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Identifying chickpea (*Cicer arietinum* L.) genotypes rich in ascorbic acid as a source of drought tolerance

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Drought stress induces a range of physiological changes in plants, including oxidative damage. Ascorbic acid (AsA), commonly known as vitamin C, is a vital non-enzymatic antioxidant capable of scavenging reactive oxygen species and modulating key physiological processes in crops under abiotic stresses like drought. Chickpea (*Cicer arietinum* L.), predominantly cultivated in drought-prone regions, offers an ideal model for studying drought tolerance. We explored the potential of AsA phenotyping to enhance drought tolerance in chickpea. Using an automated phenomics facility to monitor daily soil moisture levels, we developed a protocol to screen chickpea genotypes for endogenous AsA content. The results showed that AsA accumulation peaked at 30% field capacity (FC)—when measured between 11:30 am and 12:00 noon—coinciding with the maximum solar radiation (32 °C). Using this protocol, we screened 104 diverse chickpea genotypes and two control varieties for genetic variability in AsA accumulation under soil moisture depletion, identifying two groups of genotypes with differing AsA levels. Field trials over two consecutive years revealed that genotypes with higher AsA content, such as BDNG-2018-15 and PG-1201-20, exhibited enhanced drought tolerance and minimal reductions in yield compared to standard cultivars. These AsA-rich genotypes hold promise as valuable genetic resources for breeding programs aimed at improving drought tolerance in chickpea.

Keywords *Cicer arietinum* L., Plant phenomics, Endogenous ascorbic acid, Field capacity and drought tolerance

Chickpea (*Cicer arietinum* L.) is the third most important legume globally[1], with its evolutionary origin in the hot, arid regions of Turkey and northern Syria[2]. Chickpea is cultivated in over 50 countries, primarily in semi-arid environments, with India accounting for 70% of global production[3–5]. In eastern and southern Africa, Ethiopia, Tanzania, and Sudan are major producers, with significant potential for expansion in Sub-Saharan Africa. However, climate extremes such as unpredictable rainfall, drought, and heat stress pose major challenges to chickpea cultivation worldwide[4,6], with drought alone responsible for 40–45% of yield losses[7].

Soil moisture deficit can lead to an overproduction of reactive oxygen species (ROS), such as hydrogen peroxide, singlet oxygen, superoxide anion radicals, and hydroxyl and organic peroxyl radicals[8], which damage plant cells by oxidizing lipids, carbohydrates, proteins, enzymes, and DNA, often resulting in plant death[9,10]. Plants have evolved various defense mechanisms, including enzymatic and non-enzymatic antioxidants, to counteract these stressors[11,12]. Non-enzymatic defenses include the synthesis of metabolites, such as ascorbic acid (AsA), glutathione, phenolic acids, alkaloids, flavonoids, carotenoids, α-tocopherol, and nonprotein amino acids[13,14]. Among the non-enzymatic defenses, AsA is a key antioxidant that detoxifies ROS[15,16] and regulates essential processes like photosynthesis, cell division, and cell differentiation[17,18]. It also acts as a signaling molecule in stress response pathways[10].

With drought stress projected to worsen under climate change, there is an urgent need to develop drought-tolerant cultivars to ensure sustainable agricultural production[19–21]. One approach is to harness plant traits like

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AsA accumulation, which could enhance stress tolerance while maintaining productivity[22]. We hypothesized that phenotyping chickpea for AsA content would provide valuable insights into drought tolerance. Because AsA levels are highly sensitive to soil moisture stress, we aimed to determine the optimal moisture level for AsA assessment, allowing us to distinguish between drought-tolerant and drought-sensitive genotypes. Leveraging a phenomics platform equipped with precise soil moisture monitoring systems, we screened chickpea genotypes at this optimal soil moisture level and evaluated the significance of AsA accumulation for chickpea productivity under limited irrigation.

Results

Protocol standardization for estimating endogenous ascorbic acid

The initial AsA content in well-watered plants was 203.94 μg g^{-1} FW at 60% FC. As the soil moisture level decreased, AsA content increased, peaking at approximately 30 ± 1% FC with measured 284.97 μg g^{-1} FW (Fig. 1C). When soil moisture dropped to 20% FC, the plants did not survive despite rewatering. A repeated experiment confirmed that leaf AsA peaked at 30 ± 1% FC (Fig. 2).

Efficacy of method for differentiating drought-tolerant and drought-sensitive genotypes

Drought-tolerant Vijay accumulated more AsA than drought-sensitive Vishal under well-watered and water-stressed conditions (Fig. 1B). At 60% FC, Vijay had 234.29 μg g^{-1} FW AsA, while Vishal only had 173.59 μg g^{-1} FW. Both check varieties exhibited a similar AsA accumulation pattern from 60% FC to 30 ± 1% FC, peaking at 324.67 μg g^{-1} FW for Vijay and 245.27 μg g^{-1} FW for Vishal (Fig. 1C) before declining at 20% FC, without recovery after rewatering.

Genetic variation in endogenous AsA accumulation

Leaves

Among the 104 chickpea genotypes, significant genetic variation in endogenous leaf AsA content was observed at 30% FC (Supplementary Table 2). Two AsA-rich genotypes, BDNG-2018-15 and PG-1201-20, with high AsA contents (367.12 and 361.25 μg g^{-1} FW, respectively), and two AsA-poor genotypes, C-19159 and C-19294, with low AsA contents (179.87 and 186.28 μg g^{-1} FW, respectively), were selected for further investigation (Fig. 3A).

Seeds

The seed AsA content of the 104 genotypes varied significantly (Supplementary Table 2). Seeds had about 3.26 times lower AsA contents than fresh leaves. Genotypes PG-1201-20 and BDNG-2018-15 had the highest seed AsA contents (133.57 and 132.99 μg g^{-1} FW, respectively), while C-19294 and C-19159 had the lowest (46.31 and 47.79 μg g^{-1} FW, respectively). The AsA-rich and AsA-poor genotypes followed similar genetic variation trends for leaf and seed AsA contents (Fig. 3B).

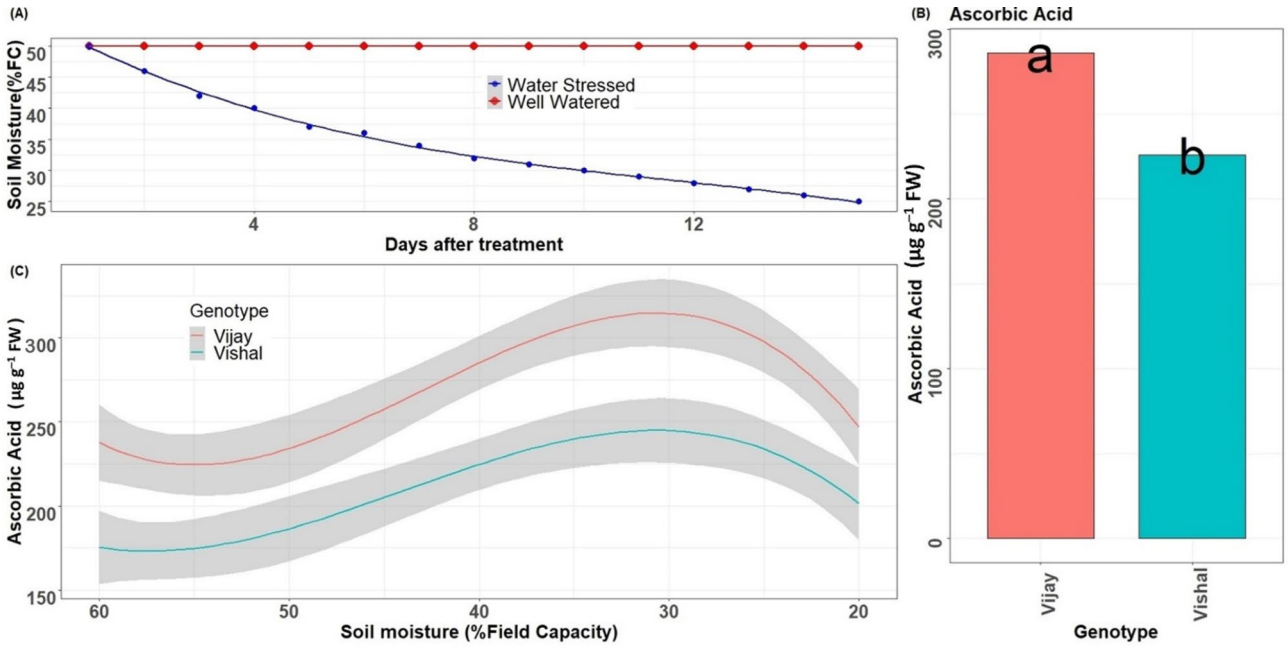

Fig. 1. Ascorbic acid (AsA) response to soil moisture depletion. (**A**) Soil moisture level as indicated by % field capacity in well-watered and water-stressed plants. Each point in the plot indicates mean values for all genotypes in each treatment. (**B**) Maximum content in drought-tolerant and drought-sensitive checks. (**C**) AsA response to depleting soil moisture levels. AsA content peaked at 30 ± 1% field capacity. Solid lines indicate mean values; shaded areas indicate 95% confidence intervals.

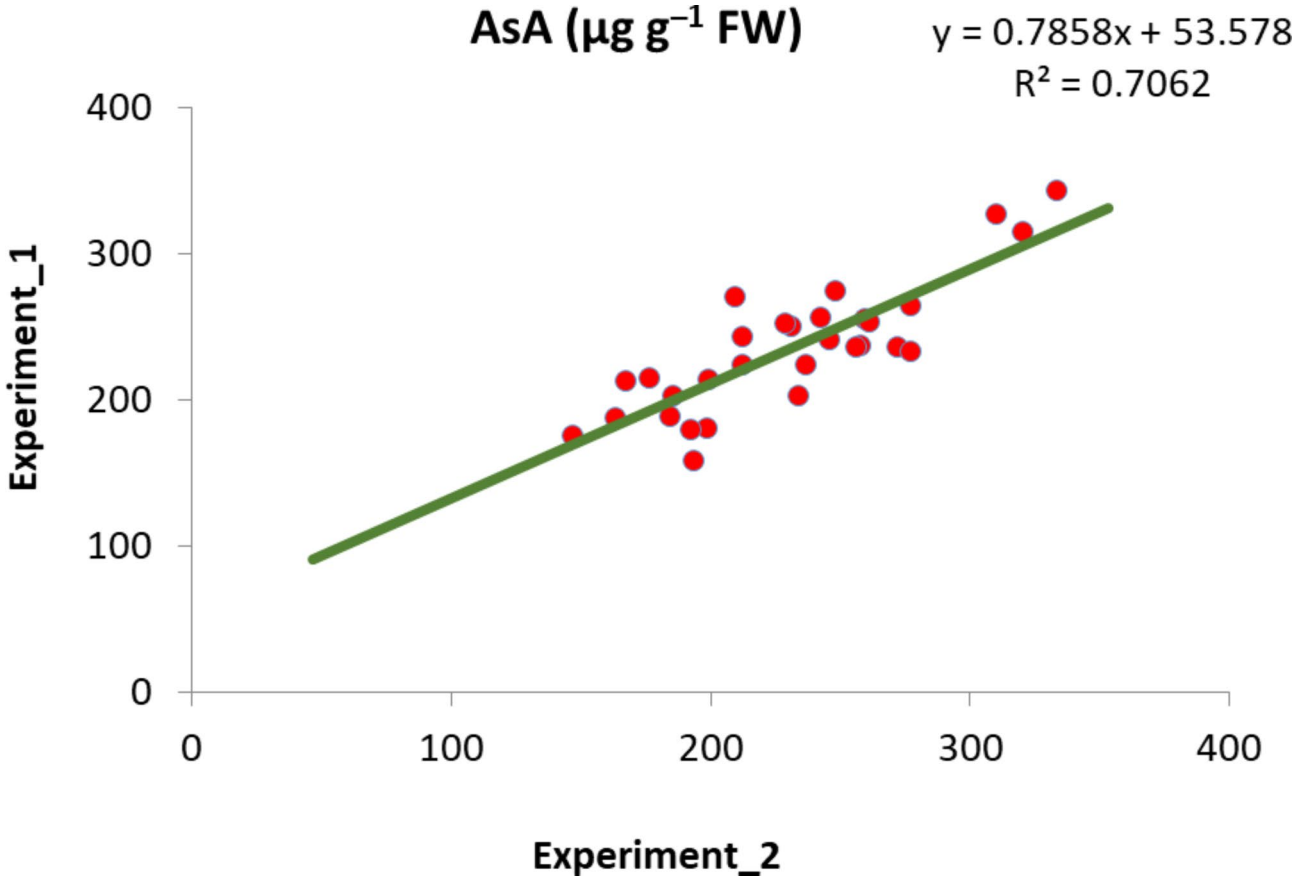

Fig. 2. Validation of ascorbic acid (AsA) content in two contrasting chickpea genotypes by comparing Experiments 1 and 2 at depleting soil moisture levels.

Yield attributes under field conditions

Significant genetic variation in yield attributes occurred across the six genotypes under well-watered and water-stressed conditions, with water stress adversely affecting these attributes in both years. Dry biomass production ranged from 30.77 to 17.47 g plant^{-1} under well-watered conditions and 29.52–14.89 g plant^{-1} under water-stressed conditions. AsA-rich PG-1201-20 and BDNG-2018-15 produced significantly more biomass than the AsA-poor genotypes (C-19159 and C-19294), Vijay, and Vishal under well-watered and water-stressed conditions (Fig. 4). Drought stress significantly decreased dry biomass production in all genotypes, particularly C-19294, which declined by 14.73% (Table 1).

Water stress also decreased pod production, from 43.55 to 67.90 pods plant^{-1} under well-watered conditions to 37.89–64.50 pods plant^{-1}. AsA-rich PG-1201-20 had significantly more pods plant^{-1} than the other genotypes (Fig. 5) and experienced the smallest decline (5%) under water stress, whereas Vishal decreased the most (16%) (Table 1).

Water stress notably reduced grain pod^{-1} specially in AsA-poor genotypes.Under well-watered conditions, the grains pod^{-1} were similar across most genotypes, except for C-19294, which had significantly fewer grains plant^{-1}. Under water stress, BDNG-2018-15 and Vijay produced significantly more grains pod^{-1} (1.11) than C-19294 (1.02) (Fig. 6).

The 100-seed weight ranged from 15.14 to 27.06 g under well-watered conditions and 13.83–26.52 g under water-stressed conditions (Fig. 7). BDNG-2018-15 consistently had the highest 100-seed weight, while C-19294 had the lowest, regardless of moisture levels. Water stress decreased 100-seed weight more significantly in AsA-poor C-19294 (7.89%) than AsA-rich BDNG-2018-15 (2.23%) (Table 1). Higher AsA levels positively correlated with increased 100-seed weight.

Water stress also adversely affected seed yield plant^{-1}, particularly in AsA-poor genotypes. PG-1201-20 had the highest seed yield plant^{-1} under both conditions. Despite performing slightly lower under well-watered conditions, AsA-rich BDNG-2018-15 showed better resilience under water-stressed conditions than AsA-poor genotypes, except for Vishal (Fig. 8). Water stress had a relatively small impact on BDNG-2018-15's seed yield (Table 1).

Stress indices in AsA-rich and AsA-poor genotypes

Multivariate analysis, incorporating yield-related parameters and stress indices (Table 2) over two years, revealed distinct patterns between AsA-rich and AsA-poor genotypes. The first two principal components explained 99.8%

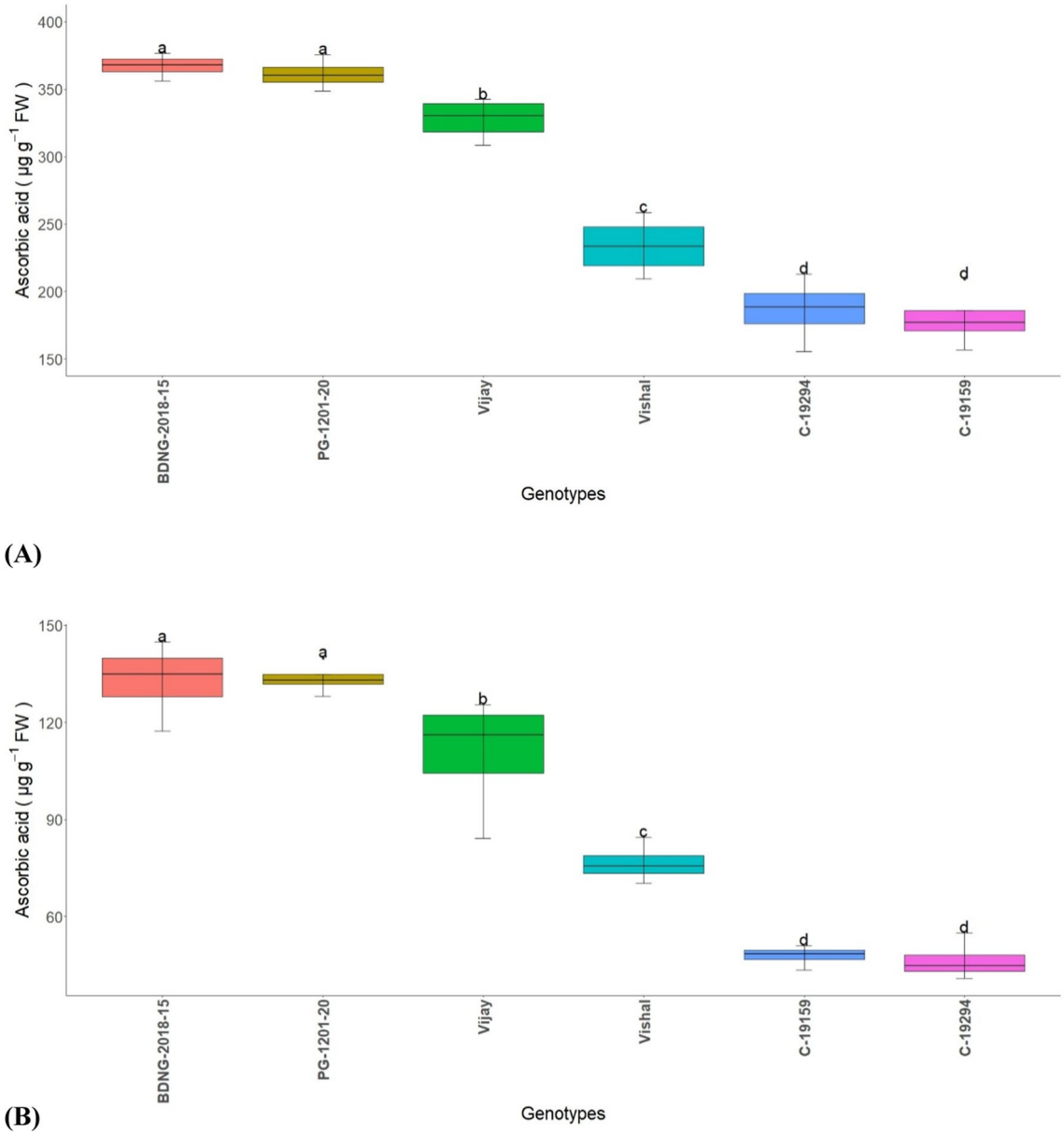

Fig. 3. Genetic variation in ascorbic acid (AsA) content in (**A**) leaves and (**B**) seeds of six contrasting chickpea genotypes [three AsA-rich (on the left) and three AsA-poor (on the right)] screened from 104 chickpea genotypes. Box edges represent the upper and lower quartiles; horizontal lines indicate median values; upper and lower whiskers are the extreme values; letters above bars were derived from Duncan's multiple range test at a 95% confidence interval; genotypes with common letters do not significantly differ.

of the variability, effectively separating the genotypes into two groups (Fig. 9). AsA-poor genotypes clustered together, with relatively high index values for YRR, PYR, SSI, SDI, DSI, SI, TOL, SSPI, and ATI, indicating higher drought sensitivity. Conversely, AsA-rich genotypes were more drought tolerant and associated with other indices. Among them, BDNG-2018-15 and Vijay had high values for GM, RDI, YSI, DTE, and DRI, while PG 1201-20 had high values for DI, HM, GMP, REI, MRP, and STI.

Water stress significantly affected APX activity in AsA-rich and AsA-poor genotypes. Under well-watered conditions, all genotypes exhibited similar APX activity except for C-19294, which had significantly lower activity (169.77 mg^{-1} protien min^{-1}). Water stress drastically increased APX activity in all genotypes, with AsA-rich genotypes showing significantly higher values—BDNG-2018-15 (450.32 mg^{-1} protien min^{-1}), PG-1201-20 (446.58 mg^{-1} protien min^{-1}), and Vijay (438.40 mg^{-1} protien min^{-1}) than AsA-poor genotypes- Vishal (329.14 mg^{-1} protien min^{-1}), C-19159 (323.94 mg^{-1} protien min^{-1}) and C-19294 (298.43 mg^{-1} protien min^{-1}) (Supplementary Fig. 1).

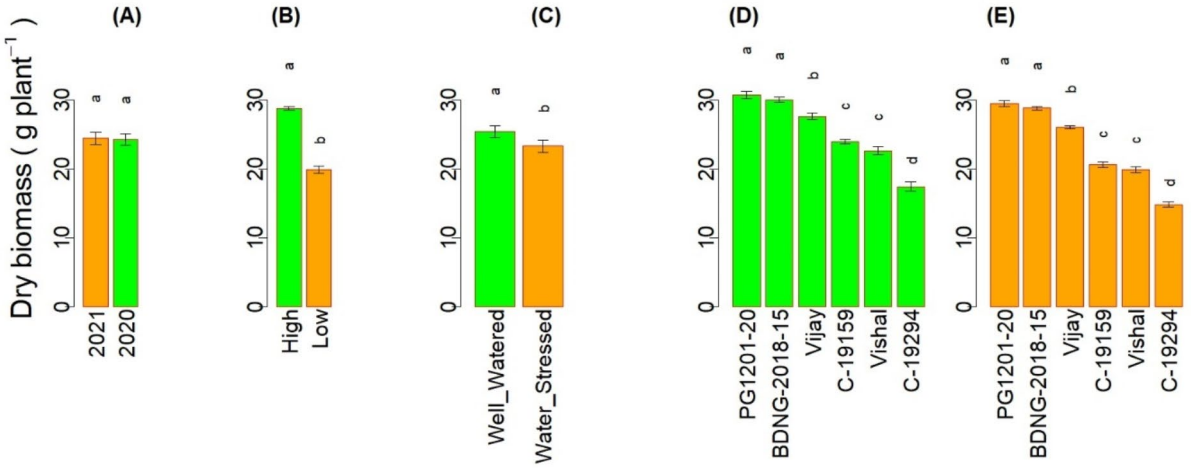

Fig. 4. Effect of crop season (**A**) year effect, (**B**) tissue ascorbic acid (AsA) content, and (**C**) soil water regime (treatment effect) on dry biomass (g plant^{-1}) of selected AsA-rich and AsA-poor chickpea genotypes. Genotype effects on dry biomass (**D**) without stress (well-watered) and (**E**) with stress (water-stressed). Different letters above bars within a figure indicate significant differences among mean values as computed by Duncan's multiple range test at a 95% confidence interval.

	BDNG-2018-15	PG-1201-20	Vijay	C-19294	C-19159	Vishal
DM	3.97	4.04	5.80	14.73	13.71	10.73
PP	5.62	5.01	11.52	12.99	15.51	15.69
GP	2.62	6.82	1.33	2.07	8.90	7.55
SW	2.23	3.82	3.82	7.89	5.58	7.84
YP	12.37	12.60	15.53	30.95	43.28	38.09

Table 1. Percent decrease in yield attributes of selected six chickpea genotypes under water stress in the field relative to well-watered conditions. DM, dry biomass (g); PP, pods plant^{-1}; GP, grains pod^{-1}; SW, 100-seed weight (g); YP, yield plant^{-1} (g); YH.

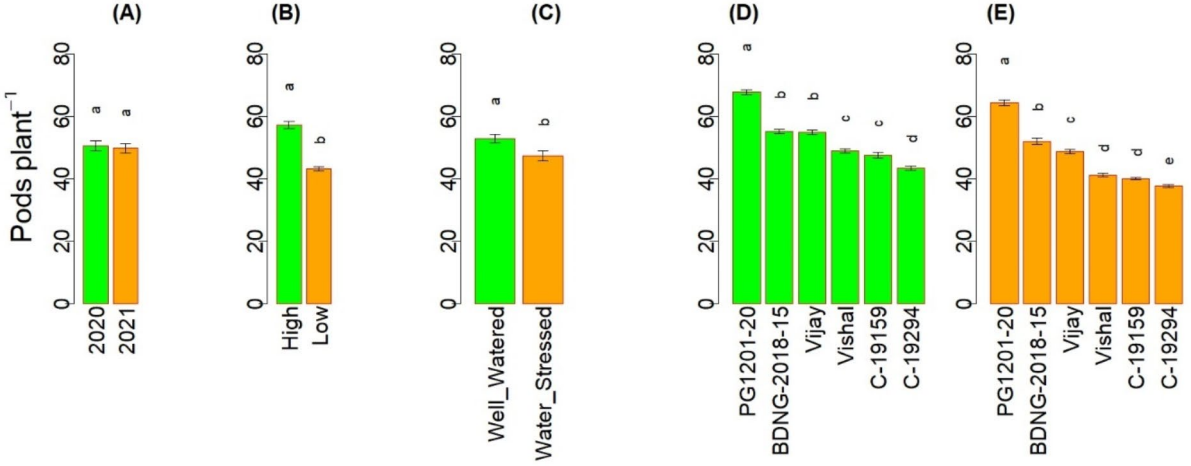

Fig. 5. Effect of crop season (**A**) year effect, (**B**) tissue ascorbic acid (AsA) content, and (**C**) soil water regime (treatment effect) on pods plant^{-1} of selected AsA-rich and AsA-poor chickpea genotypes. Genotype effects on pods plant^{-1} (**D**) without stress (well-watered) and (**E**) with stress (water-stressed). Different letters above bars within a figure indicate significant differences among mean values as computed by Duncan's multiple range test at a 95% confidence interval.

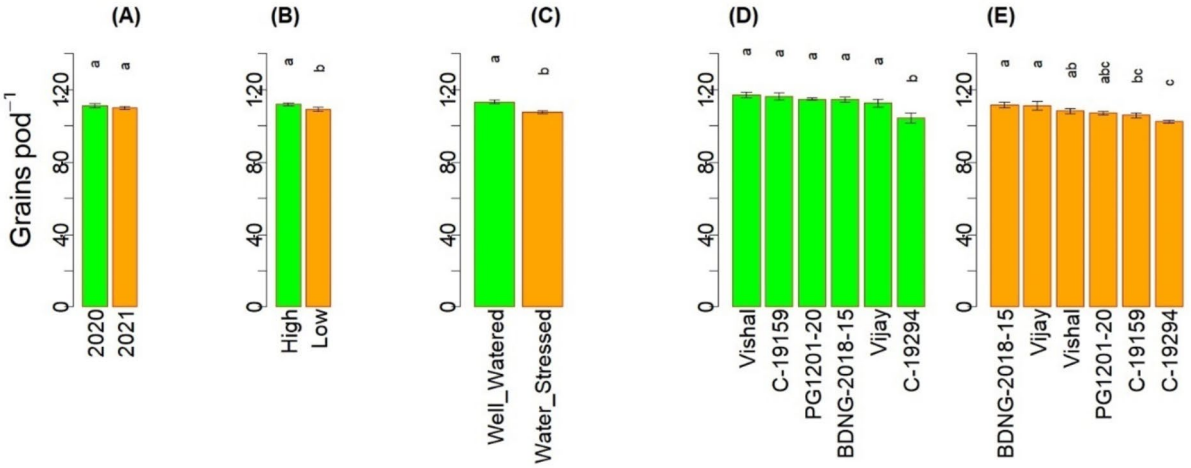

Fig. 6. Effect of crop season (**A**) year effect, (**B**) tissue ascorbic acid (AsA) content, and (**C**) soil water regime (treatment effect) on grains pod^{-1} of selected AsA-rich and AsA-poor chickpea genotypes. Genotype effects on grains pod^{-1} (**D**) without stress (well-watered) and (**E**) with stress (water-stressed). Different letters above bars within a figure indicate significant differences among mean values as computed by Duncan's multiple range test at a 95% confidence interval.

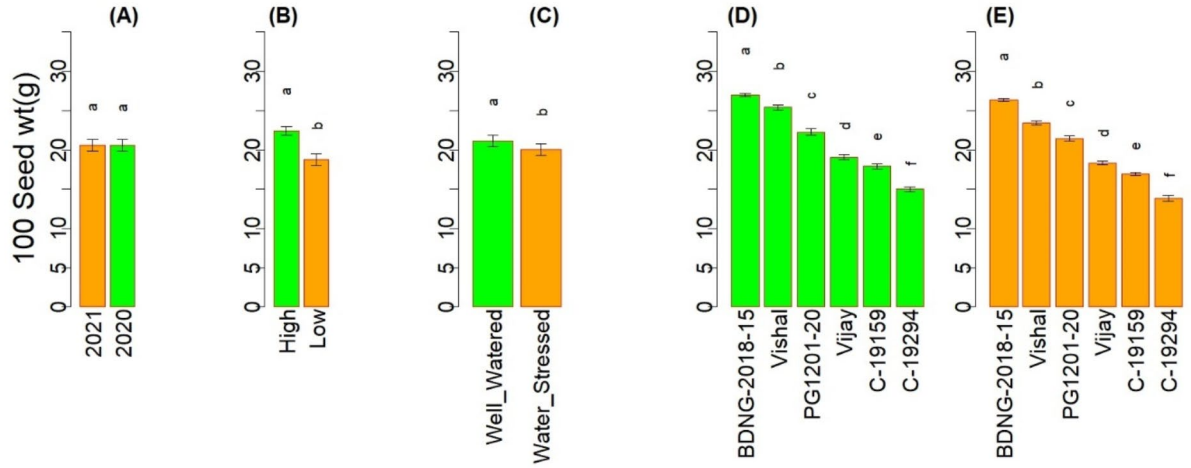

Fig. 7. Effect of crop season (**A**) year effect, (**B**) tissue ascorbic acid (AsA) content, and (**C**) soil water regime (treatment effect) on 100-seed weight (g) of selected AsA-rich and AsA-poor chickpea genotypes. Genotype effects on 100-seed weight (**D**) without stress (well-watered) and (**E**) with stress (water-stressed). Different letters above bars within a figure indicate significant differences among mean values as computed by Duncan's multiple range test at a 95% confidence interval.

Discussion

Integrating AsA estimation into the phenotyping protocol for chickpea drought tolerance

Changes in AsA levels in plants under drought conditions are widely recognized as an adaptive response, primarily due to its role in antioxidant defense mechanisms[40,41]. While AsA has been applied in drought mitigation through seed priming techniques, its use in genetic enhancement has been limited due to the lack of standardized phenotyping protocols. This study aimed to establish a protocol for incorporating AsA levels into the selection process for breeding drought-tolerant chickpea cultivars.

The study began by estimating AsA for screening chickpea genotypes based on the soil moisture level where intrinsic AsA peaks. Using automated watering and weighing systems to monitor soil moisture daily, we established that peak AsA levels in chickpea occur when soil moisture drops to 30 + 1% FC. The differences in AsA levels between drought-tolerant (Vijay) and drought-sensitive (Vishal) chickpea cultivars also peaked at this stage. A similar study involving three chickpea genotypes (Akcin, Gokce, and Uzunlu-99) reported peak

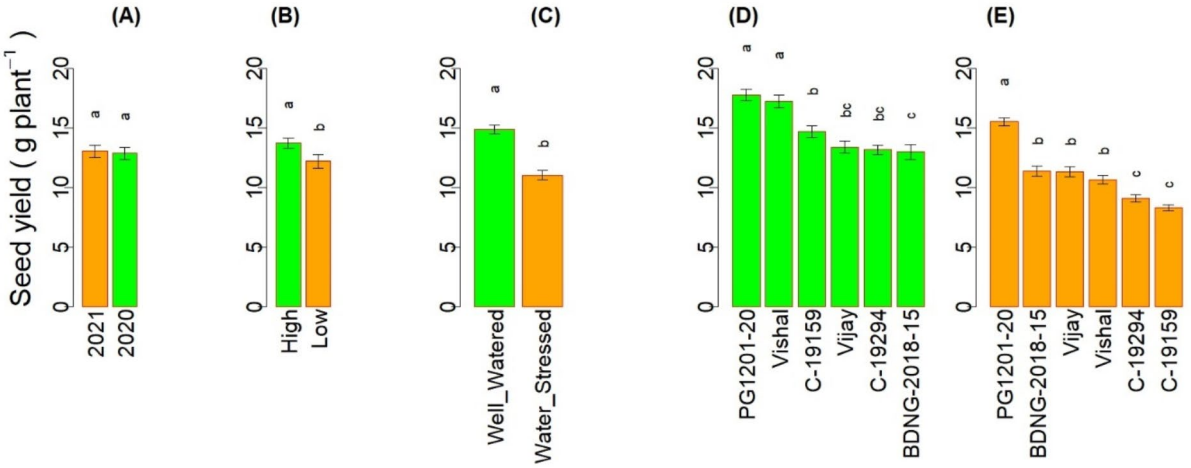

Fig. 8. Effect of crop season (**A**) year effect, (**B**) tissue ascorbic acid (AsA) content, and (**C**) soil water regime (treatment effect) on seed yield (g plant^{-1}) of selected AsA-rich and AsA-poor chickpea genotypes. Genotype effects on seed yield (**D**) without stress (well-watered) and (E) with stress (water-stressed). Different letters above bars within a figure indicate significant differences among mean values as computed by Duncan's multiple range test at a 95% confidence interval.

Index	Formula	References
Stress tolerance (TOL)	Yp−Ys	[23]
Geometric mean productivity (GMP)	√Yp×Ys	[24]
Stress susceptibility index (SSI)	[1−(Ys/Yp)]/SI	[25]
Stress index (SI)	1−(Ys/Yp)	[25]
Stress tolerance index (STI)	(Yp×Ys)/(Yp^2)	[24]
Yield index (YI)	Ys/Ys	[26]
Yield stability index (YSI)	Ys/Yp	[27]
Drought resistance index (DI)	(Ys×(Ys/Yp))/Ys	[28]
Harmonic mean (HM)	[2(Ypx×Ys)]/[Yp +Ys]	[29]
Relative drought index (RDI)	(Ys/Yp)/(Ys/Yp)	[30]
Stress susceptibility percentage index (SSPI)	[(Yp−Ys)/2(Yp)]×100	[31]
Mean relative performance (MRP)	(Ysi/Ys)+(Ypi/YP)	[32]
Relative efficiency index (REI)	(Ysi/Ys)×(Ypi/Yp)	[32]
Modified stress tolerance index 1 (MSTIk1))	[(Ypi)^2/(Yp)^2]×STI	[33]
Modified stress tolerance index 2 (MSTIk2)	[(Ysi)^2/(Ys)^2]×STI	[33]
Sensitivity drought index (SDI)	(Ypi−Ysi)/Ypi	[34]
Golden mean (GM)	(Ypi+Ysi)/(Ypi−Ysi)	[35]
Relative decrease in yield (RDY)	100−[(Ysi/100)×Ypi]	[36]
Drought tolerance efficiency (DTE)	(Ysi/Ypi)×100	[37]
Abiotic tolerance index (ATI)	[(Ypi−Ysi)/(Yp/Ys)]×(√Ypi×Ysi)	[31]
Yield reduction ratio (YRR)	1−(Yp/Ys)	[38]
Percent yield reduction (PYR)	[(Yp−Ys)/Yp]×100	[39]
Drought susceptibility index (DSI)	[(1−Ys)/Yp]/(Ys/Yp)	[37]

Table 2. Drought tolerance indices, formulas, and references. Yp and ys are the grain yield of each genotype under well-watered and water-stressed conditions, respectively.

AsA levels at 40% FC, relative to the control (60% FC), under early and late drought stress[42]. Other studies have shown similar results[43], where significant increases in antioxidant activity at 30% FC in oilseed rape, and maximum AsA content at 40–60% FC in quinoa compared to 100% FC (control), with a decline at 20% FC[44]. Additionally, a study found that endogenous AsA activity in sweet pepper plants increased significantly from 60 to 30% FC[45]. These findings underscore the importance of integrating AsA estimation into drought tolerance

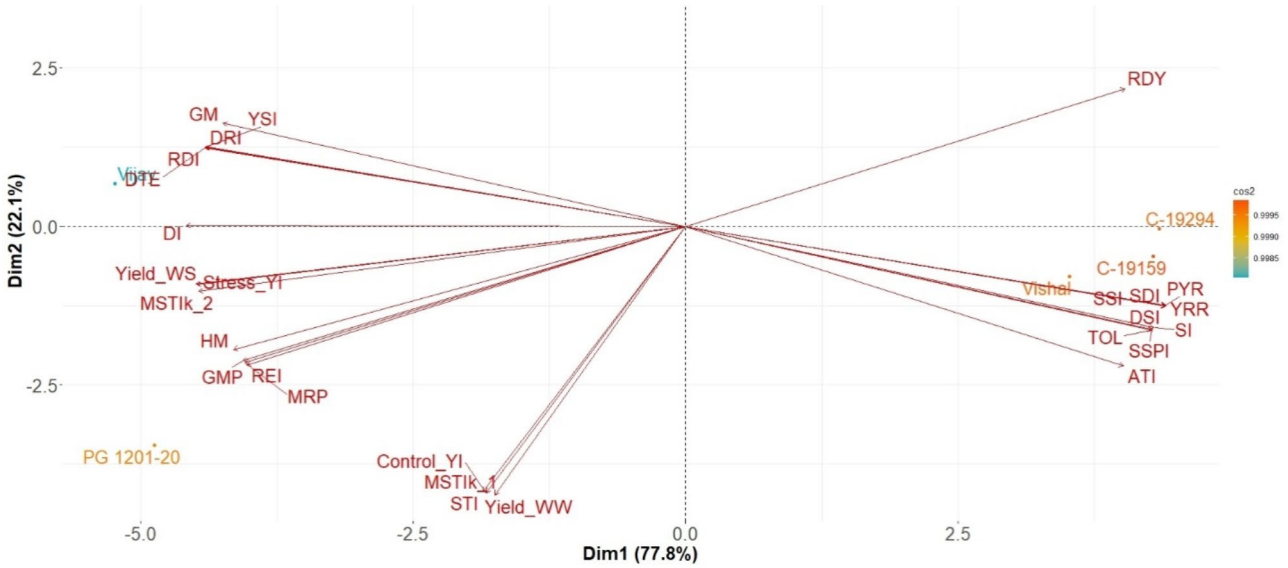

Fig. 9. Principal component analysis biplot of drought tolerance indices in six chickpea genotypes under well-watered and water-stressed conditions for yield-attributing traits. Genotypes are dispersed in different ordinates based on their dissimilarity. The length and color intensity of a vector in the biplot indicate the quality of representation and the contribution of the traits, respectively, based on the principal components. The angles between the vectors derived from the middle point of biplots exhibit positive or negative interactions of studied traits.

screening protocols, offering a more precise approach than earlier methods that relied on measurements taken only at a few time points[43–45].

The study also examined the impact of light conditions—specifically, hours of illumination and light intensity—on ascorbate metabolism, highlighting the importance of sampling leaf tissues for AsA estimation during periods of peak AsA accumulation[46,47]. Ascorbic acid levels in chickpea peaked during high light intensity periods, decreasing later in the day. Therefore, we restricted leaf sampling for AsA estimation to between 11:00 a.m. and 12.00 noon, when light intensity (PAR) was about 174 W m^{-2} in the phenomics facility and 247 ± 7 W m^{-2} in the screenhouse (Supplementary Fig. 2, Supplementary Table 3), close to peak values in the 24 h cycle. Similar findings have been reported in horseradish, where increased light exposure elevated AsA levels in young and mature leaves[48]. Additionally, light intensity and time of day at harvest influenced AsA levels in field-grown *Brassica juncea* L[49]. and spinach[50]. By restricting leaf sampling to periods of high light intensity, we minimized the potential influence of solar radiation variations on AsA levels.

Despite the efficiency of HPLC methods for AsA estimation, we opted for a spectrophotometric approach due to its affordability and accessibility. Limited resources often hinder plant breeders from using advancements in abiotic stress biology. Without cost-effective phenotyping methods, breeders tend to focus on crop productivity, overlooking critical stress tolerance traits. Thus, adopting low-cost technologies is crucial for advancing trait-based selection, particularly in developing countries. Total AsA content, including its recycled forms like ascorbate (AsC) and DHA, is vital in evaluating the antioxidative capacity of genotypes. We confirmed a strong correlation between AsC/AsA and DHA across several crops like apple (AsC vs. total AsA, $R^2 = 0.93$)[34], spinach (AsA vs. DHA, $R^2 = 0.86$)[35] and tomato (AsA vs. DHA, $R^2 = 0.90$)[36], we confirmed a close relationship between AsA and total AsA, suggesting the feasibility of utilizing AsA alone as a selection criterion, indicating that AsA alone can serve as a reliable selection criterion. Moreover, the study[51] demonstrated that calorimetrically estimated AsA in sweet potato, taro, and giant taro aligned closely with total AsA measured by HPLC[52].

Genetic variation in AsA content

Ascorbic acid content positively correlates with drought stress tolerance in many crop species, with drought-tolerant varieties typically showing higher AsA accumulation even under drought stress[53]. This study aligns with these findings, as the drought-tolerant Vijay exhibited significantly higher AsA contents than the drought-sensitive Vishal under depleting soil moisture levels. Similar results have been reported in other drought-tolerant chickpea varieties (Akcin, Aydin-92, and Uzunlu-99)[42], as well as in wheat[54], and cotton[55] cultivars.

Phenotyping of 104 diverse chickpea genotypes using our optimized protocol at 30 ± 1% FC revealed significant genetic variation in endogenous leaf AsA content, ranging from 179.87 to 367.12 µg g^{-1} FW. These values are comparable to those reported for mung bean under well-watered conditions 17 days after flowering (199.10 ± 1.70 µg g^{-1} FW)[56].

Ascorbic acid concentrations vary significantly between plant tissues[57,58]. In our study, fresh leaves exhibited higher AsA concentrations than water-soaked seeds, as high AsA levels are typically found in meristematic plant tissues[59,60]. As chickpea sprouts, AsA levels increase linearly[61]. For example the AsA in dry seeds (8 µg g^{-1} DW) increased almost ten-fold (99 µg g^{-1} DW) in germinating chickpea seeds[62], mainly due to the reactivation of

AsA biosynthesis[63]. Also observed similar increases in AsA during chickpea germination[64]. With their higher carbon and reductant assimilation and biosynthetic capacity, leaves are generally more capable of producing and maintaining AsA than sink organs like seeds[65]. Despite this, the seed AsA content across different genotypes followed similar trends to leaf AsA content in our study. A similar trend was found in drought-tolerant chickpea genotypes exhibited higher antioxidant expression in leaves and seeds than drought-sensitive genotypes[64]. This finding suggests that genetic factors influencing AsA accumulation in leaves also influence seed AsA content, with genotypes exhibiting higher leaf AsA content also tending to have higher seed AsA content. However, the weak correlation between leaf AsA and seed AsA levels indicates that leaf AsA should be prioritized when assessing drought tolerance.

Performance of AsA-rich chickpea genotypes under field conditions

Our study supports the hypothesis that chickpea genotypes with higher levels of AsA perform better under water stress due to the antioxidative properties of AsA[66]. Water stress significantly decreased dry biomass across all genotypes. However, AsA-rich genotypes had significantly higher biomass than AsA-poor genotypes, aligning with previous research showing that increased AsA levels positively influence plant growth and biomass production under drought conditions. For example, higher AsA levels have been associated with increased fresh and dry biomass in drought-stressed safflower cultivars[67], stem dry weights in chickpea[68], rice growth[69], and pepper seedling growth[45].

Water stress significantly reduced pods plant^{-1}, grains pod^{-1}, 100-seed weight, and seed yield plant^{-1} across all genotypes, consistent with reports for chickpea[70], lentil and green pea[71], and soybean[72]. However, AsA-rich genotypes outperformed AsA-poor genotypes, with much smaller reductions in most yield-contributing traits under drought stress, with supporting findings[73]. Drought stress often results in reduced flower and pod numbers due to abortion and subsequent abscission[74], which may explain the reduced final productivity[70]. Notably, water stress had a more pronounced impact on grains pod^{-1}, with significant differences among genotypes. Ascorbic acid content plays a critical role in seed weight determination, with AsA-rich genotypes generally exhibiting higher seed weights than AsA-poor genotypes under well-watered and water-stressed conditions, suggesting that AsA protects against the detrimental effects of water stress on seed development and weight. Our results highlight the potential of AsA-rich genotypes to enhance seed yield under water stress, underscoring their value as donors of drought tolerance in breeding programs aimed at developing resilient crop varieties.

Overall, water stress adversely affected yield attributes in all tested genotypes, with the AsA-poor C-19294 particularly susceptible. These findings emphasize the role of AsA in mitigating the impacts of water stress on plant growth and productivity. For example, exogenous AsA application in wheat alleviated drought stress effects, enhancing biomass and yield[54]. Similar benefits have been reported for chickpea genotypes[75]. Foliar AsA application can also improve intrinsic AsA levels, helping to mitigate drought stress effects and enhance grain yield in various crops[76], including chickpea[74], pea[77], common bean[78], wheat[79] and maize[80].

Our principal component analysis revealed that AsA-rich chickpea genotypes (BDNG-2018-15, PG 1201-20, and Vijay) exhibited high values for stress indices such as GM, RDI, YSI, DTE, DRI, DI, HM, GMP, REI, MRP, and STI, indicating their superior stress tolerance compared to AsA-poor genotypes (C-19294, C-19159, and Vishal). These findings suggest that AsA-rich genotypes possess unique characteristics that enhance their ability to withstand adverse environmental conditions.

Moreover, our study underscores the importance of genetic markers associated with AsA content for facilitating breeding efforts to improve stress tolerance[81]. While markers associated with AsA content have been identified in other crops, such as melon[82], guava[83], and spinach[51], their identification in chickpea remains limited. Our study suggests that leaf AsA content could serve as a phenomics marker for chickpea breeding based on peak AsA content in response to depleting soil moisture. It is important to note that the interconversion of AsC and DHA, involving enzymes like APX, plays a significant role in the antioxidant process. Thus, relying solely on AsC estimation could result in misinterpretations. We recommend adopting our suggested protocol as a cost-effective and rapid means for screening AsC to identify potential candidates.

Additionally, AsA-rich genotypes exhibited higher APX activities than AsA-poor genotypes under water stress. This enzyme is crucial for detoxifying H_2O_2—a ROS produced under stress conditions—by converting it to DHA, which can then be regenerated to AsA by dehydroascorbate reductase[84]. Elevated APX activity in AsA-rich genotypes further supports the utility of our phenotyping protocol suggested in integrating AsA into breeding programs for drought tolerance in chickpea.

Conclusions

Our study presents an optimized method for incorporating AsA estimation into phenotyping protocols to assess drought tolerance in chickpea. We found that chickpea genotypes with a natural ability to produce AsA, an essential antioxidant, demonstrated superior yield under water stress than the other genotypes. Notably, the AsA-rich genotypes BDNG-2018-15 and PG-1201-20 showed minimal reductions in grain yield under drought conditions, highlighting their potential as valuable sources of drought tolerance for chickpea breeding programs. By harnessing genetic variation in AsA content, this study offers valuable insights for developing more resilient chickpea varieties capable of thriving in drought-prone environments.

Materials and methods

Site description

This study was conducted at two locations in India: (1) pot experiments at the National Institute of Abiotic Stress Management (NIASM), Baramati, Maharashtra (18.16° N, 74.50° E, 570 masl) from 2020 to 2021; (2)

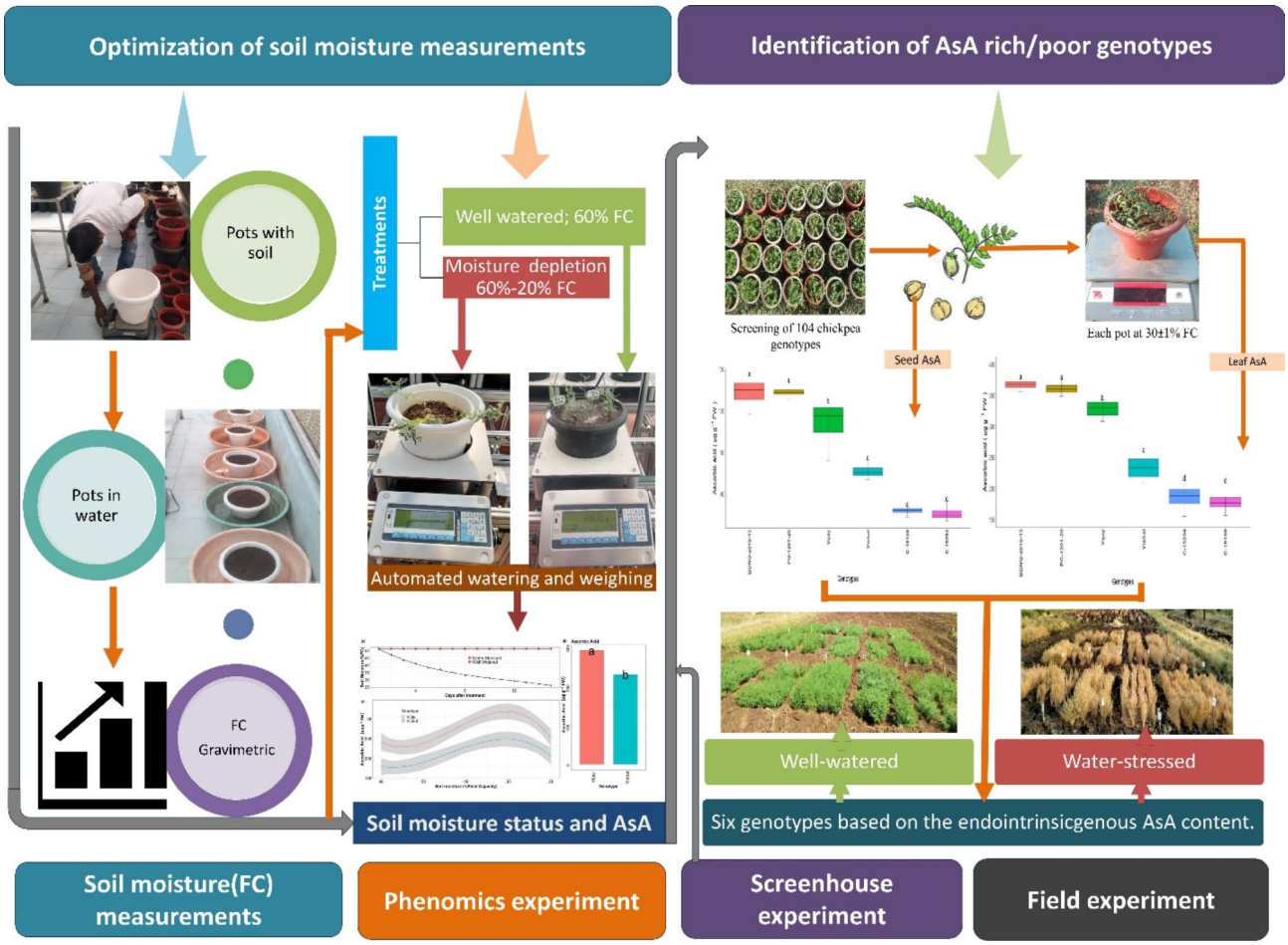

Fig. 10. Brief workflow of research methodology is summarized in the following steps: (1) Optimisation of soil moisture measurements and protocol for the endogenous ascorbic acid (AsA) estimation, (2) Genetic variation in AsA content in leaves and seeds of six contrasting chickpea genotypes screened from 104 chickpea genotypes, (3) Effect of crop season, tissue ascorbic acid content and soil water regime on yield and yield attributing traits.

field experiments at the Post Graduate Institute Farm, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra (19.33° N, 74.62° E, 559 masl) during the 2020–21 and 2021–22 *Rabi* seasons.

Plant material

The study involved 104 diverse chickpea genotypes and two popular check varieties: Vijay (AsA-rich and drought-tolerant check) and Vishal (AsA-poor and drought-sensitive check)[85,86]. These genotypes were sourced from various agricultural universities and research institutes across India (Supplementary Table 1).

Soil moisture measurements

Soil water holding capacity (FC, %) was determined using the gravimetric method[87]. Five pots with drainage holes in the bottom—filled with finely ground, oven-dried soil—were placed in a tray with water and left overnight to absorb water via capillary action. Once the topsoil became moist, surplus water was allowed to drain by carefully shifting the pots to empty trays without water (Fig. 10). The FC was calculated based on the initial dry and final pot weights. Pots were weighed daily to monitor water loss. Soil moisture stress was imposed by withholding irrigation at the seedling stage in controlled environments, while irrigation was withheld post-germination in the field.

Phenomics experiment

Two chickpea cultivars—Vijay (drought-tolerant) and Vishal (drought-sensitive)—were grown under controlled conditions. Seven seeds were sown in each plastic pot (30.48 cm top diameter and 42 cm height) filled with 13 kg clay loam soil at 80% FC. After germination, seedlings were thinned to four per pot. Twenty-four-day-old seedlings were moved to a screenhouse constructed with green mesh for two days before transfer to the National Plant Phenomics Facility. Two moisture treatments were imposed: well-watered (60% FC) and water-stressed (depletion from 60 to 20% FC), with three replicates. Soil moisture in the water-stressed treatment reached 20%

FC after 12 days. Environmental conditions included peak PAR (130–174 W m^{-2}) and day/night temperatures of 32/24°C. Leaf samples were collected from each plant within each pot at eight sampling times to analyze AsA levels, with the pots discarded after each sampling. The experiment had a randomized block design (2 cultivars × 8 sampling times × 3 replications = 48 pots). The experiment was repeated for validation.

Watering and weighing
Automated systems at the Plant Phenomics Unit (LemnaTec, GMBH, and Germany) controlled irrigation and monitored water use. Pots received a predetermined water supply via peristaltic pumps with constant and uniform distribution. Robotics activities for each pot were positioned on RFID tags marked at the bottom of moving cars to identify individual pots during the experiment, and pre- and post-watering pot weights were recorded automatically to track soil moisture changes over time[40] and determine soil moisture levels when leaf AsA contents peaked (Fig. 10).

Screenhouse experiment

A second pot experiment was conducted in a screenhouse under 25–31 °C air temperatures, a 10–12 h photoperiod, and 60–70% relative humidity to assess genetic variation for endogenous AsA in the 104 chickpea genotypes and two local cultivars used in the first pot experiment. Seven seeds were sown in each plastic pot (15 cm top diameter, 10.5 cm bottom diameter, and 15 cm height) and later thinned to four seedlings per pot. Water stress was imposed on 24-day-old seedlings by withholding water, with pots weighed daily until they reached 30 ± 1% FC. Fresh leaf samples were collected to estimate endogenous AsA levels. The experiment had a completely randomized design with four replications (106 cultivars × 4 replications = 424 pots).

Field experiment

Field trials were conducted during the 2020–21 and 2021–22 *Rabi* seasons to assess yield responses of AsA-rich (BDNG-2018-15 and PG-1201-20) and AsA-poor (C-19159 and C-19294) chickpea genotypes under drought conditions. These genotypes were selected based on endogenous AsA levels from the pot experiment. Vijay and Vishal were included as drought-tolerant and drought-sensitive checks, respectively. The experimental site has a vertisol soil type, with 18–32 °C air temperatures, an 8–10 h photoperiod, and 65–70% relative humidity during the study. The field experiment had a split-plot design and three replications. Each plot was 3.6 m^2 (1.2 m length × 3.0 m wide). Seeds were sown 10 cm apart, with rows spaced 30 cm apart. Two irrigation treatments were imposed: well-watered (recommended irrigation) and water-stressed (one irrigation at sowing).

Measurements

Yield attributes
The yield attributes assessed included dry biomass $plant^{-1}$, pods $plant^{-1}$, grains pod^{-1}, 100-seed weight (g), and seed yield (g $plant^{-1}$), measured on five randomly selected plants within each plot[88].

Drought tolerance indices
Twenty-four drought stress tolerance indices were calculated based on seed yield under water-stressed (Ys) and well-watered (Yp) conditions (Table 2).

AsA estimation spectrometric method
Ascorbic acid levels in fresh leaves and soaked seeds were measured using a modified method from Luwe[89]. Leaf samples (0.5 g) and seed samples (0.5 g) were crushed in liquid nitrogen using a mortar and pestle and homogenized in ice-cold trichloroacetic acid (TCA, 1% w/v), followed by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant was mixed with 50 µL potassium phosphate buffer solution (0.95 mL, 100 mm, pH 7.0) and 1 µL ascorbate oxidase (l μL^{-1} unit). Absorbance was measured at 265 nm in a UV-VIS spectrophotometer (serial number A124256). A standard curve was used to determine AsA concentrations.

Sample collection for AsA estimation Fresh leaf samples for AsA estimation were collected between 11.00 a.m. and 12.00 noon when the soil moisture was 30 + 1% FC in the controlled environment (seedling stage) and 33 + 2% FC in the field experiment (pod formation stage).

Crude leaf extract for ascorbate peroxidase (APX) enzyme assays
Fresh leaf tissue (~200 mg) was collected from water-stressed and well-watered plants and ground to a fine powder in a precooled mortar and pestle with liquid nitrogen. The precise weight of each powdered sample was calculated (pH 7.8 with 0.1 mM EDTA) before being thoroughly homogenized in 1.2 mL of 0.2 M potassium phosphate buffer and centrifuged at 15,000 rpm for 20 min at 40 °C. The pellet was resuspended in 0.8 mL of the same buffer after removing the supernatant, with the suspension centrifuged at 15,000 *g* for 15 min at 40 °C. Antioxidant enzyme activities were determined using the mixed supernatants kept on ice.

APX activity estimation
APX activity was assessed immediately in fresh extracts following Nakano and Asada's method[90]. The 3 mL enzyme reaction mixture contained 1.5 mL of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mL of 3 mM AsA, 0.1 mL of 3 mM EDTA, 100 µL enzyme extract, 0.7 mL pure water, and 0.1 mL of 3 mM hydrogen peroxide. The reaction was started by adding 0.1 mL of 3 mM H_2O_2. The oxidation of AsA by hydrogen peroxide was monitored by measuring the absorbance at 290 nm every 30 s until it started to decrease at 3 min. The molar extinction coefficient (2.8 mM cm^{-1}) was used to calculate the amount of oxidized ascorbate, with APX activity expressed as the amount of ascorbate oxidized mg^{-1} protein $minute^{-1}$ (nmol).

Statistical analysis

Analysis of variance (ANOVA) was conducted for both pot experiments using a completely randomized block design with three replications. Field data underwent three-way ANOVA, with year, treatment, and AsA as fixed variables. ANOVA was performed using general linear models with the 'agricolae' package in R version 4.3.1. Pairwise comparisons of means were performed using Duncan's multiple range test in R version 4.3.1.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors on demand.

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Author contributions

JR and DR conceptualized, planned, and executed experiments, analyzed data, drafted the manuscript, and undertook final editing. SG and NK contributed to the execution of experiments. DR performed the chickpea experiment and generated data from the phenomics platform. AB, JR, NVPRG, and KHMS contributed to organizing data and preparing the MS. JR, DR, NVPRG, and KHMS edited the manuscript and interpreted and curated the data.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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