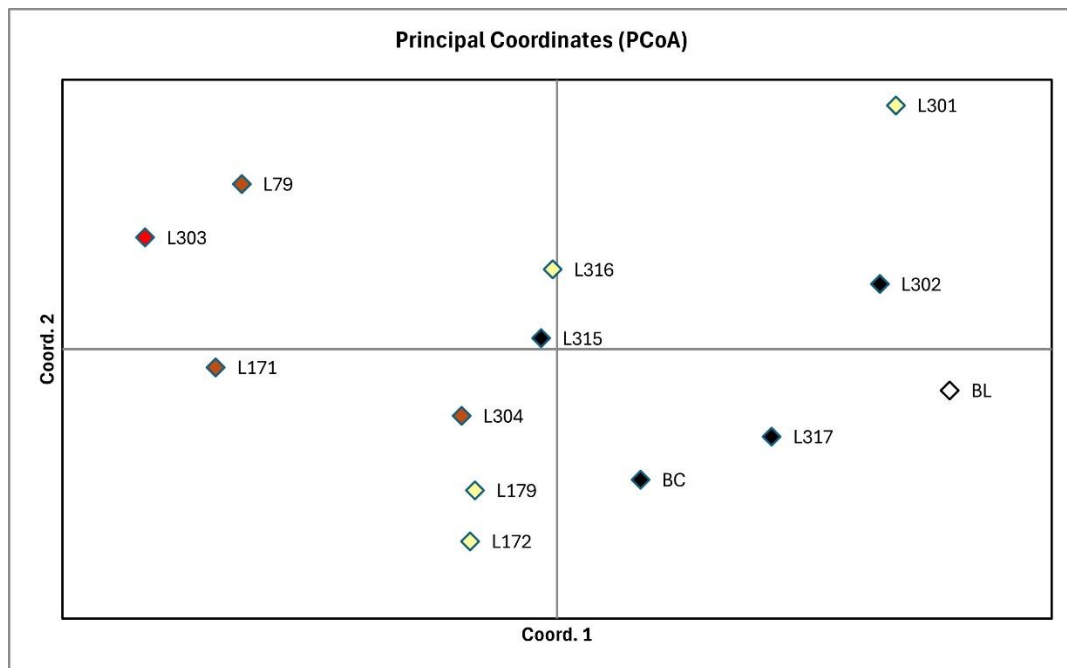


Fig. 2 Principal Coordinate Analysis (PCoA) of 13 chickpea varieties

3.3 Analysis of drought-stress related genes

3.3.1 SNPs analysis

A more targeted genetic characterization of the studied chickpeas was performed to identify potential functional single nucleotide polymorphisms (SNPs) within three genes encoding protease inhibitors (PIs). Fragments spanning the coding sequence (CDS) of the genes *CaTPI-1* and *CaKTI*, both belonging to the Kunitz trypsin inhibitors (KTI) family, and the *CaSIL* gene, encoding an α -amylase inhibitor involved in pest resistance and the response to drought stress, were amplified and sequenced. Comparative analysis of the obtained sequences from the Apulian local varieties revealed very low level of nucleotide variability, both among the local varieties and with sequences retrieved from public databases, with values ranging from 0 to 0.14% for *CaTPI-1* and *CaKTI*, while no polymorphisms were detected in *CaSIL* (Figure S1).

Regarding the *CaKTI* gene, SNPs were identified in the landraces L301 and L172 and in the reference BC. Specifically, in L301, a transversion (pyrimidine-to-purine substitution, C→A) was detected at position 379, whereas in BC a transition (purine-to-purine substitution, A→G) took place at position 672. Both variants corresponded to synonymous mutations, as they do not alter the encoded amino acid sequence. In landrace L172, three SNPs were identified. One was located within the coding region at position 485 and consisted of a purine-to-purine transition (G→A), while the remaining two variants were found within non-coding regions at positions 604 and 672, not representing any change in the CDS. The coding-region SNP represented a non-synonymous substitution, resulting in an amino acid change varying from glutamic acid (E) to serine (S), thus introducing a shift from a negatively charged residue to a polar uncharged side chain.

For the *CaTPI-1* gene, only the landrace L316 displayed a SNP at position 58, consisting of a pyrimidine-to-pyrimidine transition (C→T). This mutation was non-synonymous and led to an amino acid substitution from phenylalanine (F) to leucine (L), which can be classified as a conservative change given the preservation of hydrophobic side chain. Importantly, in both *CaKTI* and *CaTPI* proteins, none of the observed non-synonymous substitutions mapped to the reactive loop or the P1 site of the Kunitz domain. Therefore, these variants are improbable to affect the protease inhibitory activity, as they do not involve residues critical for substrate recognition or binding.

3.4.2 Gene Expression

To investigate whether drought stress could induce transcriptional changes in the selected PI-encoding genes, a gene expression analysis was performed on a sub-set of the chickpea varieties under study. The relative expression levels of *CaTPI-1* and *CaKTI* were analysed by RT-qPCR in both roots and leaves of plants subjected to water-deficient stress (S) or maintained under well-watered control conditions (C). Due to the high sequence conservation of the *CaSIL* gene and the absence of detectable SNPs, this gene was excluded from subsequent expression analysis.

CaTPI-1 is primarily transcribed in vegetative organs, and its expression is known to be influenced by light and developmental stage (Jiménez et al. 2007), whereas *CaKTI* is characterized by its antimetabolic effect on pod borer (*Helicoverpa armigera*) larvae (Srinivasan et al. 2005).

Overall, *CaTPI-1* expression was markedly higher in roots than in leaves across all genotypes (Figure 3), suggesting preferential transcriptional activation in root tissue, particularly under stress conditions. In fact, water deficiency significantly induced *CaTPI-1* mRNA accumulation relative to well-watered controls, with the most pronounced responses observed in the coloured-seed local varieties, including the black-seeded types L302 and L315 ($p < 0.01$ and $p < 0.05$, respectively) and the brown-seeded types L79, L303, and L304 ($p < 0.01$) (Figure 3a). Among these, L303 exhibited the most striking drought response, reaching the highest relative expression level recorded in this study (RQ~10.5). In accessions L302, L303, L304, L315, and L79, fold-changes in root expression ranged from 2.4-fold in L303 to 5.0-fold in L302, with intermediate values of 3.3-, 2.6-, and 2.0-fold for L304, L315, and L79, respectively. The strong induction of *CaTPI-1* in coloured-seeded varieties under drought conditions suggests a more efficient modulation of endogenous protease inhibitor activity in these genotypes, which may contribute to protein homeostasis and protect functional proteins from stress-induced degradation. In contrast, the Apulian genotypes L301 and the commercial variety BL showed significant down-regulation of *CaTPI-1* in roots under stress ($p < 0.05$ and $p < 0.01$, respectively), a pattern opposite to that observed in most local accessions, thus highlighting the genotype-dependent nature of the transcriptional response.

In leave tissue, *CaTPI-1* expression was generally lower than in roots and tended to be down-regulated under drought stress relative to the corresponding controls (Figure 3b). A notable exception was accession L171, which displayed the highest leaf expression level under control conditions (RQ~6.2, $p < 0.05$) and a significant reduction under stress, suggesting a stress-suppressed expression profile specific to this genotype. Conversely, and in contrast to the pattern observed in roots, drought stress induced the *CaTPI-1* transcription in leaves of both BL and L301 genotypes ($p < 0.05$), further underlining the tissue- and genotype-specific nature of the response.

Taken together, these results indicate a genotype- and tissue-dependent modulation of *CaTPI-1* expression in response to water deficit, with L303 emerging as the most responsive accession in roots and L171 displaying a distinctive expression pattern in leaves.

The expression profile of *CaKTI* under the same experimental conditions followed a trend similar to that of *CaTPI-1*; however, no statistically significant differences were detected among accessions for this gene (data not shown).

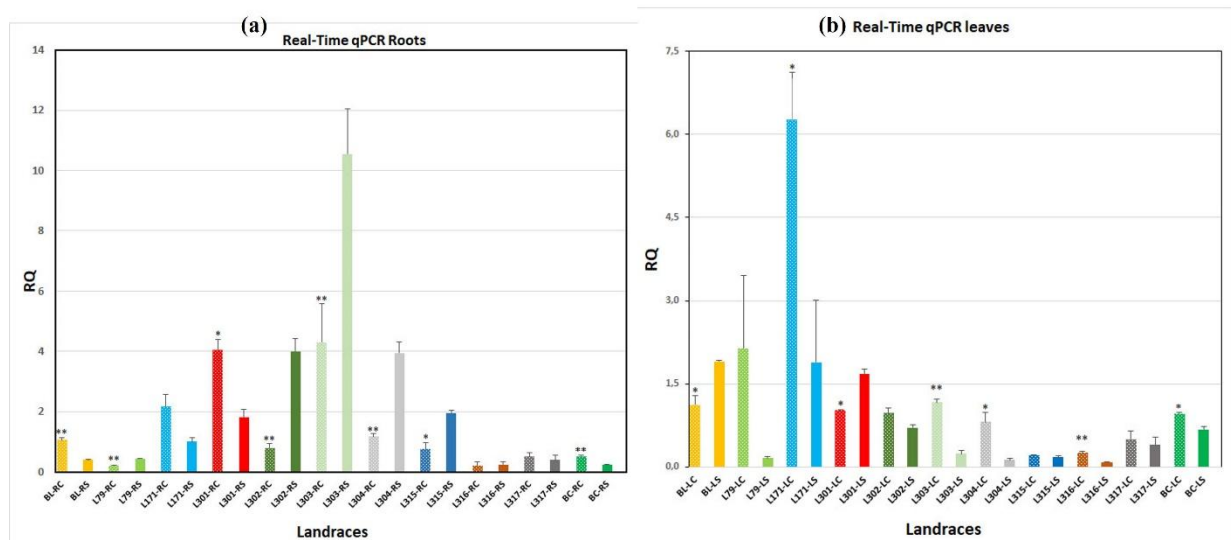


Fig. 3 RT-qPCR analysis of *C. arietinum* landraces and commercial varieties under drought stress (genotype code + S) or in well-watered control conditions (genotype code + C), based on the relative expression levels of *CaTPI-1* in roots (a) and leaves (b). Statistical analyses were performed using a homoscedastic (equal variance) Student's *t*-test between samples with solid-coloured bars and a white dotted pattern sharing the same colour. Significant differences are indicated as: $p < 0.05$ (*); $p < 0.01$ (**)

3.5 Seed composition

The seed composition of a sub-set of the local varieties under study was investigated. Although the sample size was relatively small, a wide variation was recorded across all quantified traits (Table 3). The considerable range observed for

100-seed weight (15.65–41.85 g; mean 28.06 g) and seed coat percentage (52.0–161.0 g/kg) reflects the coexistence of *desi* and *kabuli* morphotypes within the studied material, as these two categories are conventionally distinguished based on seed size. The two commercial references (BL and BC) further extended this range, with BL displaying a markedly higher 100-seed weight (58.99 g) and lower coat fraction (35.8 g/kg), consistent with a typical *kabuli* type, while BC (21.28 g; 131.4 g/kg) was more representative of the *desi* morphotype.

Similarly, the wide variation in total phenolic content (TPC; 1.60–4.55 mg GAE/g_{ss}) was largely attributable to seed coat colour. In fact, samples with brown or black-pigmented coats exhibited significantly higher TPC values compared to cream or white-coated types. The commercial variety BL, a white-seeded *kabuli* type, recorded the lowest TPC value (1.42 mg GAE/g_{ss}), while the value recorded for BC variety (3.31 mg GAE/g_{ss}) was intermediate, consistent with its black coat.

Regarding trypsin inhibitor (TI) content in seeds, a wide variation was recorded, with the lowest value of 9.23 TIU mg⁻¹ in sample L301 and the highest of 15.76 TIU mg⁻¹ in sample L315.

Pearson correlation analysis of the compositional dataset revealed several significant associations (Table 4). Seed coat percentage was negatively correlated with 100-seed weight ($r = -0.73$) and Swelling Index (SI; $r = -0.80$), but positively correlated with TPC ($r = 0.93$), TI activity ($r = 0.45$), and carotenoid content ($r = 0.54$), suggesting that the coat fraction is a key driver of antioxidant and anti-nutritional compound accumulation. Ash and P_{tot} had a strong positive correlation ($r = 0.87$), reflecting the predominantly mineral nature of phosphorus storage in legume seeds. TPC was also positively correlated with carotenoid content ($r = 0.68$) and coat percentage ($r = 0.93$), pointing to a coordinated accumulation of secondary metabolites in pigmented seed coats. There was a negative correlation between SI and seed coat percentage ($r = -0.80$), suggesting that *desi* types hydrate less efficiently than the larger, thin-coated *kabuli* types.

The Principal Component Analysis (PCA) of the biochemical data revealed that the Apulian varieties can be divided into distinct subgroups according to seed coat colour, which in turn correlates with the *desi* and *kabuli* morphotypes. In the three-dimensional PCA plot (Figure 4), a subgroup of five local varieties with brown or black seed coats clustered on the left side. The only exception was L302, a black-seeded samples that fell on the right side of the plot, clustering with the white-seeded types L301 and L316, possibly indicating biochemical similarities that transcend seed coat pigmentation. The reddish-brown accession L303 remained intermediate, not clearly associated with either subgroup. Finally, *Blanco Lechoso*, included in the study as an outgroup, appeared well separated from all Apulian chickpeas, confirming its distinct genetic and biochemical background.

Table 3. Seed quality traits of studied local and commercial varieties. The values were on dry matter base.

Work code	100-seed weight (g)	SI (%)	HI (%)	Coat (g/kg)	Cooking time (min)	Moisture (g/kg)	Protein (g/kg)	Ash (g/kg)	TI (TIU/mg)	Carotenoid (µg lut/g)	P _{tot} (mg/100g)	TPC (mg GAE/g)	P _i (mg/100g)
<i>Local varieties</i>													
L301	26.66	172.7	116.8	52.0	32-33	86.4	268.5	39.7	9.23	29.77	418.7	1.60	61.1
L316	31.29	141.7	106.3	48.0	33-34	104.0	272.8	37.6	11.09	27.54	408.1	1.65	80.9
L79	30.33	116.0	96.7	111.6	35-36	99.7	222.3	35.2	12.88	40.36	324.8	4.04	38.1
L303	34.35	142.8	102.4	94.8	39-40	89.0	199.4	36.1	11.52	38.86	399.9	2.83	54.4
L304	15.65	100.0	111.3	161.0	39-40	83.3	279.8	36.4	13.53	26.84	373.7	4.12	43.7
L302	41.85	125.0	99.5	86.8	36-37	88.3	274.5	37.0	11.41	34.99	378.5	2.72	45.5
L315	21.71	105.7	120.0	143.3	30-31	94.7	223.9	41.1	15.76	48.66	373.4	4.55	38.6
L317	22.68	118.2	102.3	139.7	31-32	95.3	220.6	36.0	9.86	30.53	404.4	3.22	42.6
Mean tot.	28.06	127.8	106.9	104.6	34.88	92.5	245.2	37.4	11.91	34.69	385.2	3.09	50.6
Std. dev.	8.199	23.709	8.363	41.991	3.461	7.055	31.716	2.024	2.099	7.550	29.751	1.109	14.544
<i>Commercial varieties</i>													
BL	58.99	134.6	97.2	35.8	33-34	78.0	241.0	3.34	12.75	7.51	251.2	1.42	36.4
BC	21.28	100.0	99.8	131.4	-----	70.0	251.3	3.36	12.79	36.12	234.3	3.31	-----

SI = Swelling Index; HI = Hydration Index; TI = trypsin inhibitor; P_{tot} = Total phosphorus; P_i = Inorganic phosphorus; TPC = Total Phenolic Compounds.

Table 4. Correlation matrix. Values in bold and underlined are significant at $p < 0.05$.

	100-seed weight	SI	HI	Coat	Cooking time	Protein	Ash	TI	Carotenoid	P _{tot}	TPC	P _i
100-seed weight	1	0,32	-0,63	<u>-0.73</u>	0,01	-0,05	<u>-0.81</u>	-0,08	-0,58	<u>-0.68</u>	-0,63	-0,12
SI		1	0,06	<u>-0.80</u>	-0,13	0,12	-0,07	<u>-0.72</u>	-0,26	0,25	<u>-0.83</u>	0,61

HI	1	0,26	-0,32	0,23	0,51	0,22	0,36	0,50	0,22	0,21
Coat		1	0,16	-0,23	0,48	0,45	0,54	0,22	0,93	-0,49
Cooking time			1	0,09	0,04	0,04	-0,02	0,00	0,14	0,00
Proteins				1	0,08	-0,18	-0,36	0,14	-0,31	0,38
Ash					1	-0,10	0,81	0,87	0,46	0,35
TI						1	0,29	-0,45	0,65	-0,51
Carotenoid							1	0,51	0,68	-0,05
P _{tot}								1	0,06	0,60
TPC									1	-0,55
P _i										1

SI = Swelling Index; HI = Hydration Index; TI = trypsin inhibitor; P_{tot} = Total phosphorus; P_i = Inorganic phosphorus; TPC = Total Phenolic Compounds.

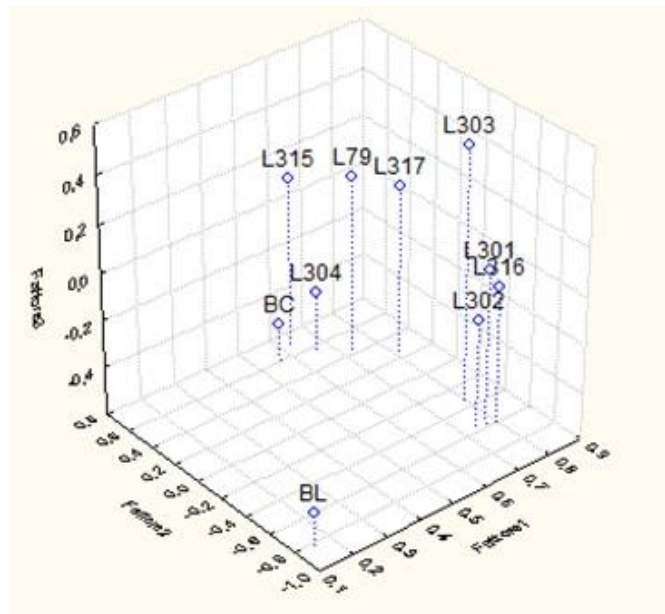


Fig. 4 Three-dimensional principal component analysis (PCA) score plot showing the distribution of samples along the first three principal components. Each point represents an individual sample (BC, BL, L301, L302, L303, L304, L315, L316, L317, L79), with dashed lines indicating projection onto the PC1-PC2 plane

4. Discussion

The present study focuses on the comprehensive characterization of some Apulian chickpea local varieties that, shaped by defined ecogeographical conditions and traditional cultural practices, may represent a valuable option within the framework of modern and sustainable agriculture to address the challenges posed by ongoing climate change. Chickpea, one of the most important food legumes worldwide, exhibits high capacity to thrive under semiarid conditions and could therefore constitute a viable alternative crop for areas affected by soil degradation and desertification risk, such as Southern Italy, which is recognized as a critical desertification hotspot in the Mediterranean basin (Hill et al. 2008; Gambella et al. 2021).

The genetic characterization of nine chickpea local varieties using SSR markers revealed a moderate but appreciable level of genetic variability despite the geographically restricted cultivation area of these local varieties and the predominantly self-pollinated nature of chickpea, with a mean of 7 alleles per locus and expected heterozygosity (He) averaging 0.72 (Table 2). These findings align with previous studies demonstrating that traditional cultivation practices maintain meaningful genetic differentiation even across limited geographic areas (Sefera et al. 2011; Torutaeva et al. 2014; Ghaffari et al. 2014; Zatybekov et al. 2025). The observation that 76.4% of loci exhibited PIC values exceeding 0.50 demonstrates the high informativeness of the SSR panel employed in this study. A particularly noteworthy finding was the genetic distinctiveness of the black chickpea accessions L315 and L317, which clustered close to the BC reference

in the PCoA plot (Figure 2), echoing the results by Pavan et al. (2017) who evidenced that, using genome-wide SNP markers, black-seeded Southern Italian chickpeas are a genetically distinct cluster separate from commercial types. Similarly, De Giovanni et al. (2017) found that genetic structure analysis of a chickpea collection from major chickpea-growing countries clearly distinguished the Italian accessions, particularly within the K3-STRUCTURE subpopulation, highlighting their unique genetic architecture. Additionally, the use of genetic characterization tools has proven highly effective in resolving cases of homonymy, synonymy, or misattribution of names in several plant species, including legumes (Oliveira et al. 2016; Haliloglu et al. 2022; Mir et al. 2022). However, as observed in the present study for the “Cece di Nardò” variety, discordant genotypic profiles cannot automatically be ascribed to contamination with foreign material. Therefore, the characterization of additional samples will be required to determine whether the observed results reflect contamination events or, rather, the presence of genetic diversity within this local variety.

To investigate the plasticity and stress responsiveness of the Apulian landraces to water deficit, we focused on the genetic and transcriptional profiles of genes encoding protease inhibitors (PIs), which participate in several physiological and metabolic pathways and are particularly abundant in legume species (Molsov and Valueva 2005). Although information about PIs in drought-exposed chickpea is limited in the literature, proteomic analyses indicate that these enzymes contribute to protein reorganization mechanisms in legumes, thus providing plant protection not only against pathogens and storage pests but also against abiotic stresses such as water deficit (Singh et al. 2023). In particular, Kunitz-type trypsin inhibitors (KTIs) encoded by the *CaTPI-1* and *CaKTI* genes have been reported to play a role in drought-stress tolerance and are often upregulated under water deficit conditions, suggesting an active function in stress adaptation (Vaseva et al. 2012; Kidrič et al. 2014; Moloi and Ngara 2023).

Analysis of *CaTPI-1* mRNA accumulation in the Apulian chickpea varieties revealed that transcripts accumulated predominantly in the roots of pigmented-seed local varieties, including the black-seeded L302 and L315, and the brown-seeded L303, L304, and L79 (Figure 3). These findings agree with previous studies demonstrating that PI genes display specialized, tissue-specific expression patterns in response to abiotic stresses such as heat, drought and salinity (Mangena 2022). Furthermore, PI genes modulate hormone-related signalling pathways during biotic stress (Sharma and Suresh 2015; Fan et al. 2019), acting preferentially in tissues directly exposed to the stress stimulus. Consistent with these observations, we detected a clear differentiation in gene expression between roots and leaves. Specifically, roots subjected to PEG treatment exhibited a rapid and pronounced transcriptional response, whereas leaves displayed a delayed and attenuated response, likely because they were not directly exposed to the osmotic stress. The upregulation of *CaTPI-1* under water deficit conditions indicates that plants activate protective mechanisms to suppress uncontrolled proteolysis by inhibiting endogenous proteases, thereby preventing degradation of essential structural and functional proteins.

Notably, the genotypes exhibiting the highest *CaTPI-1* transcript levels in root tissues were characterized by brownish or black seed coats. In line with the molecular characterization, the Apulian pigmented chickpea displayed a distinctly different behaviour from the beige types under stress conditions, suggesting significant genetic divergence between the two groups.

Beyond their potential for enhanced drought stress tolerance, the nutritional value of Apulian local varieties was also evaluated, as their genetic diversity may encompass variation in seed composition. In our study, the biochemical parameters were consistent with those reported by Choi et al. (2024), who compared 122 chickpea breeding lines, cultivars, and landraces of different geographic origins. Compared with previous studies on the nutritional profile of other Italian chickpea local varieties and breeding lines (Zaccardelli et al. 2013), Apulian chickpeas showed higher protein contents (245.2 vs 198.5 g kg⁻¹, respectively) but were characterised by the highest coat percentage, a feature that consumers do not particularly appreciate. As expected, the TPC content of black and brown local varieties significantly exceeded that of beige types. However, the black chickpea L302 showed a TPC content appreciably lower than that of the other two black types and was also characterized by a higher seed weight (Table 3). Summo et al. (2019), who compared Apulian chickpeas with those from other origins, reported similar results and suggested the presence in the Apulia germplasm of black genotypes lacking the alleles responsible for anthocyanin accumulation. Overall, the divergence between coloured and beige types was confirmed by seed compositional traits because the two subgroups (coloured vs beige) fell into different regions of the 3D PCA plot (Figure 4).

5. Conclusions

This multidisciplinary investigation combining molecular, physiological, and biochemical characterization revealed substantial genetic and functional diversity within Apulian chickpea landraces. Coloured-seeded accessions showed stronger upregulation of drought-related genes, together with higher levels of bioactive compounds, supporting their

potential as breeding material for sustainable agriculture under water-limited conditions. The maintenance of genetic polymorphism through farmer-mediated selection cycles makes these landraces particularly suitable for participatory breeding and on-farm conservation strategies. A synergistic approach integrating *ex situ* conservation, on-farm management, and participatory breeding is recommended to enhance the resilience of Mediterranean chickpea to future climate scenarios. Although consumer demand for black-seeded types is significantly lower than that for beige types, these landraces may still have a chance of success if included in cultural and gastronomic tours linked to their traditional areas of cultivation, such as some areas of the Apulia region.

Supplementary Materials:

Table S1. List of primers used for gene amplification and RT-qPCR assays.

Name	Sequence (5'-3')	Ta (°C)	Amplicon size (bp)
<i>Primers for gene amplification</i>			
CaSIL_1_For	TCCTTGCTCAACTGTGGCTT	55	473
CaSIL_1_Rer	GCACACCTCAGTAGGACACC		
CaKTI_For	GGAACGAAGATGTTGAACAAG	55	600
CaKTI_Rev	AACAACAGATTTAACAATTCC		
CaTPI-1_For	GCTATTGAGCAAGTGTGGATACAAATGG	58	809
CaTPI-1_Rev	CTAAACTGACGCATCAAATCCACC		
<i>Primers for RT-qPCR</i>			
CaCqPCR-HK_For	CATGGACTAGACCACCAATTCA	60	109
CaCqPCR-HK_Rev	AACAGTGTGTACCCGCTCTTT		
CaTPI-1_qPCR_For	AGCTTTCTCAAACGAAGATGTTGA	62	106
CaTPI-1_qPCR_Rev	ACCTGGAGGTCCACGAATTG		
B-CaKTI_qPCR_For	TCAGAGTGTCCAGTTACCGT	60	125
B-CaKTI_qPCR_Rev	AACTCGATTTCAATTGGTGTACC		

Table S2. Microsatellites used to analyse the chickpea collection. Locus type, primer sequence, linkage group, annealing temperature and amplicon size range are indicated. An M13 tail (TGTAACGACGGCCAGT) was added at the 5' end of each forward primer.

SSR locus	Primer sequence 5'-3'	LG	Ta (°C)	Amplicon size range (bp)	Reference
H3C06	Fw: AATTCGTAATCATTAATAATAGAGG Rv: CACATGACTATCTAGACATTTATTTATC	LG3	60	188-220	Lichtenzveig et al. (2005)
H4D08	Fw: TGTCTTTATTTCTTAAGCACACAT Rv: GAGATGGATGTTATTGGACTCATC	LG2	60	202-232	Lichtenzveig et al. (2005)
H1F14	Fw: GAGAGAGAGGAAGGGAACG Rv: TCCTAACTTGCTCCTTAACCTTG	LG3	59	217-223	Lichtenzveig et al. (2005)
H3F08	Fw: AAACACCCGTGATTCTCTAAAGTT Rv: TGACACCTAATTTATTCGGTTTTT	LG3	td 65-52	214-272	Lichtenzveig et al. (2005)
ICCM0069	Fw: TCTTCTTGCTATCTGTCTCGC Rv: TGCATGTCAAACATTAGACAACTTT	LG4	59	220-258	Nayak et al. (2010)
TR29	Fw: GCCCACTGAAAAATAAAAG Rv: ATTTGAACCTCAAGTTCTCG	LG3	td 64-52	194-246	Admas et al. (2021)
H4H01	Fw: TAGTATTTTCTTTCATTTCTTCGTT Rv: TTGCTTAATTCATTATGGTATATTTATG	LG6	td 65-52	282-296	Lichtenzveig et al. (2005)
H5E11	Fw: ATTTATTTAGTGGCCTCACTCTCC Rv: GATTTCAAAATCCCTTTGTTCTAGTTA	LG7	td 64-52	217-279	Lichtenzveig et al. (2005)
GM37999	Fw: GCAAGGCTAGTGCATCAACA Rv: TTGTACCCCAATTTTGTCCG	Ca8	td 65-52	252-294	Mir et al. (2022)
H6C07	Fw: CAAAAGTGAATTAAGCCTACATAATA	LG3	td 65-52	216-242	Lichtenzveig et al. (2005)

	Rv: CCATTTTGTTTTAACACATATTTAACG				
ICCM0068	Fw: TCTTCTTTGCTATCTGTCTCGC Rv: TGCATGTCAAACATTAGACAACCTTT	LG4	td 68-55	220-260	Nayak et al. (2010)
ICCM0076	Fw: CTCATCGAATAGAACCTACCGA Rv: CCGCTACACCTACAACGGTAA	LG5	td 65-52	270-310	Nayak et al. (2010)
CESSRDB47	Fw: ACGAAGAAAGTTCCTGTGAA Rv: ACCGAAAACCTGATTCATTA	EST	td 62-52	256-268	Choudhary et al. (2009)
ICCM0249	Fw: TTTCTTCGCATGGGCTTAAC Rv: GGAGATTTGTTGGGTAGGCTC	LG4	td 62-52	143-167	Nayak et al. (2010)
NCPGR21	Fw: TCTACCTCGTTTTTCGTGCC Rv: TTGCTCCTTCAACAAAACCC	LG4	td 64-52	162-184	Sethy et al. (2006)
NCPGR127	Fw: CATAATGCAAGGCAATTAG Rv: CTCTTATCTTCATGTTGCCG	LG4	td 64-52	198-206	Sethy et al. (2006)
STMS11	Fw: TCTCACAGCAGTGGTCCTCTT Rv: AATGTCAAATTGAAGCCACCT	LG2	td 62-52	149-153	Winter et al. (1999)

Figure S1. Alignment of the cloned and sequenced CaTPI-1, CaKTI, and CaSIL gene fragments encoding Kunitz-type trypsin inhibitor-like proteins from *C. arietinum*, using an NCBI reference sequence (shown as the last sequence in each alignment).

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