BMC Plant Biology

RESEARCH Open Access

Field and biochemical evaluation of glyphosate tolerant chickpea (*Cicer arietinum* L.) mutants developed through induced mutagenesis

Mariam Ilyas[1], Amjad Hameed[1*] and Tariq Mahmud Shah[1]

Abstract

Weed control in chickpea (*Cicer arietinum* L.) is challenging due to narrow genetic base of available germplasm and limited herbicide options. In this view, present research was focused on induced mutagenesis in chickpea for development of herbicide (glyphosate) tolerant mutants and subsequent screening under field conditions. Further, objective was to analyze the defence response and biochemical adjustments in selected glyphosate tolerant chickpea mutants. Initially, 376 chickpea mutants (M_6 populations developed through EMS and gamma rays) were screened for glyphosate tolerance under filed conditions and scored on a 1 to 5 scale based on plant injury related traits. Among tested mutants, 40 were found highly tolerant (score = 5), 32 as tolerant (score = 4) and 20 as highly sensitive (score = 1) to glyphosate. Chickpea mutants with variable glyphosate tolerance also differed significantly (Tukey test, $p < 0.05$) in leaf biochemical profiles. For instant, lowest total oxidant status (4175.µM/g f. wt.) was detected in glyphosate tolerant mutant developed from desi chickpea genotype "D3009" using 0.3% EMS and in highly tolerant mutant (1775. µM/g f. wt.) developed from kabuli genotype "K709" using 0.2% EMS. In general, highly tolerant chickpea mutants exhibited highest antioxidant potential (SOD, POD, CAT, TAC) that contributed in glyphosate tolerance. Desi i.e. D1M1HT-2 and Kabuli i.e. KM3HT-2 type mutants with highest seed yield had maximum catalase activity (4200 Units/g f. wt and 540 Units/g f. wt.). Mutants developed from desi type genotypes were comparably superior to mutants derive from Kabuli in terms of herbicide tolerance.

Keywords Glyphosate tolerant, EPSPS, Chickpea mutants, Antioxidant enzymes

*Correspondence:
Amjad Hameed
amjad46pk@yahoo.com
[1]Nuclear Institute for Agriculture and Biology College, Pakistan Institute of Engineering and Applied Sciences (NIAB-C, PIEAS), Jhang Road, Faisalabad 38000, Pakistan

Introduction

Plant growth is directly or indirectly be affected by non-ideal environmental conditions. In the field, biotic and abiotic variables have varying degrees of impact on plant growth, development, and reproduction. These diverse climatic factors may include severe drought, heat, salinity, weeds infestation, radiation, water logging, mineral deficiency or excess, etc [1]. Although consideration of all these stresses have crucial role in plant growth and development system, more emphasis of the researchers can be viewed on annual crop yield losses due to weeds infestation. Weeds cause serious restraint in crop yields potential and production worldwide [2]. Weeds seek nutrients, water, light, space and cause damage due to insect pests and diseases in crop plants. They also cause obstacles during harvesting. During the duration of early crop growth, weed infestation can cause yield losses of up to 95%[[3]. However, effective control of weeds can increase the yield of chickpea from 17 to10% [3].

Overall, weeds caused the highest (34%) potential loss of agriculture production and consume the food of almost 1 billion populations. The world's population is expected to increase to 9.1 billion by 2050, a 34% increase from current levels. In order to meet the demands of a shifting global food supply, crop productivity must increase by 100–110%, with the use of herbicides as one tool [4]. Chickpea yield losses can range from 24 to 63%, contingent on the extent of weed infestation. However, yield losses of up to 88% can occur if weeds are not well controlled during the crucial growth period of chickpea plants, which is 35–60 days following emergence [5].

Glyphosate (N-(phosphonomethyl) glycine) a broad spectrum, non-selective post emergent herbicide is extensively used for weed management in agronomic practices. Annually, $5 billion and $11 billion are spent on products containing glyphosate in both the USA and worldwide. After 2,4-D, glyphosate has become the most significant herbicide. This significance was increased in 1996 with the advent of transgenic, glyphosate-resistant (GR) crops. Glyphosate resistance is present in over 80% of the transgenic crops grown on expanding farming area dedicated to these crops. Canola, sugar beet, maize, soybeans, and cotton are examples of GR crops [3]. Glyphosate has been utilized worldwide for over 40 years in weed management [2]. Different herbicides are used in multiple agronomic practices above all glyphosate based herbicides are renowned worldwide and broadly used to control the growth of perennial weeds [6]. Herbicides are mainly and frequently used in various advance-cropping systems for the better management and crop production.

To improve the crop growth and yield the inhibition of weeds is unavoidable; the use of herbicide is mainly recommended to tackle this problem for multiple crops including oilseeds (soybean, canola, etc.), cereals (wheat, barley, oat, corn, sorghum, etc.), pulses (beans, peas, chickpea, lentils, etc.) and pseudo cereals (buckwheat, quinoa, etc.) [6].

Glyphosate (N-phosphonomethyl glycine) is a chemical derived from glycine that is used in the form of herbicide to overcome weed infestation in many agriculture practices [7]. The efficiency of glyphosate-based herbicides is at risk due to glyphosate resistant weeds. In 1998, the first glyphosate resistant weed was reported which has increased in number thereafter. Presently, 36 species have been nominated as glyphosate resistant. HRW have many harmful effects on field and surrounding environment. Consequently, the effectiveness of the world's primary herbicide reserve has been lowered due to the emergence of glyphosate-resistant weeds [8].

The plant defense system is highly affected or altered by herbicide applications. Many physiological systems in plants have been demonstrated to be impacted by glyphosate, and these effects may be connected to glyphosate's herbicidal properties [9]. Reactive oxygen species (ROS) are important products of metabolic reactions produced in plant cells [10, 11]. The following catalog contains the fundamental processes of herbicide resistance: The four main ways that herbicides can be modified chemically are through conjugation or degradation; (1) substitution of amino acids that alter herbicide interactions at the target enzyme; (2) metabolism; (3) physical or physiological exclusion of the herbicide from the target, achieved through active transporters or enhanced cuticular and other structural barriers, a fourth and important mechanism called avoidance, which refers to the biochemical capacity to manage the toxic agent generated by the pesticide and prevent a hazardous outcome [12].

The viability of organisms is damaged when ROS are produced in excess amount exclusive of enough removal their decomposition is unavoidable. Stress caused by biotic and abiotic factors usually leads to an increase in ROS production. In reaction to excess ROS generation, the plant's defense mechanism scavenges harmful chemicals and restores cellular homeostasis. However, when ROS overproduction rises above the antioxidant scavenging system's capacity, ROS accumulates. Excess ROS in susceptible plant cells causes lipidic peroxidation (LPO) and membrane damage resultantly plant dies while in herbicide resistant plants antioxidant system activates and overcome the effect of herbicide damage [13]. Nature has produced ROS-decomposing enzymes to escape the damage e.g. catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD). The activity of these enzymes are regularly monitored in plants and may be affected with application of herbicide [7]. One example of herbicide avoidance is the overexpression of superoxide dismutase as a paraquat rescue agent [12].

The United Nations declared 2016 as "International Year of pulses" with the main emphasis on increasing production and utilization of pulses by 10% by 2020 and assuring the benefits of pulses [14]. Pakistan grows pulses on an area of 1.5 Mha. Among these pulses chickpea covers up a total of 73% of the area under pulses with the production of 76% with an average consumption of 4.18 kg/person/year [15]. Chickpea ranked second as a food legume crop worldwide after beans [16]. As an important source of protein it's nutritional quality plays a vital role in human nutrition around the world [17]. It is also called as poor man's crop. Chickpea has narrow genetic base to compete with weeds because of sluggish growth rate and partial leaf area development at early stages of crop development [18]. Weeds strive for nutrients, water, light, space and cause damages due to insect- pests and diseases in chickpea. They also cause hindrances during harvesting [19]. Therefore, high quality of produce and maximum yield of chickpea is only possible with weed management.

To date no post-emergence herbicide is recommended for weed control in South Asia where bulk of chickpea is grown. This is mainly because of the sensitivity of available chickpea cultivars to herbicides [20, 21]. Glyphosate inhibits the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) a regulatory enzyme crucial for the synthesis of aromatic amino acids in the 'shikimate pathway'.

The first gene responsible for herbicide tolerance has been reported in 1984 [3]. This achievement is a key to success for the development of herbicide resistant variety in chickpea [22]. To date, no chickpea mutant with mutation in target glyphosate tolerant gene (EPSPS) has reported.

Various researches have been performed to understand the morphological and biochemical changes induced in crop plants to study the effect of different herbicides; however, no information is available on the morphological and biochemical responses of chickpea under the influence of glyphosate (herbicide) application. To our knowledge, this is very first report of chickpea mutants having significant field tolerance for glyphosate. Therefore, present study was performed with objectives (1) to screen most promising chickpea mutants with herbicide tolerance bassed on physical plant injury under field conditions and (2) to examine the biochemical mechanisms involved in herbicide tolerance of chickpea mutants.

Table 1 Details of chickpea genotypes, mutagen and doses used in the study

Type	Genotype	Mutagen/Dose Rate	No. of mutants (Lines)
Desi	D3009	400 Gy	2
	D3009	500 Gy	78
	D3009	0.3% EMS	3
	D3009	0.4% EMS	1
	CM1036/09	0.3% EMS	45
	CM1036/09	0.4% EMS	57
Kabuli	K709	200 Gy	60
	K709	300 Gy	10
	K709	0.1% EMS	60
	0K709	0.2% EMS	60

Materials and methods

The present study was conducted to identify and characterize the herbicide tolerant chickpea mutant lines. The work involved phenotypic evaluation followed by biochemical testing to identify antioxidant enzymes involved in tolerance mechanism. The materials and methods followed to conduct the present study are described here.

Screening of herbicide tolerant mutants under field conditions (1st Year)

Experimental location

The present study was conducted at Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. The field experiment for phenotyping and herbicide response was conducted during post rainy season (Nov-Feb) of 2018–2019.

Chickpea mutants (M_6) used in study

A set of 376 mutants (M_6 population) developed at Plant Breeding and Genetics Division, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan were used. These mutants were selected due to their apparent glyphosate tolerance during preliminary field screening trials conducted over past five years. These chickpea mutants (now at M_6 population stage) were initially developed through induced mutation by EMS and gamma rays using two Desi i.e., D3009, CM-1036/09 and one Kabuli line i.e., K709 as parental genotypes. Details are presented in Table 1.

Experimental design and setup

The field experiment was conducted in RCBD design with two replicates. A distance of 2 m was maintained for sprayer access between the strips keeping one plot as treated and the other as control. A total of 376 chickpea mutants were screened against herbicide along with three parental genotypes i.e. D3009, CM-1036/09 and K709 as susceptible checks. To determine the herbicide tolerance/resistance, the treatment plots were sprayed with the glyphosate (Roundup), 30 days after sowing using a shoulder-mounted hand operated knapsack sprayer. Glyphosate herbicide was sprayed according to the recommended dose of 850 ml/acre.

Phenotypic characterization

For phenotypic screening and rating of the mutants, the data from plots was recorded three times using a visual injury rating scale (Table 2) after an interval of 10 days post herbicide application. Glyphosate tolerance ratings based on plant injury on a 1–5 scale was used for this purpose as described earlier [23]. Out of 376 mutants, 72 mutant plants were selected on plant injury basis. Leaf samples of the selected plants were collected and stored at -40°C for determination of biochemical activities.

Screening and selection of herbicide tolerant mutants under field conditions (2nd Year)

Experimental location

The 2nd year screening was also conducted at Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. The 2nd year field experiment for phenotyping and herbicide response was conducted during post rainy season (Nov-Feb) of 2019–2020.

Chickpea mutants (M_7) used in study

Out of 72 chickpea mutants selected during 1st year field trial, 27 mutants were selected for 2nd year field evaluation. The seeds of single plant progenies of these selected 27 promising chickpea mutants (Table 3) were grown in field.

Experimental design and setup

The 2nd year field experiment was also conducted in RCBD design with two replicates and other parameters same as described in 1st year experiment. A total of 27 chickpea mutants were screened against herbicide along with three parental genotypes i.e. D3009, CM-1036/09 and K709 as susceptible checks. To determine the herbicide tolerance/resistance, the treatment plots were sprayed with the glyphosate (Roundup), 30 days after sowing using a shoulder-mounted hand operated knapsack sprayer. Glyphosate herbicide was sprayed according to the recommended dose of 850 ml/acre (850 mL in 20 L = 2.295% solution).

Phenotypic screening for herbicide tolerance

For phenotypic screening and rating of the mutants, the data from plots was recorded three times using a visual injury rating scale (Table 2) after an interval of 05 days post herbicide application. Glyphosate tolerance ratings based on plant injury on a 1–5 scale was used for this purpose as described earlier [23]. The herbicide induced damage was visually observed after spray with an interval of 5 days. The treated plants showed no damage, chlorosis on less than 50% leaves, some plants showed more than 50% chlorosis and even some found dead. The percent survival of plants was calculated in control and herbicide treated plots in percentage.

Morphological parameters

The mutants from both desi and kabuli genotypes were also evaluated for quantitative morphological traits. Plant height was measured from the base of the seedling to the tip of the longest leaf. Plant height was measured in cm. Number of primary branches, number of pods per plant and number of seeds per plant were aslso recorded.

Determination of biochemical activities

Collection of leaf samples

To measure the different oxidant and antioxidant activities, leaf samples of mutants were collected from field and stored in zipper bags at -20°C to ensure integrity till further analysis. All analysis was performed at Marker Assisted Breeding Lab-1 Plant Breeding and Genetics Division, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan.

Estimation of antioxidant activities

Sample extraction

Leaves were grinded to prepare extract in different buffers as required. Weighed 0.15 g of each leaf sample in Eppendorf tubes and then added 1 ml of 50mM potassium phosphate Buffer (pH 7.4). To homogenize the mixture, all the samples were first vortexed and then centrifuged at 14,000 rpm for 10 min at 4°C. Successively, the supernatant was separated and used for the estimation of enzymatic and non-enzymatic activities.

Enzymatic antioxidants

Peroxidase assay

To measure peroxidase activity, reaction mixture containing 535 µl water, 100 µl of 200 mM Guaiacol (enzyme), 100 µl of 400 mM H_2O_2 (substrate), 250 µl of 0.2 M potassium phosphate Buffer and 15 µl enzyme extract was added. The reaction was initiated by adding the enzyme extract. Peroxidase activity was determined by using the modified method of Chance and Maehly [24]. Increase in absorbance was measured after adding enzyme extract for 1 min at 470 nm. One unit POD activity means an absorbance change of 0.01 units per minute.

Ascorbate peroxidase assay

APX activity was measured by the method of Dixit et al., 2001 [25]. For APX estimation, firstly prepared assay buffer containing 6.8 ml of 10 mM ascorbic acid, 20 ml Ethylene Diamine-Tetra-Acetic acid (EDTA), 50 ml of 0.2 M potassium phosphate buffer and make the volume up to 100 ml. APX assay required 1 ml assay buffer, 1 ml 4 mM H_2O_2 and 50 µl enzyme extract. The reaction was initiated by adding H_2O_2 and measured the oxidation rate of ascorbic acid by subsequent decrease in absorbance at 290 nm for 1 min APX activity was recorded using the method described by Dixit et al. [25].

Table 2 Scale used for visual injury ratings for glyphosate tolerance [23]

Score	Plant State	Type of reaction	Snaps
1	Complete chlorosis leading to mortality	Highly Sensitive	
2	Poor plant appearance, severe chlorosis	Sensitive	
3	Fair plant appearance, moderate chlorosis	Moderately tolerant	
4	Good plant appearance, minor chlorosis	Tolerant	
5	Excellent Plant appearance, no chlorosis	Highly tolerant	

Catalase assay

To check catalase activity in chickpea samples was measured using the method of Beers and Sizer [26]. The reaction mixture constituting 2 ml of 50 mM potassium phosphate buffer, 100 µl H_2O_2 (59 mM) and 100 µl enzyme extract. Reaction started after adding enzyme extract that's why decrease in absorbance was measured at 240 nm within 1 min. One enzyme unit means 0.01 absorbance change per minute.

Superoxide dismutase assay

Superoxides produced by white light and quench color. The activity of SOD was examined by evaluating its capacity to prevent the photochemical decrease

Table 3 List of 27 selected mutants and their parental genotypes (03) used in 2nd year field evaluation

Sr. No.	Mutant	Prenatage (mutagen used)/response to glyphosate
1.	D1d1-p3	D3009(400 Gy) Highly Tolerant
2.	D1d2-p1	D3009(500 Gy) Highly Tolerant
3.	D1d2-p2	D3009(500 Gy) Highly Tolerant
4.	D1d2-p3	D3009(500 Gy) Highly Tolerant
5.	D1d2-p4	D3009(500 Gy) Highly Tolerant
6.	D1d3-p1	D3009(0.3%EMS) Highly Tolerant
7.	D1d3-p2	D3009(0.3%EMS) Highly Tolerant
8.	D1d3-p3	D3009(0.3%EMS) Highly Tolerant
9.	D1d3-p4	D3009(0.3%EMS) Highly Tolerant
10.	D1d4-p1	D3009(0.4%EMS) Highly Tolerant
11.	D1d4-p2	D3009(0.4%EMS) Highly Tolerant
12.	D1d4-p3	D3009(0.4%EMS) Highly Tolerant
13.	D1d4-p4	D3009(0.4%EMS) Highly Tolerant
14.	D3009	Parent genotype (sensitive check)
15.	D2d1-p2	CM1036/09(0.3%EMS) Highly Tolerant
16.	D2d1-p3	CM1036/09(0.3%EMS) Highly Tolerant
17.	D2d2-p2	CM1036/09(0.4%EMS) Highly Tolerant
18.	D2d2-p3	CM1036/09(0.4%EMS) Highly Tolerant
19.	CM 1036/09	Parent genotype (sensitive check)
20.	K1-p1	K709(200GY) Highly Tolerant
21.	K1-p3	K709(200GY) Highly Tolerant
22.	K1-p4	K709(200GY) Highly Tolerant
23.	K2-p1	K709(300GY) Highly Tolerant
24.	K2-p3	K709(300GY) Highly Tolerant
25.	K3-p1	K709(0.1%EMS) Highly Tolerant
26.	K3-p2	K709(0.1%EMS) Highly Tolerant
27.	K4-p1	K709(0.2%EMS) Highly Tolerant
28.	K4-p3	K709(0.2%EMS) Highly Tolerant
29.	K2-p2	K709(300GY) Highly Tolerant
30.	K709	Parent genotype (sensitive check)

of nitrobluetetrazolium (NBT) following the method of Dixit et al. [25]. Reaction mixture was Prepared having 400 µl water, 250 µl potassium phosphate buffer (200mM), 100µL L-methane, 100 µl Triton X, 50 µl Nitroblue Tetrazolium (NBT) and 50 µl enzyme extract. At the end riboflavin was added under white light and placed for 10 min. Blank was prepared same but contained potassium phosphate buffer instead of enzyme extract. Blank reading was taken at the end to get maximum absorbance value then samples at 560 nm. One unit of SOD activity means the extent of enzymes which produced 50% inhibition of photochemical reduction of NBT.

Non- enzymatic antioxidants

Total phenolic content (TPC)

Total phenolic contents were measured following the protocol established by Ainsworth and Gillespi [27] in which Folin-Ciocalteau (F-C) reagent was used. For determination of TPC 0.5 g of leaf samples were homogenized in 500 µl ice cold 95% methanol using an ice cold mortar and pestle. The samples were incubated in dark at room temperature for 48 h. The samples were then centrifuged at 14,462 × g for 5 min at room temperature. The supernatant was taken and used for TPC measurement. A 100 µl of supernatant was mixed with 100 µl of 10% (v/v) F-Creagent, vortexed thoroughly, and then 800 µl of 700mM Na_2CO_3 was added. The samples were then incubated at room temperature for 1 h. The blank corrected absorbance of samples was recorded at 765 nm. A standard curve was made using various concentrations of gallic acid and a linear regression equation was calculated. Phenolic content (gallic acid equivalents) of samples was measured using linear regression equation.

Total flavonoid content (TFC)

The total flavonoid content was determined according to the aluminum chloride colorimetric method Lin and Tang [28].The sample (400µ+1.6mLd H_2O) was mixed with 0.1mL of 10% aluminum chloride hexahydrate, 0.1mL of 1Mpotassiumacetate and 2.8mL of deionized water. After 40-min incubation at room temperature, the absorbance of the reaction mixture was measured by spectrophotometer at 415 nm. Rutin was used as a standard (concentration range:0.005 to 0.1 mg/mL) and the total flavonoid content was expressed as a microgram per mL of the sample.

Ascorbic acid

To measure reduced ascorbic acid, then previously described method by Hameed et al. [29].was followed. In this method, vitamin C converts a molecule of DCIP into $DCIPH_2$ and this conversion can be detected by a decrease in absorbance at 520 nm. A standard curve was prepared using a series of known ascorbic acid concentrations then a simple linear regression equation was used to find the ascorbate concentration in unknown samples.

Alpha-amylase activity

For the estimation of leaf alpha-amylase activity, a previously described method of Varavinit [30]was followed with some minor modifications. In this method, there are two reagents: 3,5 dinitrosalicylic acid (DNS) and 1% starch solution. The DNS solution was prepared by adding 96 mM DNS (1 g DNS in 50 mL of distilled water)+sodium potassium tartrate (30 g)+2 N NaOH (20 mL) and making the final volume 100 mL by using distilled water. The reaction mixture contained 0.2 mL of sample, 1.8 mL of distilled water, and 1 mL of 1% starch solution. The reaction mixture was incubated for 3 min. Then 1 mL of DNS reagent was added to each sample mixture, placed in water for 15 min at 100 °C, and allowed to cool to room temperature. Finally, 9 mL of distilled water was added to each sample mixture,

and the absorbance was measured at 540 nm using a spectrophotometer.

Other biochemical parameters

Pigment analysis

The pigments such as total carotenoids, β-carotene, lycopene, total chlorophyll (Chla & b) and provitamin A were determined by the method of Lichtenthaler and Wellburn [31] Leaf samples (0.075) were grind well and extracted in 80% acetone, mixed and centrifuged at 10,000 rpm for 10 min. Through supernatant absorbance was measured at 663 nm, 645 nm, 505 nm, 470 nm and 453 nm by spectrophotometer. The concentration of total chlorophyll (*a* and*b*) and carotenoids were calculated by the following formulae:

Chl*a* (mg/g f. wt.) = [12.7 (O.D 663) -2.69 (OD 645) × V/1000 × W]

Chl*b* (mg/g f. wt.) = [22.9 (O.D 645) -2.69 (OD 643) × V/1000 × W]

Carotenoids (mg/g f. wt.) = [Acar/EM] × 1000 Acar = OD 480 + 0.114 (OD 663) – 638(OD 645).

Where V is the volume of the sample, W is the weight of the leaf sample and EM is 2500.

Malondialdehyde (MDA) content

The level of lipid peroxidation in leaf was measured in terms of malondialdehyde (MDA, a product of lipid peroxidation) content determined by the thiobarbituric acid (TBA) reaction using measured using the method of Heath and Packer, with slight modifications elaborated by Dhindsa [32]. (Dhindsa et al., 1981). To check MDA content, 20% trichloroacetic acid (TCA) and 0.05% TBA required. Reaction mixture was prepared containing 250 µl of TBA + TCA solution and 125 µl of sample and placed in water bath at 95 °C for 30 min and then cool down at room temperature in ice cold water. Absorbance was measured at 532 nm and 600 nm.

Tannin

Tannins were calculated from leaf samples by following the method of Ainsworth and Gillespie [27].According to this assay, tannin level decreased by adding 0.1 g Polyvinylpyrrolidone (PVPP) in TPC samples and then centrifuged at 14,000 rpm for 10 min. Absorbance was measured at 765 nm by taking 1 ml supernatant.

Total oxidant status

Total oxidant status of seed samples was measured using the method of Erel [33]. The amount of oxidants present in the samples, which convert ferrous ions to ferric ions in an acidic medium, was measured by using xylenol orange. In this assay, reagent 1 + reagent 2 and sample extract were used. The reagent R1 was the stock xylene orange solution containing 75 µL xylenol orange dye (0.38 g xylenol orange in 500 µL of 25 mM H2SO4), 0.409 g of NaCl, 500 µL of glycerol, and the final volume was made up to 50 mL with 25 mM H2SO4. The reagent 2 (R2) contained 0.0317 g of o-dianisidine and 0.0196 g of ferrous ammonium sulfate in 10 mL of 25 mM H_2SO_4. After adding 900 µL of reagent 1, 140 µL of sample, and 44 µL of reagent 2, the reaction mixture was incubated for 5 min. Then the absorbance of the reaction mixture was measured at 560 nm by using a spectrophotometer. After that, a standard curve for H2O2 was prepared. The results for TOS were expressed in µM H_2O_2 eq/L.

Statistical analysis

All the data was presented as mean ± SD. XL-STAT software version 2021.5.1.1223, copyright Addinsoft 2022 (http://www.xlstat.com) was used for all the statistical analysis. Descriptive statistics was applied to organize and analyse the resulting data. Data were analyzed using two-way ANOVA with replications. Tukey (HSD) Test at $p < 0.05$ and analysis of variance (ANOVA) were used to test the significance of the data. Mean values of the data were also subjected to perform Principal component Analysis using the same software.

Results

Field performance of 376 chickpea mutants was evaluated during two consecutive years. Representative pictures from field screening trails of mutants showing variable response to herbicide are presented as Fig. 1 (1st year) and Fig. 2 (2nd year). Representative figures showing field view of chickpea mutants under control and herbicide treated conditions (Fig. 3) and Plant survival rate after herbicide treatment (Fig. 4) are provided.

Biochemical and morphological response of chickpea mutants

Total 72 chickpea mutants, including 30 mutants developed from desi genotype D3009 while 14 from desi genotype CM1036/09 and 28 mutants of kabuli genotype K709 were tested for different biochemical activities and morphological traits. Resulting data is present in two different ways. Firstly, mean data showing performance of individual mutants (all 72) was presented. Secondly, to evaluate the comparative effectiveness and superiority of mutation source (400 Gy and 500 Gy 0.3% and 0.4% EMS) and parent chickpea genotype, means were computed for different mutation treatment sources and for each parental genotype used in the study and were compared statistically.

Fig. 1 Field screening of chickpea mutants for herbicide tolerance during 1st Year (2018-19). Where **a**: highly tolerant mutant, **b**: tolerant mutant, **c**: moderately tolerant mutants, **d**: sensitive mutants and **e**: highly sensitive mutants

Comparative effectiveness and superiority of mutation source and parent chickpea genotype

Enzymatic antioxidants

Superoxide dismutase (SOD) activity The mutants of genotype D3009 at 400 Gy and 500 Gy that were placed in highly tolerant category showed higher activity of SOD enzyme with a mean value of 202.7Units/g f. wt.) at 400 Gy while some mutants of genotype D3009 categorized as tolerant showed lowest SOD with a mean value of 122 Units/g f. wt.) (Fig. 5a). The mutants of same genotype at 500 Gy showed low activity of SOD in HT category mutants with a mean value of 141.8 Units/g f. wt.) while high activity is observed with a mean value of 165.6 Units/g f. wt. in tolerant category. The mutants of genotype D3009 developed by using 0.3% and 0.4% that were also placed in highly tolerant and tolerant categories. The mutants treated at 0.3%EMS showed low SOD activity with a mean value of 168.6 Units/g f. wt. in HT category while high activity with a mean value of 272.1 Units/g f. wt. in tolerant category. Similarly, some mutants of the same genotype at 0.4% EMS showed low SOD activity in HT mutants with a mean value of 171 Units/g f. wt. while high SOD activity with a mean value of 238.3 Units/g f. wt. was seen in the mutants of tolerant category. The mutants of genotype CM1036/09 treated using 400 Gy and 500 Gy was also categorized as highly tolerant and tolerant mutants. Some of the mutants at 400 Gy showed low SOD activity in highly tolerant category with a mean value of 146 Units/g f. wt. and high activity in tolerant category with a value of 231.4 Units/g f. wt. (Fig. 5a). Similarly, some of the mutants of same genotype showed low SOD activity in HT category at 500 Gy while high activity was observed in tolerant category. While the mutants of kabuli genotype K709 developed by using 200 Gy and 300 Gy showed low concentration of SOD in highly tolerant category and high in tolerant category (Fig. 5b). While the mutants of the same genotype developed by using 0.1% EMS showed high SOD in highly tolerant category and low in tolerant category. Whereas, the mutants of the same genotype developed by using 0.2%EMS showed low SOD contents in highly tolerant category and high in tolerant category in terms of mean.

Catalase activity The mutants of genotype D3009 at 400 Gy and 500 Gy that were placed in highly tolerant category showed higher concentration of catalase activity with a mean value of (1096Units/g f. wt.) at 400 Gy while some mutants of genotype D3009 categorized as tolerant showed lowest catalase activity with a mean value of

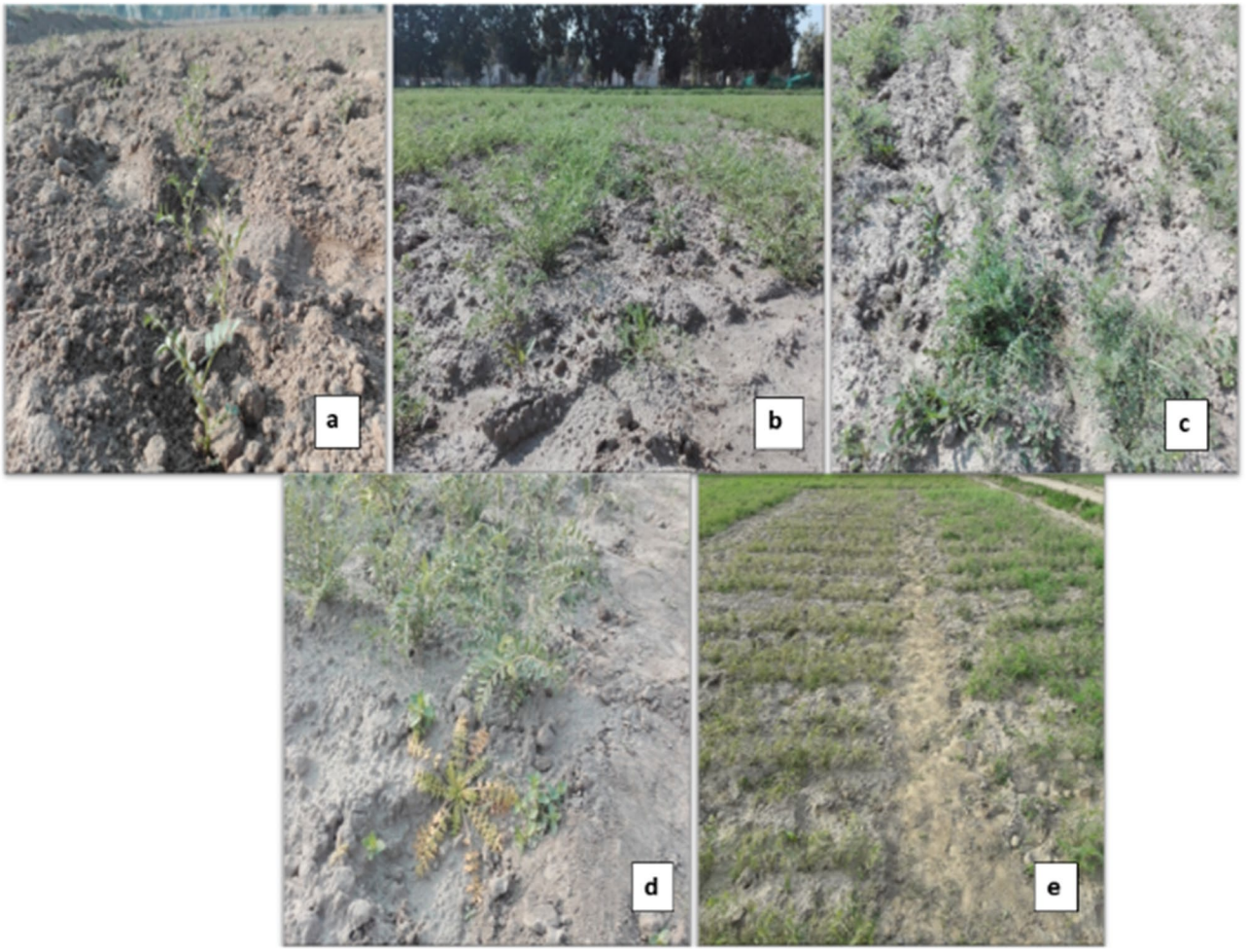

Fig. 2 Field screening of chickpea mutants for herbicide tolerance during 2 Year (2019-20). **a**) Mutants at seedling stage **b**) mutants before herbicide treatment **c**) mutants with weeds before spray, **d**) tolerant mutant with killed weeds after spray **e**) behavior of plants and weeds after spray

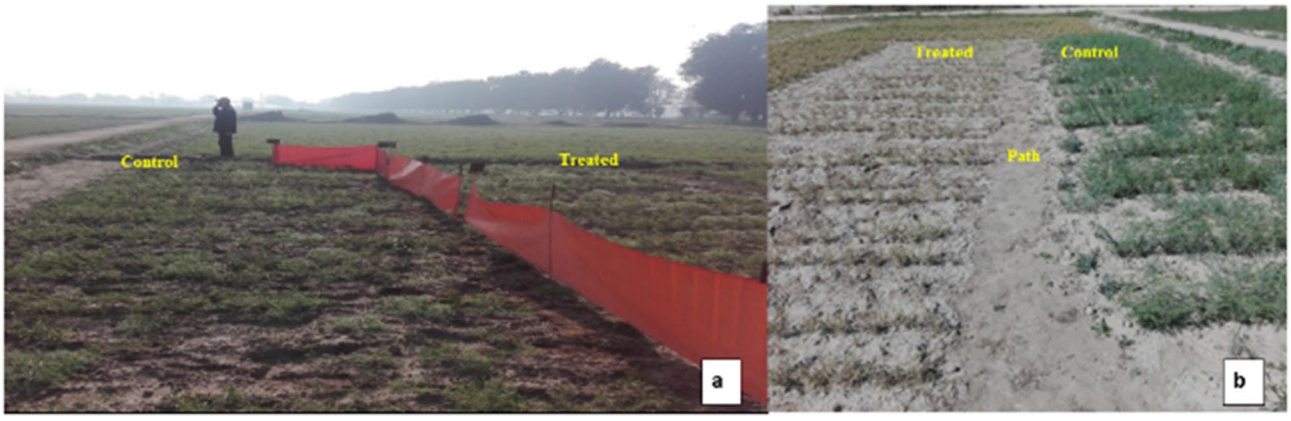

Fig. 3 Field view of chickpea mutants under control and herbicide treated conditions. **a**) Field view immediately after spray of herbicide **b**): Effect of herbicide treatment clearly evident in treated plot as compared with control plot

(306.7 Units/g f. wt.) (Fig. 5c). The mutants of same genotype at 500 Gy showed high catalase activity in HT category mutants while low activity is observed in tolerant category. The mutants of genotype D3009 developed by using 0.3% and 0.4% that were also placed in highly tolerant and tolerant categories. The mutants treated at 0.3%EMS showed higher catalase activity with a mean value of (1010 Units/g f. wt.) in HT category while low

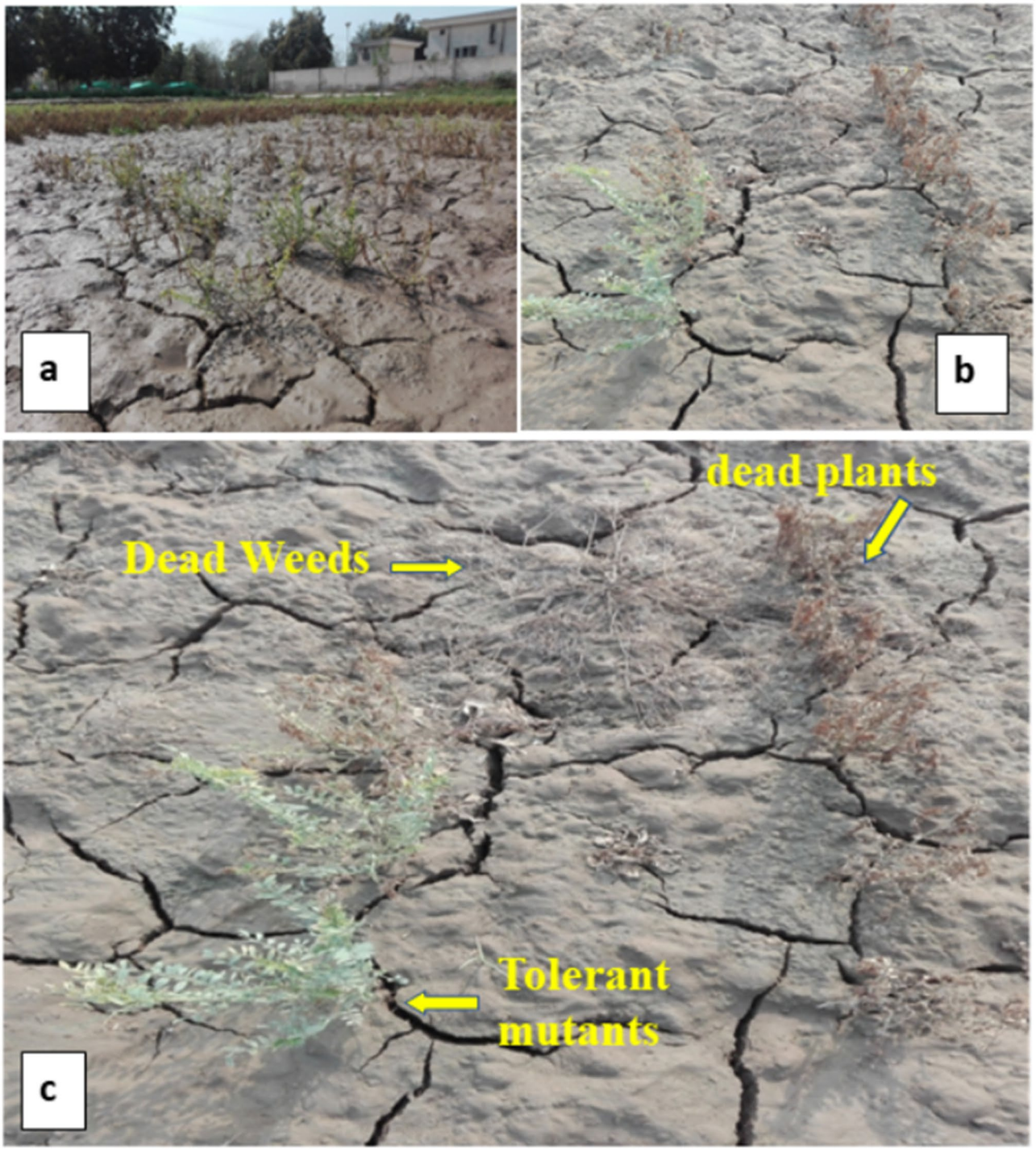

Fig. 4 Survival rate of plants after herbicide treatment. **a**: Field view of chickpea mutants three weeks after herbicide treatment, **b**: herbicide tolerant and sensitive (wilted) mutants three weeks after treatment, **c**: field view of dead weeds, dead mutants, alive and healthy herbicid tolerant mutants growing side by side

activity with a mean value of (300 Units/g f. wt.) in tolerant category. Similarly, at 0.4% EMS high catalase activity was observed in HT mutants of the same genotype while low activity was seen in the mutants placed in tolerant category. The mutants of genotype CM1036/09 treated using 400 Gy and 500 Gy was also categorized as highly tolerant and tolerant showed low catalase activity in highly tolerant category and high catalase activity in tolerant category at 400 Gy (Fig. 5c). Similarly, some of the mutants of same genotype showed high catalase activity in HT category at

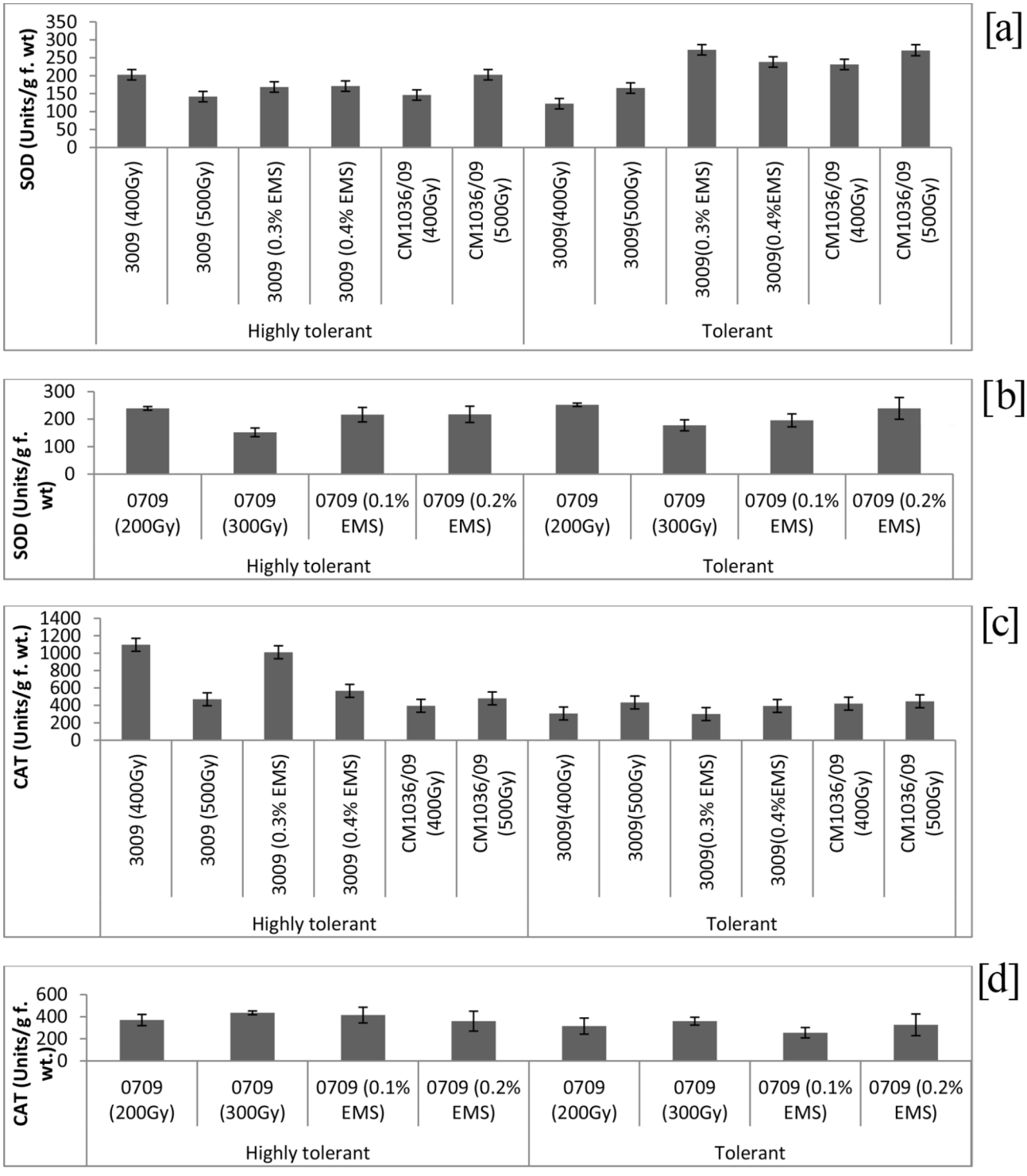

Fig. 5 **a**) Variation in SOD activity of desi genotypes 3009 and CM1036/09, **b**) SOD of kabuli 0709, **c**) CAT activity of desi genotypes 3009 and CM1036/09 and **d**) CAT activity of kabuli 0709 in different chickpea mutants

500 Gy while low activity was observed in tolerant category. While the mutants of kabuli genotype K709 developed by using 200 Gy and 300 Gy showed high catalase activity in highly tolerant category and low in tolerant category (Fig. 5d). Similarly, the mutants of the same genotype developed by using 0.1%EMS and 0.2%EMS showed high catalase activity in highly tolerant category and low in tolerant category in terms of mean.

Peroxidase (POD) activity The mutants of genotype D3009 at 400 Gy and 500 Gy that were placed in highly tolerant category showed low POD with a mean value of 12414.2 Units/g f. wt.) at 400 Gy while some mutants of genotype D3009 categorized as tolerant showed high POD with a mean value of 17849 Units/g f. wt.) (Fig. 6a). The mutants of same genotype at 500 Gy showed high value of POD in HT category mutants while low activity was observed in tolerant category. The mutants of genotype D3009 developed by using 0.3% and 0.4% that were also placed in highly tolerant and tolerant categories. The mutants treated at 0.3%EMS showed high POD activity with a mean value of 14419 Units/g f. wt.) in HT category while low activity with a mean value of 2198 Units/g f. wt.) in tolerant category (Fig. 6a). Similarly, some mutants of the same genotype at 0.4% EMS showed low POD activity in HT mutants while high POD activity was seen in the mutants of tolerant category. The mutants of genotype CM1036/09 treated using 400 Gy and 500 Gy was also categorized as highly tolerant and tolerant mutants. Some of the mutants at 400 Gy showed low POD activity in highly tolerant category high activity in tolerant category. Similarly, some of the mutants of same genotype showed high POD activity with a mean value of 28355 Units/g f. wt.) (Fig. 6a) in HT category at 500 Gy while low activity with a mean value of 18714.600 was observed in tolerant category. While the mutants of kabuli genotype K709 developed by using 200 Gy showed low concentration of POD in highly tolerant category and high in tolerant category (Fig. 6b). While the mutants of the same genotype developed by using 300 Gy showed high POD in highly tolerant category and low in tolerant category. The mutants of the same genotype developed by using 0.1%EMS showed high POD contents in highly tolerant category and low in tolerant category. Whereas, the mutants of the same genotype developed by using 0.2%EMS showed low POD contents in highly tolerant category and high in tolerant category in terms of mean.

Ascorbate peroxidase (APX) activity The mutants of genotype D3009 at 400 Gy and 500 Gy that were placed in highly tolerant category. The mutants of this genotype showed low APX at 400 Gy in HT category while some mutants of genotype 3009 categorized as tolerant showed high contents of APX (Fig. 6c). The mutants of same genotype at 500 Gy showed high value of APX in HT category mutants while low contents were observed in tolerant category. The mutants of genotype D3009 developed by using 0.3% and 0.4% that were also placed in highly tolerant and tolerant categories. The mutants treated at 0.3%EMS showed low contents of APX in HT category while higher in tolerant category with a mean value of 9800 Units/g f. wt. (Fig. 6c). Similarly, some mutants of the same genotype at 0.4% EMS showed low APX in HT mutants while high level of APX was seen in the mutants of tolerant category. The mutants of genotype CM1036/09 treated using 400 Gy and 500 Gy was also categorized as highly tolerant and tolerant mutants (Fig. 6c). Some of the mutants at 400 Gy showed high APX in highly tolerant category while low contents were observed in tolerant category. Some of the mutants of same genotype showed high APX in HT category at 500 Gy while low in tolerant category. While the mutants of kabuli genotype K709 developed by using 200 Gy and 300 Gy showed high concentration of APX in highly tolerant category and low in tolerant category (Fig. 6d). Similarly, the mutants of the same genotype developed by using 0.1%EMS and 0.2% showed high APX in highly tolerant category and low in tolerant category in terms of mean.

Non-enzymatic antioxidants

Total phenolic content (TPC)

The mutants of genotype D3009 at 400 Gy that were placed in highly tolerant category showed high concentration of total phenolics while some mutants of genotype D3009 categorized as tolerant showed low TPC (Fig. 7a). The mutants of genotype D3009 at 500 Gy showed low contents of total phenolics with a mean value of 63388μM/g f. wt. in HT category and high contents with a mean value of 657\ μM/g f. wt. in tolerant category. Similarly, the mutants of genotype D3009 developed using 0.3% and 0.4% EMS that were placed in highly tolerant category showed high concentration of total phenolics while the mutants of same genotype showed low total phenolic contents in tolerant category. The mutants of genotype CM1036/09 treated by 400 Gy and 500 Gy was also categorized as highly tolerant and tolerant showed high concentration of TP in both the highly tolerant category at 400 Gy and 500 Gy and low concentration in tolerant category. While the mutants of kabuli genotype K709 at 200 Gy showed high TPC contents in highly tolerant category and low in tolerant category (Fig. 7b). While the mutants of the same genotype at 300 Gy showed low TPC contents in highly tolerant category and high in tolerant category. The mutants of the genotype developed by using 0.1%EMS and 0.2%EMS showed low TPC contents in highly tolerant category and high contents were observed in tolerant category.

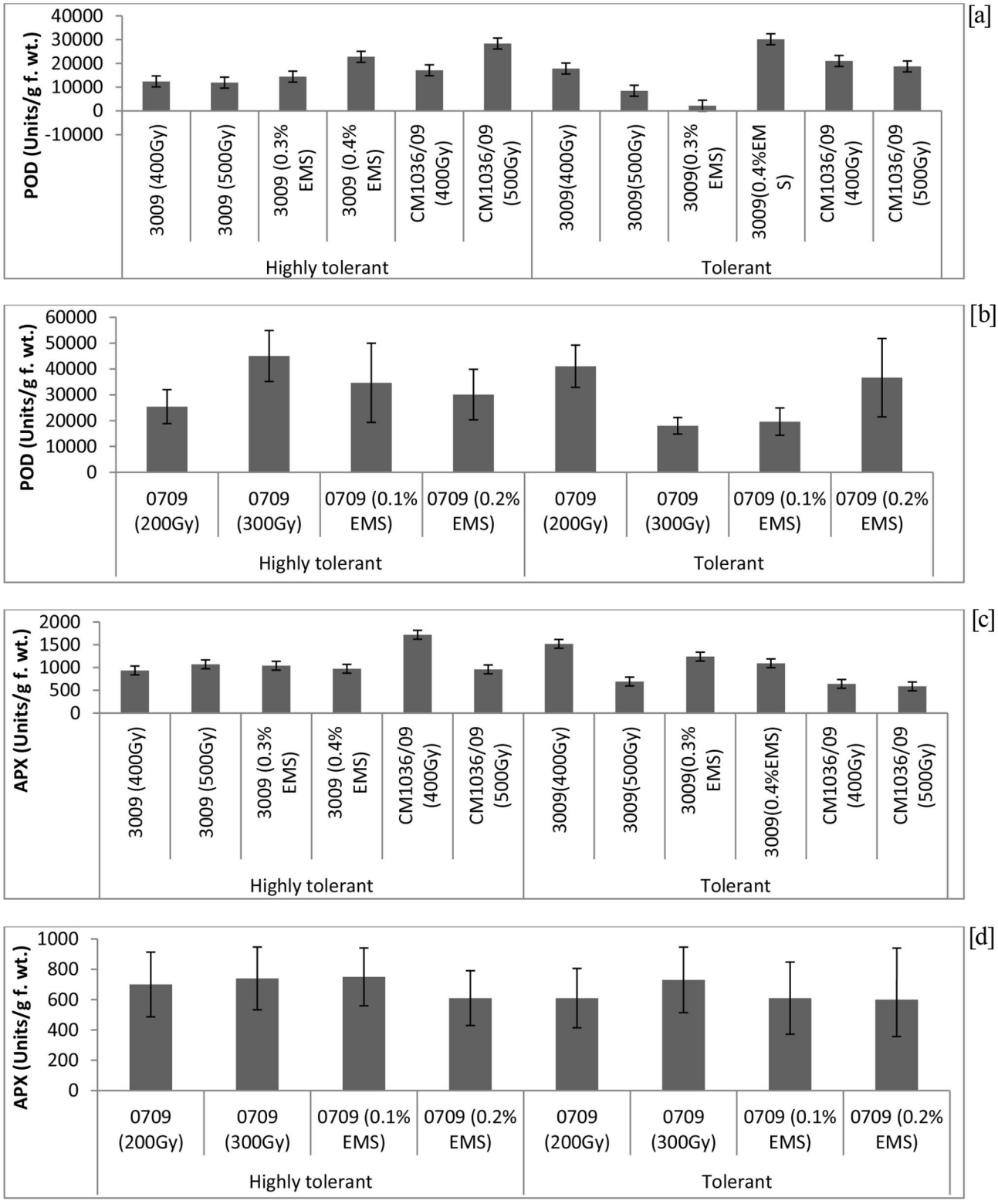

Fig. 6 **a**) Variation in POD activity of desi genotypes 3009 and CM1036/09, **b**) POD of kabuli 0709, **c**) APX activity of desi genotypes 3009 and CM1036/09 and **d**) APX activity of kabuli 0709 in different chickpea mutants

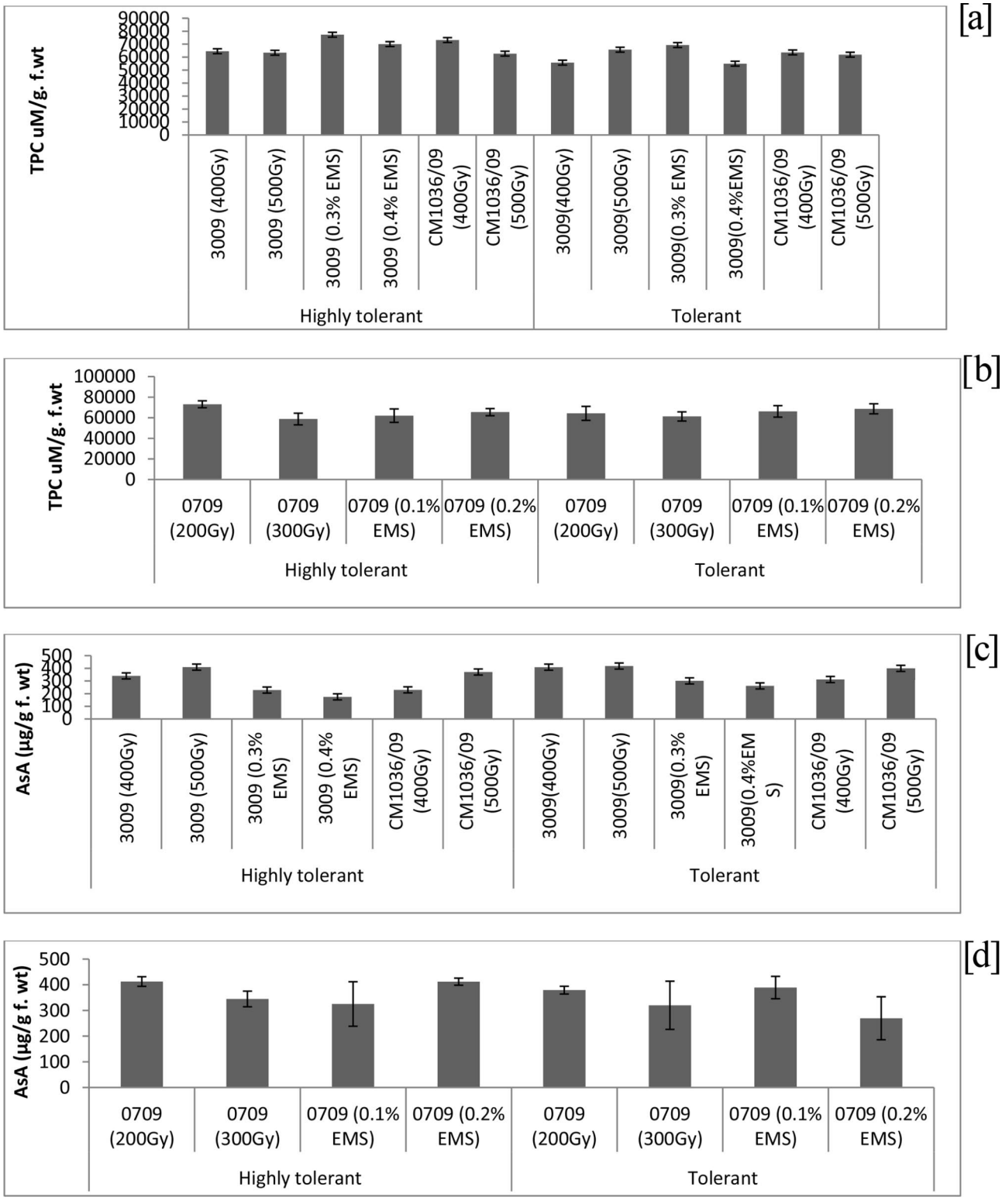

Fig. 7 **a**) Variation in TPC of desi genotypes 3009 and CM1036/09, **b**) TPC of kabuli 0709, **c**) AsA contents of desi genotypes 3009 and CM1036/09 and **d**) AsA contents of kabuli 0709 in different chickpea mutants

Ascorbic acid (AsA) content

The mutants of desi genotype D3009 showed low ascorbic acid at 400 Gy in HT category while some mutants of genotype D3009 categorized as tolerant, showed high contents of ascorbic acid (Fig. 7c). The mutants of same genotype at 500 Gy showed low value of ascorbic acid in HT category mutants while high contents were observed in tolerant category. The mutants of genotype D3009 developed by using 0.3% and 0.4% that were also placed in highly tolerant and tolerant categories. The mutants treated at 0.3%EMS showed low contents of ascorbic acid in HT category while higher in tolerant category. Similarly, some mutants of the same genotype at 0.4% EMS showed low ascorbic acid in HT mutants while high level of ascorbic acid was seen in the mutants of tolerant category. The mutants of genotype CM1036/09 treated using 400 Gy and 500 Gy was also categorized as highly tolerant and tolerant mutants. Some of the mutants at 400 Gy showed low ascorbic acid in highly tolerant category while high contents were observed in tolerant category. Some of the mutants of same genotype showed low ascorbic acid in HT category at 500 Gy while higher in tolerant category. While the mutants of genotype K709 developed by using 200 Gy and 300 Gy showed high concentration of ascorbic acid in highly tolerant category and low in tolerant category (Fig. 7d). Similarly, the mutants of the same genotype developed by using 0.1%EMS showed low ascorbic acid in highly tolerant category and high in tolerant category. While the mutants of the same genotype developed by using 0.2%EMS showed high ascorbic acid in highly tolerant category and low in tolerant category in terms of mean.

Alpha amylase activity

The alpha amylase activity is significantly higher in the mutants of desi genotype D3009 (HT category) developed at 500 Gy while significantly lower in the same genotype in T category (Fig. 8a). Some of the mutants of genotype D3009 (0.3%EMS) (T category) showed significantly lower amylase activity. The mutants of same genotype at 500 Gy showed high value of amylase activity in HT category mutants while low activity was observed in tolerant category. The mutants of genotype D3009 developed by using 0.3% and 0.4% that were also placed in highly tolerant and tolerant categories. The mutants treated at 0.3%EMS showed high value of amylase activity in HT category while low in tolerant category. Similarly, some mutants of the same genotype at 0.4% EMS showed high amylase activity in HT mutants while low level of amylase activity was seen in the mutants of tolerant category. The mutants of genotype CM1036/09 treated using 0.3% EMS and 0.4% EMS was also categorized as highly tolerant and tolerant mutants. Some of the mutants at 0.3% EMS showed higher amylase activity in highly tolerant category while low activity was observed in tolerant category. Some of the mutants of same genotype showed low amylase activity in HT category at 0.4% EMS while high amylase activity was observed in tolerant category. While the mutants of kabuli genotype K709 developed by using 200 Gy and 300 Gy showed high amylase activity in highly tolerant category and low in tolerant category (Fig. 8b). Similarly, the mutants of the same genotype developed by using 0.1%EMS showed low amylase activity in highly tolerant category and high in tolerant category. while the mutants of the same genotype developed by using 0.2%EMS showed high amylase activity in highly tolerant category and low in tolerant category in terms of mean.

Total flavonoids content (TFC)

The mutants of genotype D3009 at 400 Gy and 500 Gy that were placed in highly tolerant category showed low concentration of total flavonoids at 400 Gy while some mutants of genotype D3009 categorized as tolerant showed high total flavonoids contents (Fig. 8c). The mutants of the same genotype showed high value of TF at 500 Gy in HT category while low contents in tolerant category were observed. Contrary to this, some mutants of genotype D3009 developed using 0.3% and 0.4% EMS showed high concentration of total flavonoids in HT category at 0.3% EMS while the mutants of same genotype showed low concentration of total flavonoids in tolerant mutants. The mutants developed by 0.4% EMS showed high concentration of total flavonoids in HT category while low concentration of total flavonoids in tolerant mutants. The mutants of genotype CM1036/09 treated using 400 Gy and 500 Gy was also categorized as highly tolerant and tolerant showed high concentration of total flavonoids in highly tolerant category and low contents in tolerant category at 400 Gy. Similarly, the mutants of the same genotype showed high contents in HT category at 500 Gy while low flavonoid contents were observed in tolerant category. While the mutants of kabuli genotype K709 at 200 Gy showed low total flavonoids contents in highly tolerant category and high in tolerant category (Fig. 8d). While the mutants of the same genotype at 300 Gy showed low total flavonoids contents in highly tolerant category and high in tolerant category. The mutants of the genotype developed by using 0.1%EMS showed low TF contents in highly tolerant category while low contents were observed in tolerant category. Whereas, the mutants developed at 0.2%EMS showed high TF contents in highly tolerant category while low contents were observed in tolerant category in terms of mean.

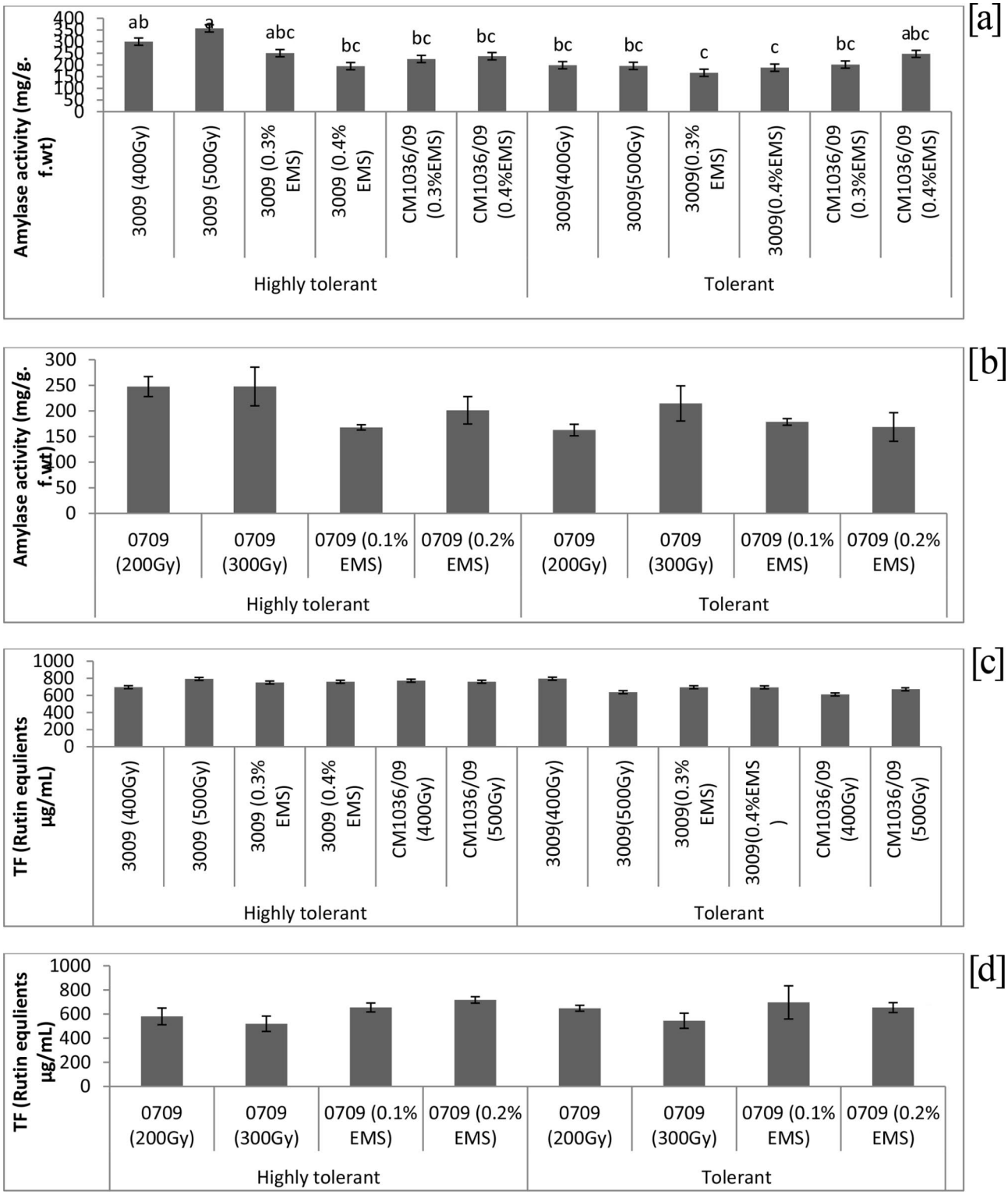

Fig. 8 **a**) Variation in amylase activity of desi genotypes 3009 and CM1036/09, **b**) amylase activity of kabuli 0709, **c**) Total flavonoid contents of desi genotypes 3009 and CM1036/09 and **d**) TF contents of kabuli 0709 in different chickpea mutants

Other biochemical parameters

Total oxidant status (TOS)

The mutants of genotype D3009 at 400 Gy and 500 Gy that were placed in highly tolerant category. The mutants of this genotype showed low TOS at 400 Gy in HT category while some mutants of genotype D3009 categorized as tolerant showed high contents of TOS (Fig. 9a). The mutants of same genotype at 500 Gy showed low value of TOS in HT category mutants while high contents were observed in tolerant category. The mutants of genotype D3009 developed by using 0.3% and 0.4% that were also placed in highly tolerant and tolerant categories. The

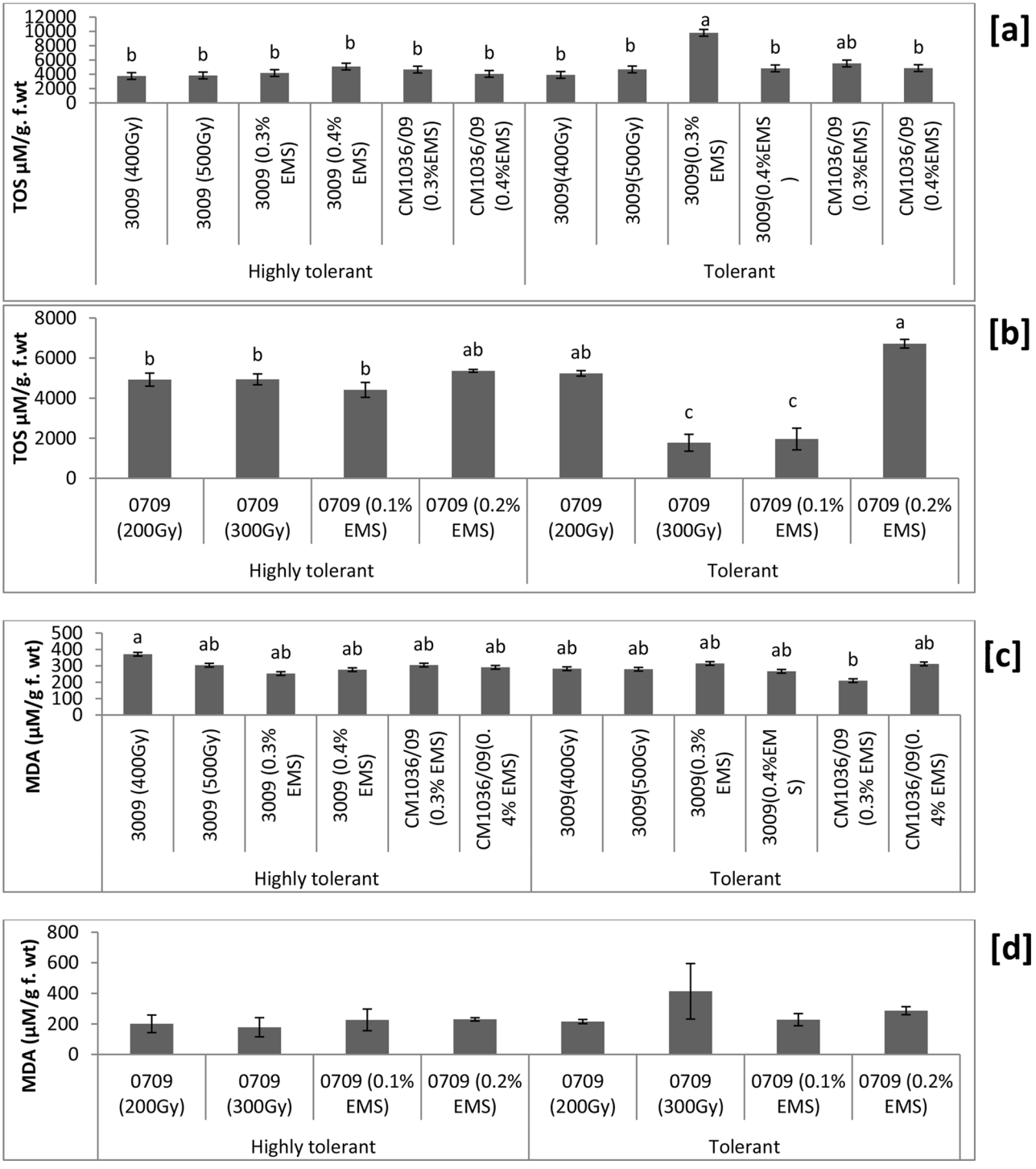

Fig. 9 **a**) Variation in TOS activity of desi genotypes 3009 and CM1036/09, **b**) TOS activity of kabuli 0709, **c**) MDA of desi genotypes 3009 and CM1036/09 and **d**) MDA of kabuli 0709 in different chickpea mutants

mutants treated at 0.3%EMS showed low contents of TOS in HT category with a mean value of 4175µM/g. f. wt. while higher in tolerant category with a mean value of 9800 µM/g. f. wt. Similarly, some mutants of the same genotype at 0.4% EMS showed low TOS in HT mutants while high level of TOS was seen in the mutants of tolerant category. The mutants of genotype CM1036/09 treated using 400 Gy and 500 Gy was also categorized as highly tolerant and tolerant mutants. Some of the mutants at 400 Gy showed low TOS in highly tolerant category while high contents were observed in tolerant category. Some of the mutants of same genotype showed low TOS in HT category at 500 Gy while higher in tolerant category. While the mutants of kabuli genotype K709 developed by using 200 Gy showed low concentration of TOS in highly tolerant category and high in tolerant category (Fig. 9b). While, the mutants of the same genotype developed by using 300 Gy showed high TOS in highly tolerant category and low in tolerant category. The mutants of the same genotype developed by using 0.1%EMS showed high TOS in highly tolerant category and low in tolerant category. Whereas, the mutants of the same genotype developed by using 0.2%EMS showed low TOS in highly tolerant category while high contents were observed in tolerant category in terms of mean.

Malondialdehyde (MDA) content

The mutants of genotype D3009 (400 Gy) from highly tolerant category showed significantly higher MDA contents (370.8µM/gf. wt.) (Fig. 9c). The mutants of genotype CM1036/09 (0.3%EMS) from tolerant category showed significantly lower MDA value. The mutants of same genotype at 500 Gy showed high value of MDA in HT category mutants while low contents were observed in tolerant category. The mutants of genotype D3009 developed by using 0.3% EMS and 0.4% EMS that were also placed in highly tolerant and tolerant categories. The mutants treated at 0.3%EMS showed low concentration of MDA in HT category while high contents were observed in tolerant category. Similarly, some mutants of the same genotype at 0.4% EMS showed high MDA contents in HT mutants while low level of MDA was seen in the mutants of tolerant category. The mutants of genotype CM1036/09 treated at 0.3%EMS and 0.4% EMS was also categorized as highly tolerant and tolerant mutants. Some of the mutants at 0.3%EMS showed high concentration of MDA with a mean value of 304.6 µM/g. f.w in highly tolerant category and low concentration with a value of 209.5 µM/g. f.w in tolerant category. Similarly, some of the mutants of same genotype showed low concentration of MD in HT category at 0.4% EMS while high MDA concentration was observed in tolerant category. While the mutants of Kabuli genotype K709 developed by using 200 Gy and 300 Gy showed low concentration of MDA in highly tolerant category and high in tolerant category (Fig. 9d). Similarly, the mutants of the same genotype developed by using 0.1%EMS and 0.2%EMS showed low concentration of MDA in highly tolerant category and high in tolerant category in terms of mean.

Reducing sugars

The mutants of genotype D3009 at 400 Gy showed high concentration of reducing sugars in HT category while some mutants of genotype D3009 categorized as tolerant showed low at 400GY. Some mutants of the same genotype at 0.4% EMS showed low concentration of reducing sugars with a mean value of 9.381 mg/g f. wt. (Fig. 10a) in HT mutants while high level of reducing sugars was seen with a mean value of 14.071 in the mutants of tolerant category. While the mutants of Kabuli genotype K709 developed by using 200 Gy and 300 Gy showed high concentration of reducing sugars in highly tolerant category and low in tolerant category (Fig. 10b). Similarly, the mutants of the same genotype developed by using 0.1%EMS and 0.2%EMS showed high concentration of reducing sugars in highly tolerant category and low in tolerant category in terms of mean.

Tannins

The mutants of genotype D3009 at 400 Gy and 500 Gy that were placed in highly tolerant category showed low concentration of tannins while some mutants of genotype D3009 categorized as tolerant showed high tannins contents. Contrary to this, some mutants of genotype D3009 developed using 0.3% EMS that were placed in highly tolerant category showed low concentration of tannins with a mean value of 21962.5 (µM/g. f. wt.) (Fig. 10c) while the mutants of same genotype showed high tannins contents with a mean value of 33,225 (µM/g. f. wt.). The mutants of genotype D3009 developed using 0.4% EMS showed low contents in HT category and high concentration of tannins in tolerant category. The mutants of genotype CM1036/09 treated using 400 Gy and 500 Gy was also categorized as highly tolerant and tolerant showed high concentration of tannins in highly tolerant category and low contents in tolerant category at 400 Gy while this genotype behaved different at 500 Gy as low contents of tannins in HT category and high contents in tolerant category. While the mutants of kabuli genotype K709 at 200 Gy showed low tannins contents in highly tolerant category and high in tolerant category (Fig. 10d). While the mutants of the same genotype at 300 Gy showed high tannins contents in highly tolerant category and low in tolerant category. The mutants of the genotype developed by using 0.1%EMS and 0.2%EMS showed high tannins contents in highly tolerant category while low contents were observed in tolerant category in terms of mean.

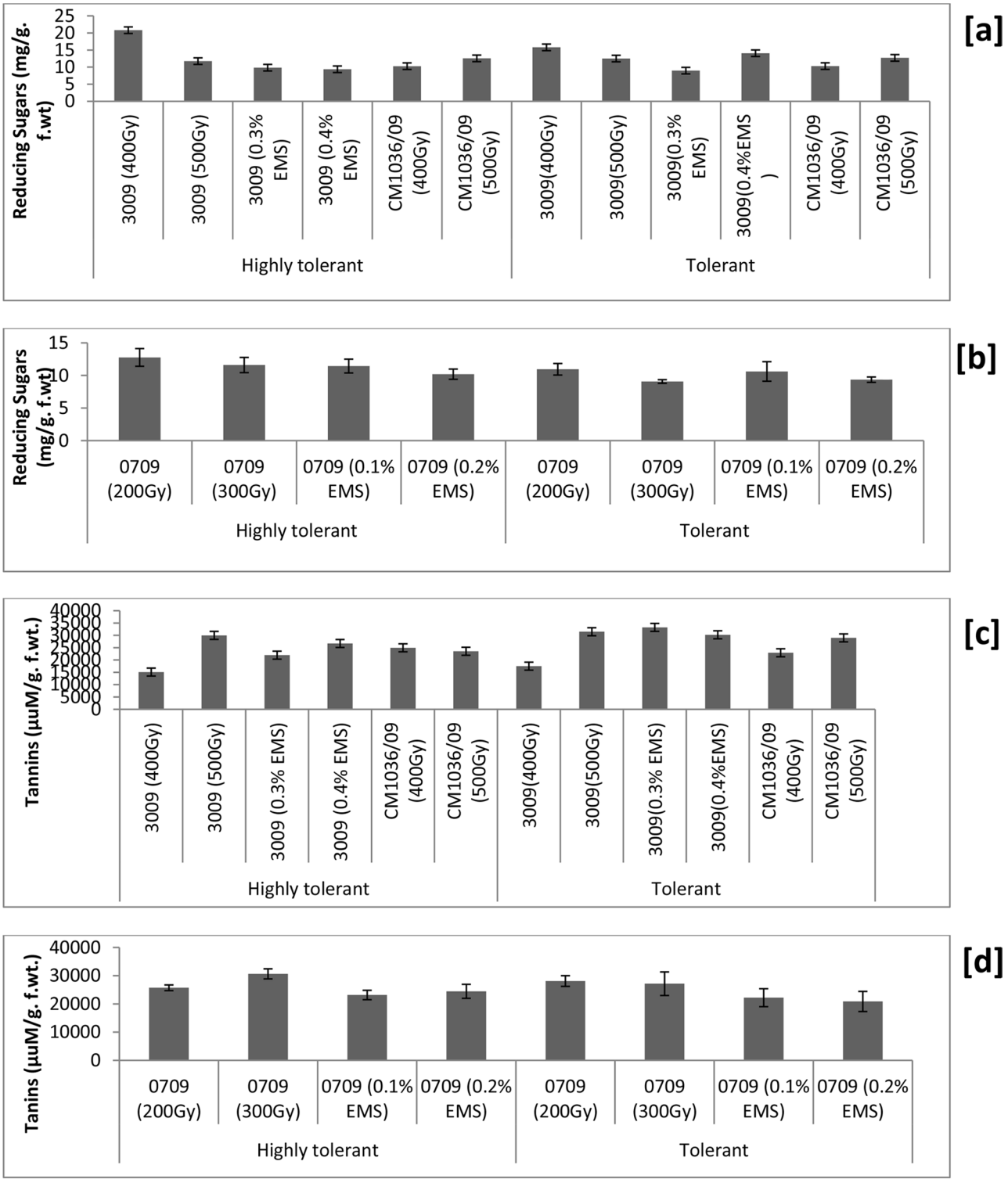

Fig. 10 **a**) Variation in reducing sugars of desi genotypes 3009 and CM1036/09, **b**) reducimg sugars of kabuli 0709, **c**) tanins of desi genotypes 3009 and CM1036/09 and **d**) tanins of kabuli 0709 in different chickpea mutants

Pigments analysis

Lycopene

The mutants of genotype D3009 (at 400 Gy and 500 Gy) that were placed in highly tolerant category showed low contents of lycopene while some mutants of genotype D3009 categorized as tolerant showed higher lycopene contents. Similarly, the mutants of genotype D3009 developed using 0.3% and 0.4% EMS that were placed

in highly tolerant category showed low concentration of lycopene. The mutants of same genotype showed higher lycopene contents in tolerant category. The mutants of genotype CM1036/09 treated by 400 Gy and 500 Gy was also categorized as highly tolerant. The tolerant category showed low concentration of lycopene in highly tolerant category at 400 Gy and high concentration in tolerant category (Fig. 11a). Similarly, the lycopene contents in the mutants of same genotype at 500 Gy showed almost same concentration in both the categories. While the mutants

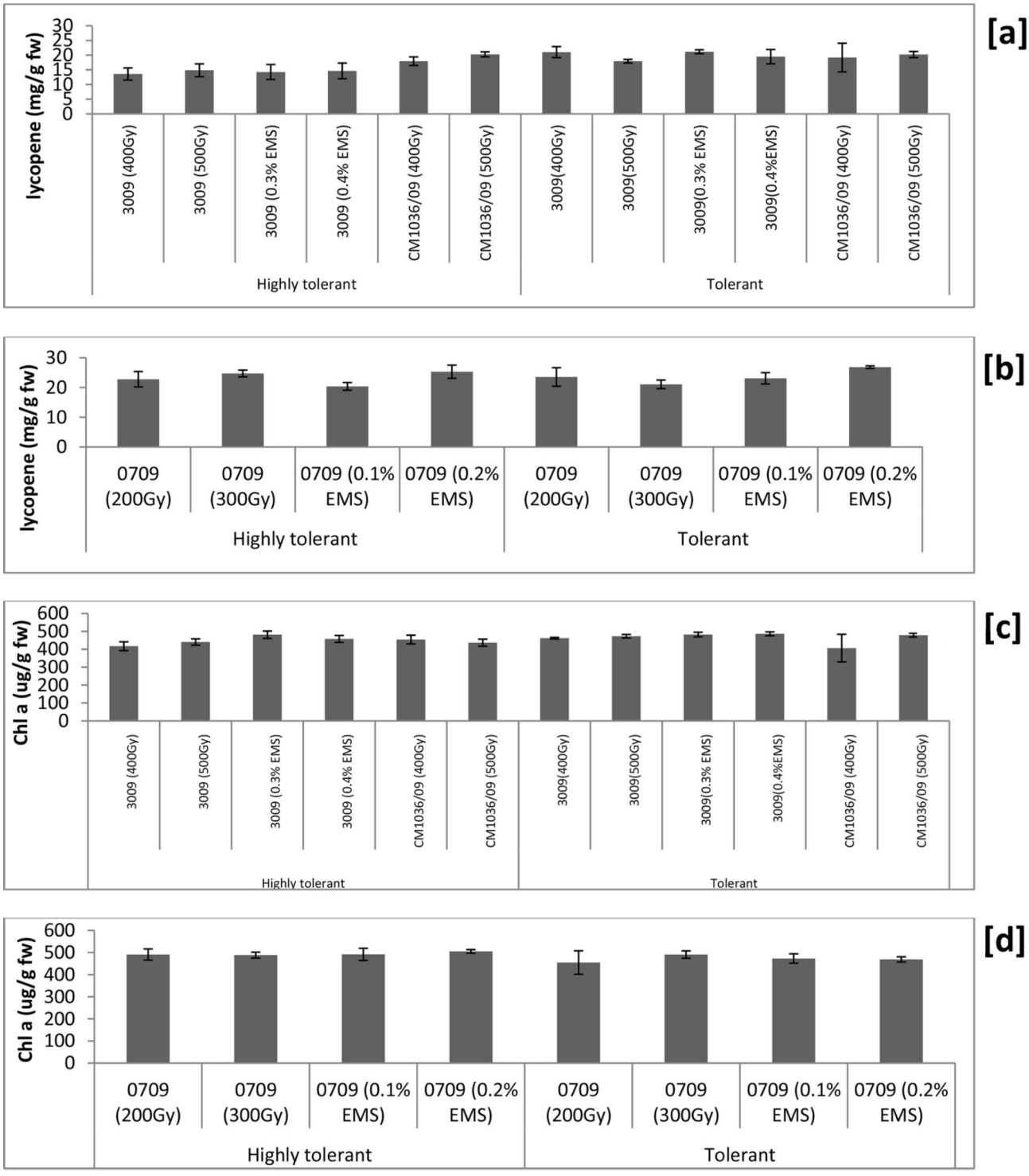

Fig. 11 **a**) Variation in lycopene contents of desi genotypes 3009 and CM1036/09, **b**) lycopene contents of kabuli 0709, **c**) chla of desi genotypes 3009 and CM1036/09 and **d**) chl a of kabuli 0709 in different chickpea mutants

of kabuli genotype K709 (at 200 Gy and 300 Gy) placed in highly tolerant and tolerant category based on presence of lycopene content showed no significant difference in terms of mean (Fig. 11b). Similarly, the mutants of the same genotype developed by using 0.1%EMS and 0.2%EMS that were also placed in highly tolerant and tolerant category showed no significant difference in terms of mean.

Chlorophyll a

The mutants of genotype D3009 (at 400 Gy and 500 Gy) that were placed in highly tolerant category showed low contents of chlorophyll a. Some of the mutants of genotype D3009 categorized as tolerant showed higher chlorophyll a content (Fig. 11c). Similarly, the mutants of genotype D3009 developed using 0.3% and 0.4% EMS that were placed in highly tolerant category showed low concentration of chlorophyll a while the mutants of same genotype showed higher chlorophyll a content in tolerant category. The mutants of genotype CM1036/09 treated by 400 Gy and 500 Gy was also categorized as highly tolerant and tolerant. A high concentration of chlorophyll a with a mean value of 454.3(µg/g fw) was shown in highly tolerant category at 400 Gy and low concentration with a mean value of 406.4 (µg/g fw) in tolerant category. Similarly, the chlorophyll a contents in the mutants of same genotype at 500 Gy showed high chlorophyll a contents in HT category and low concentration in tolerant category. While the mutants of kabuli genotype K709 at 200 Gy and 300 Gy showed high chl a contents in highly tolerant category and low in tolerant category (Fig. 11d). Similarly, the mutants of the same genotype developed by using 0.1%EMS and 0.2%EMS showed high chl a contents in highly tolerant category and low in tolerant category.

Chlorophyll b

The mutants of genotype D3009 (at 400 Gy and 500 Gy) that were placed in highly tolerant category showed low contents of chlorophyll b while some mutants of genotype D3009 categorized as tolerant showed higher chlorophyll b contents. Similarly, the mutants of genotype D3009 developed using 0.3% and 0.4% EMS placed in highly tolerant category showed low concentration of chlorophyll b while the mutants of same genotype showed higher chlorophyll b contents in tolerant category (Fig. 12a). The mutants of genotype CM1036/09 treated by 400 Gy and 500 Gy showed high concentration of chlorophyll b with a mean value of (446.7 µg/g f. wt.) in highly tolerant category at 400 Gy and low concentration with a mean value of (443.3 µg/g f. wt.) in tolerant category. Similarly, the chlorophyll b contents in the mutants of same genotype at 500 Gy showed high chlorophyll b contents in HT with a mean value of (584 µg/g f. wt.) category and low concentration in tolerant category with a mean value of (570.7 µg/g f. wt.) The mutants of genotype K709 at 200 Gy and 300 Gy showed high chlb contents in highly tolerant category and low in tolerant category. Similarly, the mutants of the same genotype developed by using 0.1%EMS showed low chlb contents in highly tolerant category and high in tolerant category (Fig. 12b). The mutants of the genotype K709 at 0.2%EMS showed low chl b contents in highly tolerant category and high in tolerant category.

Total carotenoids

The mutants of genotype D3009 (at 400 Gy and 500 Gy) that were placed in highly tolerant category showed low concentration of total carotenoids while some mutants of genotype D3009 categorized as tolerant showed higher total carotenoids contents (Fig. 12c). Similarly, the mutants of genotype D3009 developed using 0.3% and 0.4% EMS that were placed in highly tolerant category showed low concentration of total carotenoids while the mutants of same genotype showed higher total carotenoids contents in tolerant category. The mutants of genotype CM1036/09 showed low concentration in highly tolerant category at 400 Gy and high concentration in tolerant category. Similarly, the total carotenoids contents in the mutants of same genotype at 500 Gy showed low total carotenoids contents in HT category and high concentration in tolerant category. The mutants of Kabuli genotype K709 at 200 Gy and 300 Gy showed high total carotenoids contents in highly tolerant category and low in tolerant category (Fig. 12d). Similarly, the mutants of the same genotype developed by using 0.1%EMS and 0.2%EMS showed high total carotenoids contents in highly tolerant category and low contents were observed in tolerant category.

Total chlorophyll contents

The mutants of genotype D3009 at 400 Gy that were placed in highly tolerant category showed high concentration of total chlorophyll while some mutants of genotype D3009 categorized as tolerant showed low total chlorophyll contents (Fig. 13a). The mutants of genotype D3009 at 500 Gy showed slight difference as some mutants showed low contents of total chlorophyll with a mean value of 63,388 µg/g f. wt. in HT category and high contents with a mean value of 65,750 g/g f. wt. Similarly, the mutants of genotype D3009 developed using 0.3% and 0.4% EMS that were placed in highly tolerant category showed high concentration of total chlorophyll while the mutants of same genotype showed low total chlorophyll contents in tolerant category. The mutants of genotype CM1036/09 was treated by 400 Gy and 500 Gy. The mutants showed high concentration of total chlorophyll in highly tolerant category at 400 Gy and low concentration in tolerant category. Similarly, the total

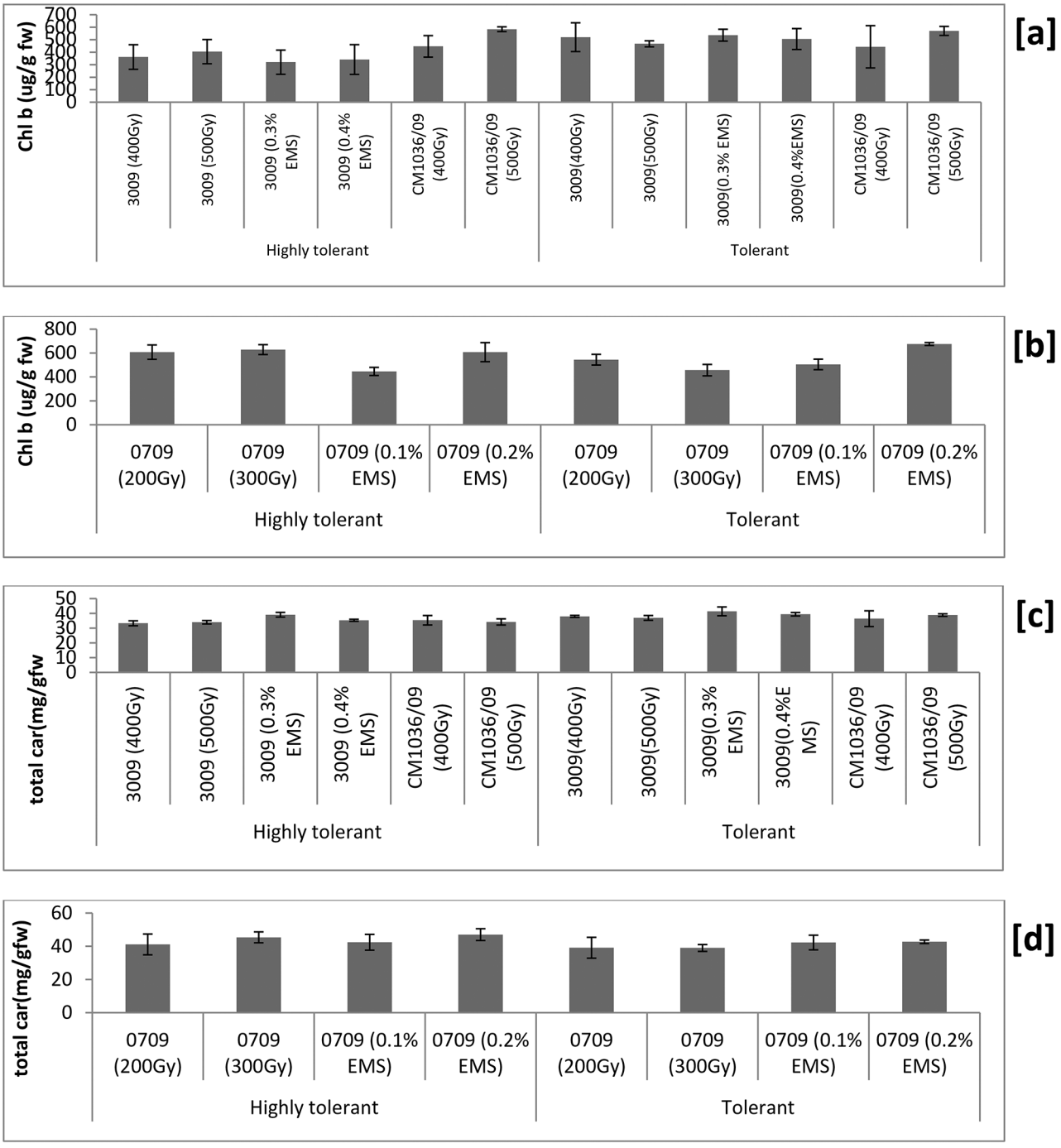

Fig. 12 **a**) Variation in chl b contents of desi genotypes 3009 and CM1036/09, **b**) chl b contents of kabuli 0709, **c**) total carotenoids of desi genotypes 3009 and CM1036/09 and **d**) total car of kabuli 0709 in different chickpea mutants

chlorophyll contents in the mutants of same genotype at 500 Gy showed high total chlorophyll contents in HT category and low concentration in tolerant category. While the mutants of Kabuli genotype K709 at 200 Gy and 300 Gy showed higher contents in highly tolerant category and low in tolerant category (Fig. 13b). While the mutants of the same genotype developed by using 0.1%EMS and 0.2%EMS showed low total chlorophylls contents in highly tolerant category and high contents were observed in tolerant category.

Principal component analysis

Principal component Analysis is used for the conversion of data from a high-dimensional level into a

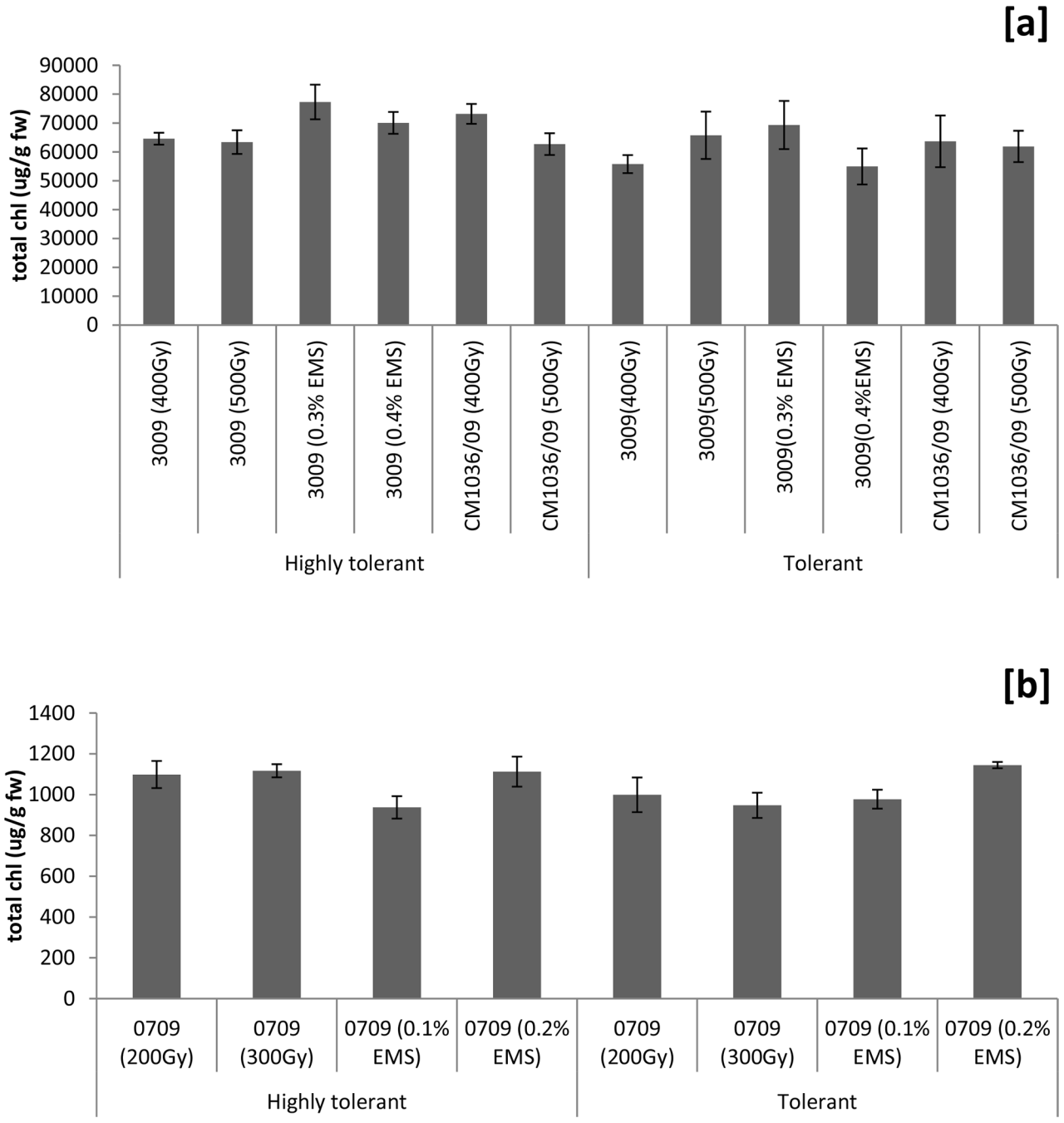

Fig. 13 **a**) Variation in total chl contents of desi genotypes 3009 and CM1036/09, **b**) total chl contents contents of kabuli 0709

low-dimensional level so that meaningful properties of the original data may retain ideally close to its intrinsic dimension. To study the variability and interrelationship among variables of all the mutants principal component analysis (PCA) was performed using all parameters under investigation. In the present study, genotypes were divided in to two categories i.e., Desi and Kabuli based on comparative values of different studied parameters. Data were subjected to principal component analysis. Scree plot (Fig. 14) showed out of the 17 principal components PC(s), five viz. PC-1, PC-II, PC-III, PC-IV, PC-V had Eigenvalues > 1 and contributed for 83.77% in case of desi genotypes of total cumulative variability among the mutants of different genotypes. The contribution of PC-I toward variability was highest (33.47%) followed by PC-II with a contribution of 18.78%, PC-III contributed 13.27%, PC-IV was 11.13% and PC-V had contributed 7.11% respectively. Similarly, data were subjected to principal

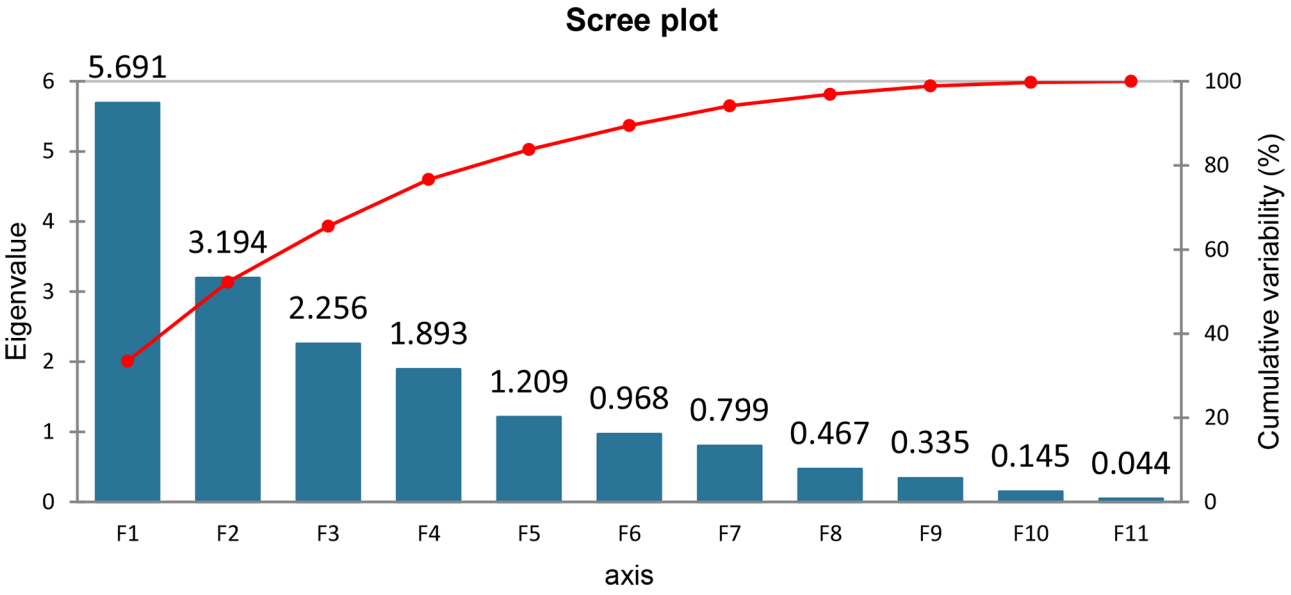

Fig. 14 Scree plot representing cumulative variability and Eigenvalues for studied parameters of desi type mutants

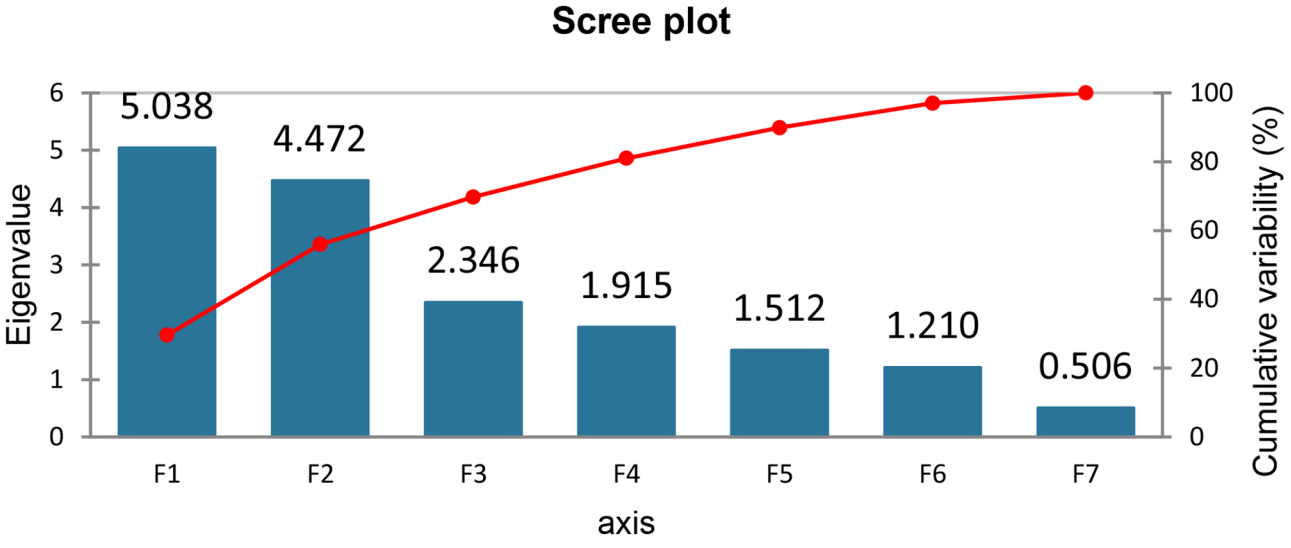

Fig. 15 Scree plot representing cumulative variability and Eigenvalues for studied parameters of Kabuli type mutants

component analysis for kabuli type. Scree plot (Fig. 15) out of the 17 principal components PC(s), six viz.PC-1, PC-II, PC-III, PC-IV, PC-V and PC-VI had Eigenvalues>1 and contributed 97% in case of Kabuli genotype of total cumulative variability among the mutants (Table S1). The contribution of PC-I toward variability was highest (29.63%) followed by PC-II with a contribution of 26.3%, PC-III contributed 13.79%, PC-IV was 11.26% and PC-V had contributed 8.89% and PC-VI had 7.11% respectively.

A genotype by trait biplot was applied in this study showing the PCI scores on x-axis against PC-II scores on Y axis for each trait and genotype (Fig. 16). A GT biplot was constructed for the mutants of both desi and kabuli genotypes separately. This statistical tool effectively explained the visual comparison and contribution of all the genotypes and traits and showed interrelationship between them. The distance of the genotypes and the angles between the vectors and from the origin of the biplot was used to extract important information. On the basis of the angle of the trait vector the correlation between the traits can be positive or negative. If the angle is <90∘ then the correlation between the traits is positive, if the angle is >90∘ then the correlation is negative. In case the angle is equal to 90∘ then there is no dependency of traits on each other. The biplot represented overall association of chickpea genotypes treated at different mutagen doses for 17 traits (Fig. 17). To observe

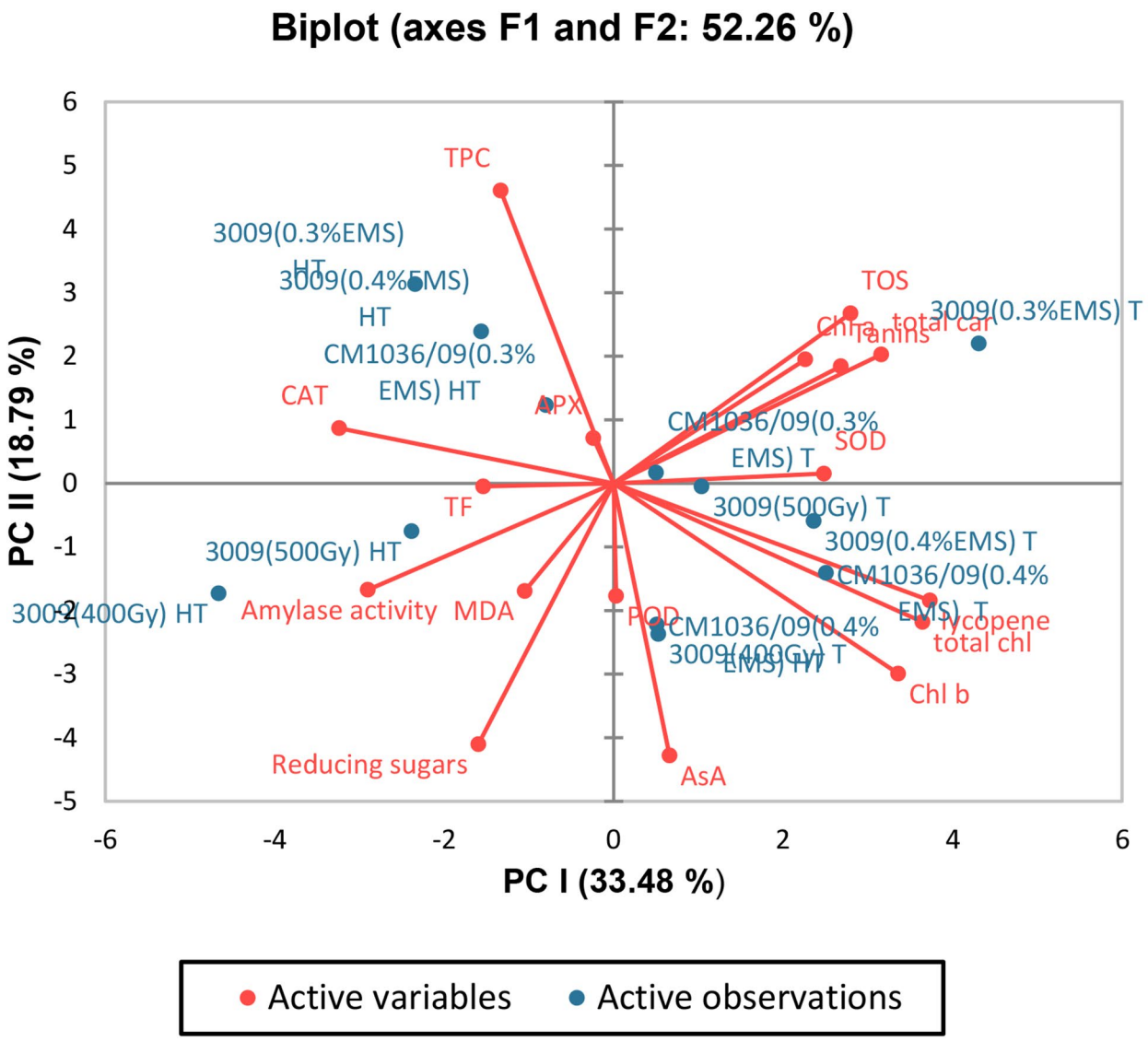

Fig. 16 Bi-plot of chickpea mutants of desi genotypes for the first two principal components

the association between different clusters the first two principal components which had contributed 52.26% (in case of desi genotypes) toward total variance were plotted as PC-I on x axis and PC-II on y-axis. Similarly, to observe the association in Kabuli type the first two principal components which had contributed 55.94% (in case of Kabuli genotypes) toward total variance were plotted as PC-I on x axis and PC-II on y-axis. The genotype by trait (G-T) biplot thus depicted 52.26% (desi genotypes) and 55.94% (Kabuli genotype) respectively of the total variation. In the genotype by trait biplot a vector line was drawn from origin to every trait to describe the interrelationship among characters.

GT biplot for desi type

On the basis of angle between the vectors, the biplot was categorically divided into four groups (A, B, C and D) for both desi and Kabuli genotypes. Group A showed a positive correlation among tannins, Chla, carotenoids, SOD, TOS, group B represented a positive correlation among Chl b, lycopene, total Chl, AsA, group C indicated a positive correlation among CAT, APX, TPC, group D showed positive correlation among amylase activity, MDA and reducing sugars. While no positive association was detected for POD and TF.

GT biplot for Kabuli type

Similarly, in Kabuli type the biplot was divided into four groups where Group A showed a positive correlation among lycopene, chl b, total chl, TOS, carotenoids,

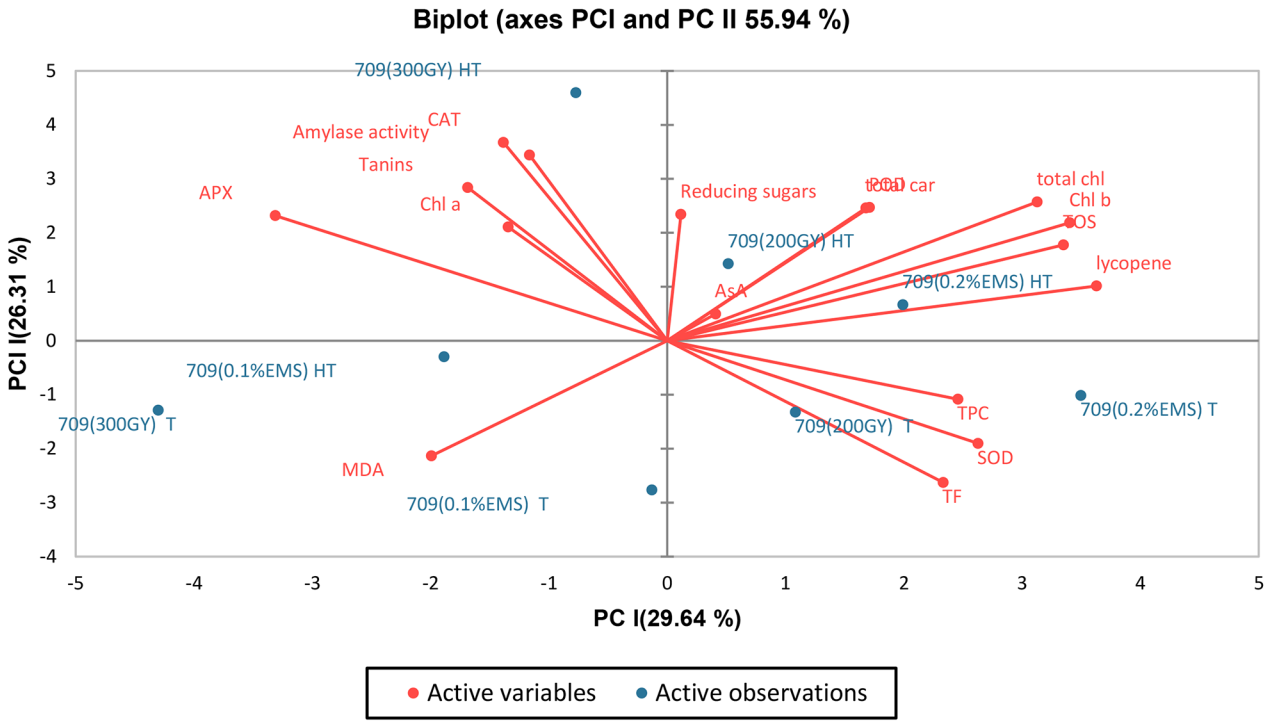

Fig. 17 Bi-plot of chickpea mutants of kabuli genotypes for the first two principal components

ASAand reducing sugars, group B represented a positive correlation among TPC, SOD and TF, group C indicated a positive correlation among amylase, catalase, tannins, chl a and APX, while in group D showed no positive association with other variables for MDA.

Performance of individual chickpea mutants

Biochemical response

Lycopene Among all 72 mutants, the highest lycopene content in desi type was present in D2M1T-1(25.81 mg/g f. wt.) and lowest content was seen n D1M1HT-5 (7.89 mg/g f. wt.). While in Kabuli type the highest contents were present in KM4HT-1 (31.22 mg/g f. wt.) and lowest contents were found in KM3HT-1 (16.53 mg/g f. wt.) (Fig. 18a).

Chlorophyll a (Chl a) The highest Chl a content in desi type were present in D1M3T-1(44.36(µg/g f. wt.) and low content were seen in D2M1HT-1 (28.2 µg/g f. wt.) while in Kabuli type the highest contents were present in KM4HT-1 (56.35 µg/g f. wt.) and lowest contents were found in KM1T-2 (28.24 µg/g f. wt.) (Fig. 18b).

Chlorophyll b (chl b) The highest Chl b contents in desi type were present in D1M3T-1(517.49 µg/g f. wt.) and low content were seen in D1M1HT-5 (321.95 µg/g f. wt.) while in Kabuli type the highest contents were present in KM1T-1 (598.18 µg/g f. wt.) and lowest contents were found in KM1T-2 (371.09 µg/g f. wt.) (Fig. 18c).

Total carotenoid content The highest total carotenoid contents in desi type were present in D1M3T-1(44.36 µg/g f. wt.) and low content were seen in D2M1HT-1 (28.2 µg/g f. wt.) while in Kabuli type the highest contents were present in KM4HT-1 (56.35 µg/g f. wt.) and lowest contents were found in KM1HT-3 (29.19 µg/g f. wt.) (Fig. 18d).

Total chlorophyll content The highest total Chl contents in desi type were present in D2M1T-1(1149.31 µg/g f. wt.) and low content were seen in D1M1HT-5 (431.03 µg/g f. wt.) while in Kabuli type the highest contents were present in KM4HT-1 (1197.29 µg/g f. wt.) and lowest contents were found in KM2T-2 (776.14 µg/g f. wt.) (Fig. 19a).

Total phenolic contents TPC The highest total phenolic contents in desi type were present in D1M3HT-3(87000 µg/g f. wt.) and low content were seen in D1M4T-3 (42550 µg/g f. wt.) while in Kabuli type the highest contents were present in KM1HT-2 (82650 µg/g f. wt.) and lowest contents were found in KM1T-4 (43950 µg/g f. wt.) (Fig. 19b).

Tannins The highest tannins in desi type were present in D2M1HT-4 (36300µM/g. f. wt.) and low content were seen in D1M1T-2 (9100 µM/g. f. wt.), while in Kabuli

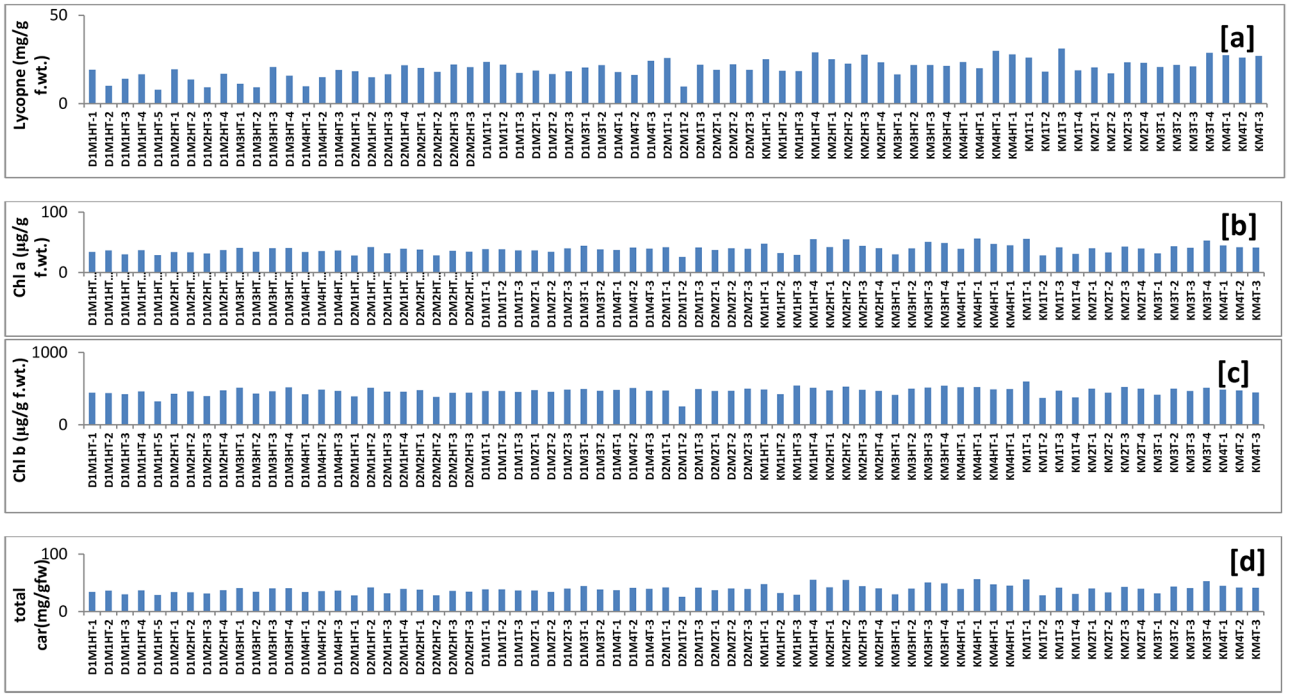

Fig. 18 **a**) Variation in lycopene contents of all 72 individual mutants of desi and kabuli genotypes., **b**) Variation in chl a, **c**) Variation in Chl b and **d**) Variation in total carotenoids in all 72 individual mutants

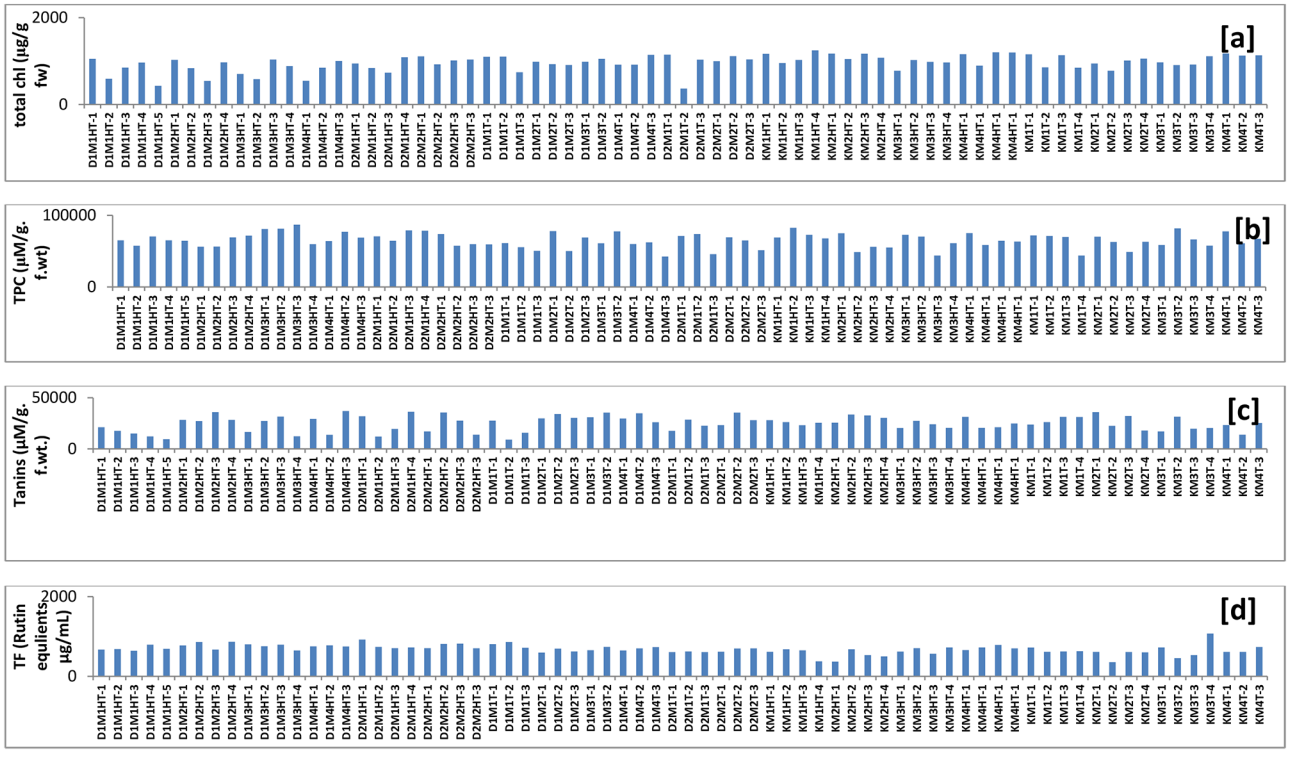

Fig. 19 **a**) Variation in total chlorophyll of all 72 individual mutants of desi and kabuli genotypes., **b**) Variation in TPC, **c**) Variation in Tanins and **d**) Variation in TF in all 72 individual mutants

type the highest contents were present in KM2T-1 (35950 µM/g. f. wt.) and lowest contents were found in KM4T-2 (13850 µM/g. f. wt.) (Fig. 19c).

Total flavonoid contents (TF) The highest total flavonoid contents in desi type were present in D2M1HT-1 (921.32 Rutin equlients µg/mL) and low content were seen in D2M1T-1 (605.41 Rutin equlients µg/mL) while in Kabuli type the highest contents were present in KM3T-4 (1071.33 Rutin equlients µg/mL) and lowest contents were found in KM2T-2 (356.29 Rutin equlients µg/mL) (Fig. 19d).

Catalase activity The highest catalase activity in desi type was present in D1M1HT-2 (4200 Units/g f. wt.) and activity was seen in D1M3HT-4 (140 Units/g f. wt.) while in Kabuli type the highest contents were present in KM3HT-2 (540 Units/g f. wt.) and lowest contents were found in KM3T-4 (120 Units/g f. wt.) (Fig. 20a).

Sodium oxide dismutase activity The highest activity of sodium oxide dismutase in desi type were present in D2M2T-1 (307 Units/g f. wt.) and low activity were seen in D1M1HT-5 (64 Units/g f. wt.) while in Kabuli type the highest activity was present in KM2HT-2 (308Units/g f. wt.) and lowest activity was found in KM4T-1 (112 Units/g f. wt.) (Fig. 20b).

Peroxidase activity The highest activity of POD in desi type were present in D2M2HT-3 (71794. 8 Units/g f. wt.) and low activity was seen in D1M1HT-5 (1665 Units/g f. wt.) while in Kabuli type the highest activity was present in KM3HT-1 (80053.2 Units/g f. wt.) and lowest activity was found in KM4HT-1 (6127.2 Units/g f. wt.) (Fig. 20c).

MDA content The highest contents of MDA in desi type were present in D1M1HT-1 (495 µM/g f. wt.) and low content were seen in D2M1T-2 (93 µM/g f. wt. while in Kabuli type the highest contents were present in KM2T-3 (957µM/g f. wt.) and lowest contents were found in KM3HT-1 (14 µM/g f. wt.) (Fig. 20d).

Reducing sugars The highest reducing sugars in desi type were present in D1M1HT-2 (56.7 mg/g. f. wt.) and low sugars were seen in D1M3T-2 (7 mg/g. f. wt.), while in Kabuli type the highest contents were present in KM1HT-4 (15.1 mg/g. f. wt.) and lowest contents were found in KM3T-2 (8.3 mg/g. f. wt.) (Fig. 21a).

Amylase activity The highest amylase activity in desi type were present in D1M2HT-1 (389 mg/g. f. wt.) and

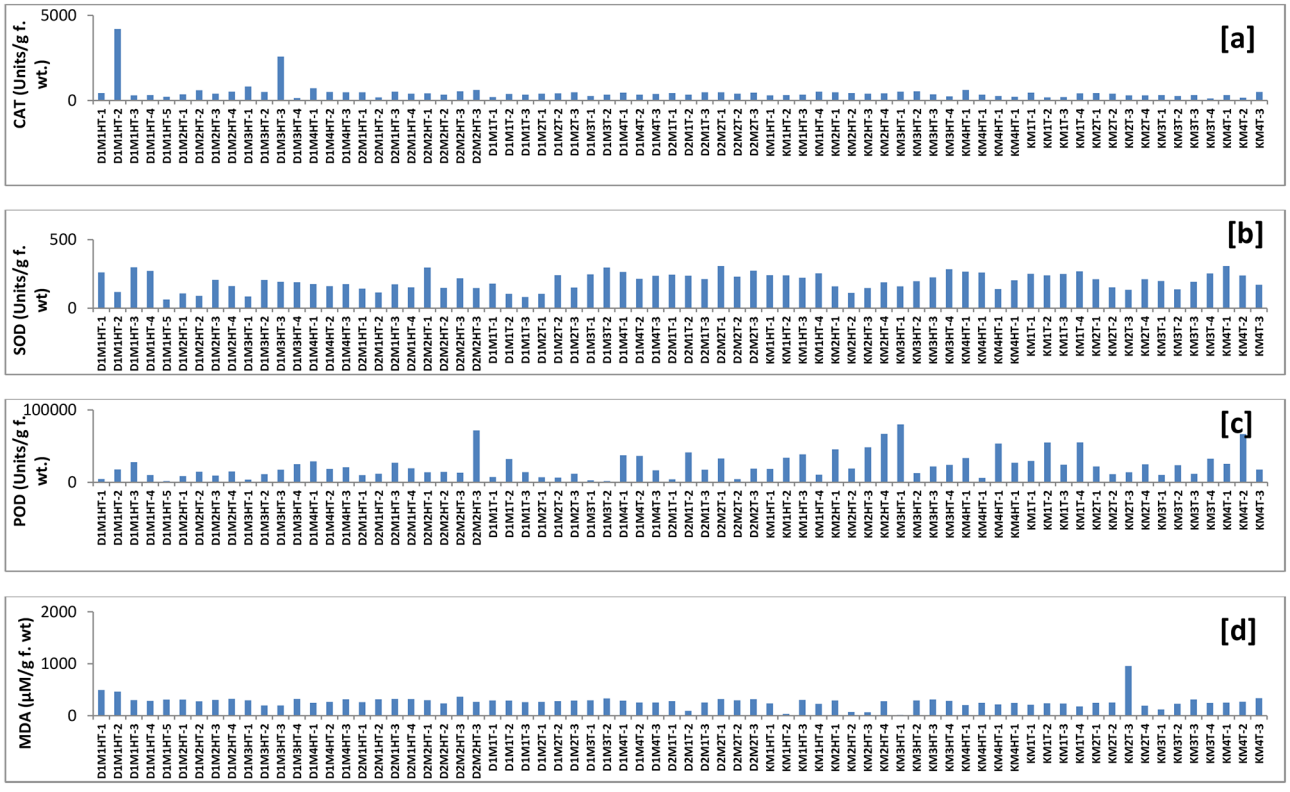

Fig. 20 Variation in enzymatic activity of all 72 individual mutants of desi and kabuli genotypes., **a**) Catalase activity **b**) Variation in SOD, **c**) Variation in POD and **d**) Variation in MDA in all 72 individual mutants

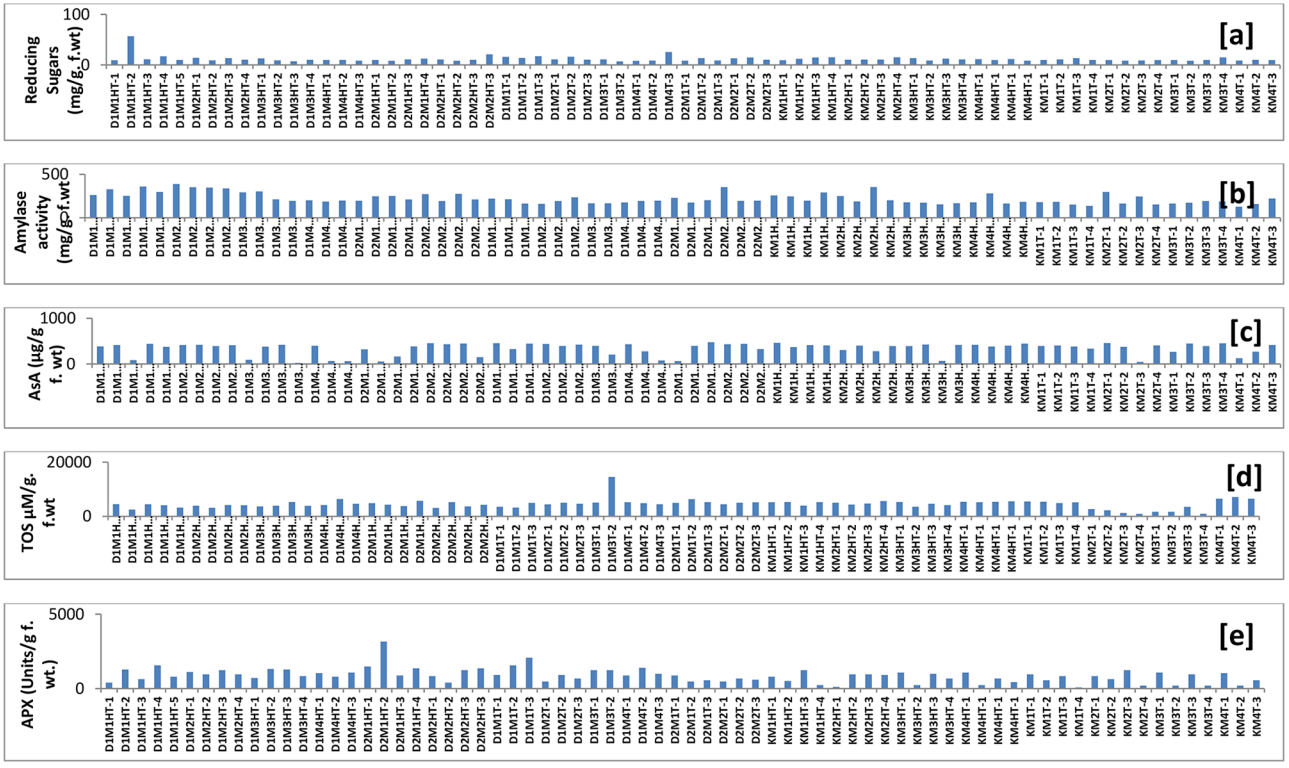

Fig. 21 **a**) Variation in reducing sugars of all 72 individual mutants of desi and kabuli genotypes., **b**) Variation in amylase activity, **c**) Variation in AsA **d**) Variation in TOS and **e**) Variation in APX in all 72 individual mutants

low activity was seen in D1M2T-1 (160 mg/g. f. wt.) while in Kabuli type the highest activity was present in KM2HT-3 (354 mg/g. f. wt.) and lowest activity was found in KM4T-1 (126 mg/g. f. wt.) (Fig. 21b).

Ascorbic acid content The highest ascorbic acid contents in desi type were present in D2M1T-3 (447 µg/g f. wt.) and low content were seen in D1M3HT-4 (22 µg/g f. wt. while in Kabuli type the highest contents were present in KM1HT-1 (462 µg/g f. wt.) and lowest contents were found in KM2T-3 (43 µg/g f. wt.) (Fig. 21c).

Total oxidant status The highest total oxidant status in desi type was present in D1M3T-2 (14550 µM/g. f. wt.) and low TOS was seen in D1M1HT-2 (2500 µM/g. f. wt.), while in Kabuli type the highest TOS was present in KM4T-2(7150 µM/g. f. wt.) and low TOS was observed nd in KM3T-4 (950 µM/g. f. wt.) (Fig. 21d).

Ascorbate peroxidase activity The highest ascorbate peroxidase activity in desi type were present in D2M1HT-2 (3160 Units/g f. wt.) and low activity was seen in D1M1HT-1 (400 Units/g f. wt.) while in Kabuli type the highest activity was present in KM1HT-3 (1240 Units/g f. wt.) and lowest activity was found in KM1T-4 (80 Units/g f. wt.) (Fig. 21e).

Cluster heat map analysis The cluster heat map analysis showed the responses of biochemical parameters of all the mutants against herbicide resistance (Fig. 22). On the basis of traits association, the heat map divided the mutants into four main dendrograms with respect to their class of tolerance highly tolerant and tolerant (HT and T) regarding to their biochemical performance. At HT two mutants of kabuli genotype 709 at 200 Gy, three mutants at 300 Gy, three mutants at 0.2% EMS and at Tone mutant at 200 Gy, two mutants at 0.2%EMS, only one mutant from desi genotype CM1036/09 at 0.3% EMS marked one cluster. This cluster represented higher pigments value > 1 including total carotenoids, chl a, chl b, total chl and lycopene contents. While at HT desi genotype D3009 at different doses of 400 Gy, 500 Gy, 0.3%EMS, and 0.4%EMS marked another cluster showed lowest pigments value <-1. Rest of the mutants marked another separate cluster, in terms of the trait association and expression against different doses class of tolerance. Similarly, the mutants showed an analogous trend of dendrograms formation at both the HT and T. All the traits demonstrated differential associations moving from positive to negative extremes in all the mutants including highly tolerant and tolerant at different doses of herbicide as demonstrated in Fig. 22.

Correlation analysis Correlation (Pearson correlation) was performed for all the studied biochemical param-

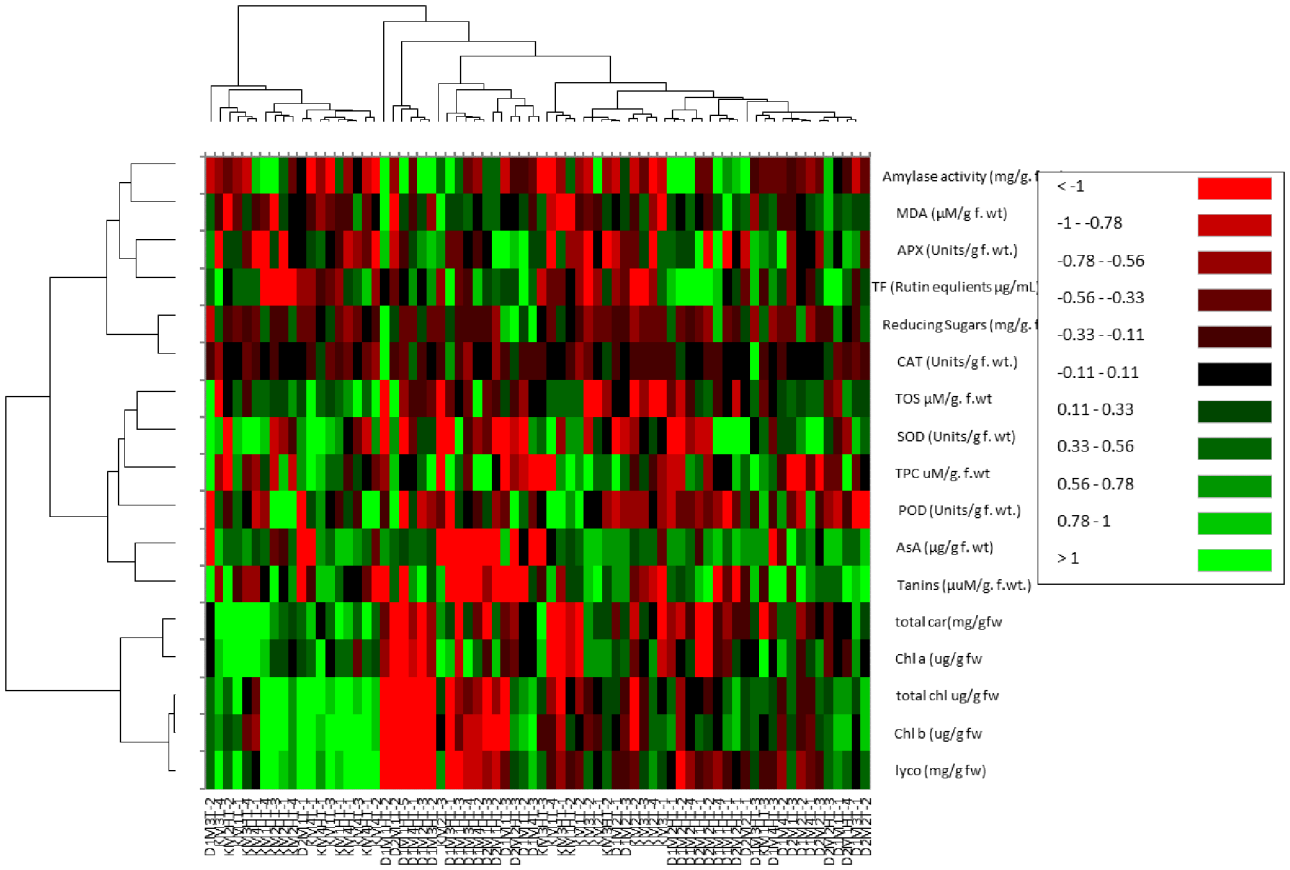

Fig. 22 Cluster heat map analysis showed the responses of biochemical parameters of all the mutants against glyphosate resistance

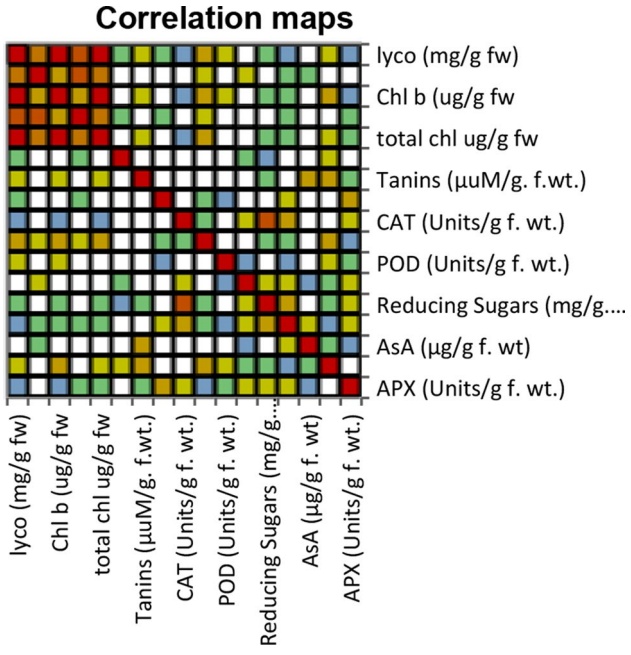

Fig. 23 Correlation (Pearson correlation) for all the studied biochemical parameters with 95% confidence of interval

eters with 95% confidence of interval. Lycopene showed significant positive correlation with chl a, chl b, total car and total chl and SOD while significant negative correlation with amylase activity and APX (Fig. 23). Chl a showed significant positive correlation with lycopene, chl b, total car and total chl. Chl b showed significant positive correlation with lycopene chl a, total, total chl and SOD. Total car showed significant positive correlation with lycopene chl a, chl b, total chl. Total chl showed significant positive correlation with lycopene chl a, chl b, total car and SOD. Tannins showed positive correlation with AsA. TF showed significant negative correlation with APX. CAT showed significant negative correlation with reducing sugars. SOD showed significant positive correlation with lycopene, chl b, total chl, TOS while negative correlation with APX. POD showed significant negative correlation with MDA. TOS showed significant positive correlation with SOD. APX showed significant positive correlation with TF and significant negative correlation with lycopene, SOD and AsA.

Morphological traits

Plant height

Among all these 72 mutants the highest plant height was observed 51 cm in D1M2HT-2 in desi genotype and lowest was 24.5 cm observed in D1M1HT-1. Similarly in case of Kabuli genotype the highest plant height was observed 56.5 cm in KM1HT-3 and the lowest was observed 24 cm in KM4HT-2 (Fig. 24a).

No. of primary branches

In desi genotype the highest No. of primary branches were observed 8 in D2M2T-1 and the lowest were 2 in D2M2T-2. Similarly in case of Kabuli genotype the lowest was observed 2 in KM3T-2 (Fig. 24b).

No. of pods/plant

The highest No. of pods/plant were observed 47 in mutant of desi genotype D1M2HT-2 and 53 were observed in kabuli genotype KM3HT-4. The lowest values were observed in D1M2T-3 and 2 in KM2T-1 (Fig. 24c).

No. of seeds /plant

Among all these 72 mutants the highest No. of seeds / plant were 57 observed 51 cm in D1M2HT-2 in desi genotype and lowest was 0 observed in multiple mutants. Similarly in case of Kabuli genotype No. of seeds /plant were observed 46 in KM3HT-2 and the lowest was 0 observed in multiple mutants due to poor pod formation and pod borer attack there were no seeds (Fig. 24d).

Seed yield/plant

Out of 72 morphologically tolerant mutants, seed was set in 34 mutants in which 24 were of Desi type and 10 were Kabuli type. The highest seed yield (13.63 g g/plant) was observed in Desi type chickpea mutant D1M2HT-2 followed by 13.52 g in D1M1HT-2, 11.26 g in D1M1HT-3 and 11.97 g in D1M2HT-3. Whereas in Kabuli type mutants, 10.992 g was observed in KM3HT-2 follwed by 7.353 g in KM4HT-1 and KM1HT-4. (Fig. 24e).

Correlation analysis

Correlation (Pearson correlation) was performed for all the studied morphological parameters with 95% confidence of interval (Table 4). Plant height showed

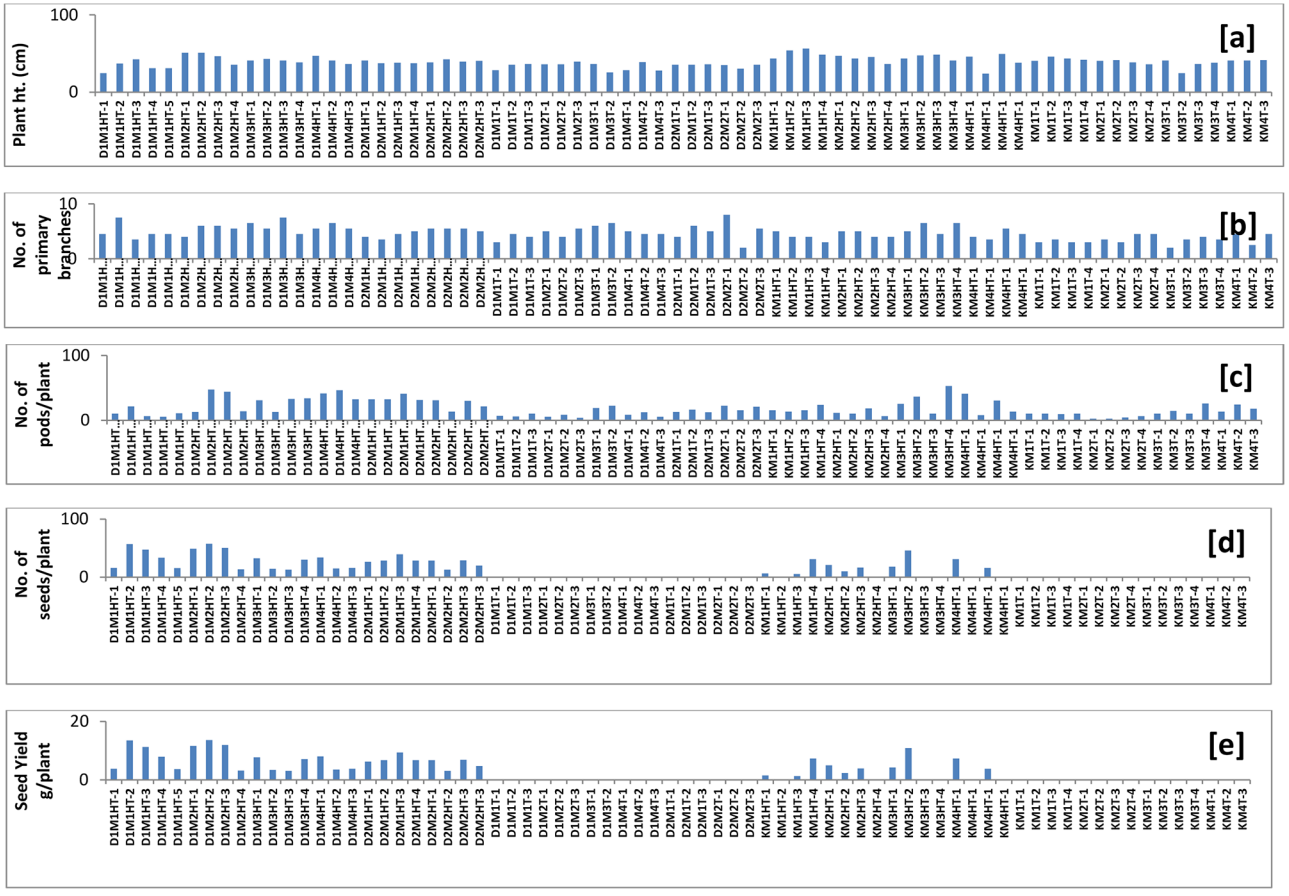

Fig. 24 Variation in different morphological parameters observed in all mutants of desi and kabuli genotypes **a**) Plant height, **b**) No. of primary branches, **c**) No. of pods/plant, **d**) No. of seeds/plant and **e**) seed yield/plant

Table 4 Correlation matrix (Pearson)

Parameters	Plant ht. (cm)	No. of Primary branches	No. of Pods/ plant	No. of seeds	Seed Yeild g/plant
Plant ht. (cm)	1	0.028	**0.296**	**0.339**	0.3386
No. of Primary branches	0.028	**1**	**0.454**	**0.302**	0.3024
No. of Pods/ plant	**0.296**	**0.454**	**1**	**0.553**	0.5533
No. of seeds	**0.339**	**0.302**	**0.553**	**1**	1.0000
Seed Yeild g/ plant	0.3386	0.3024	0.5533	1.0000	1

Values in bold are different from 0 with a significance level alpha = 0.05

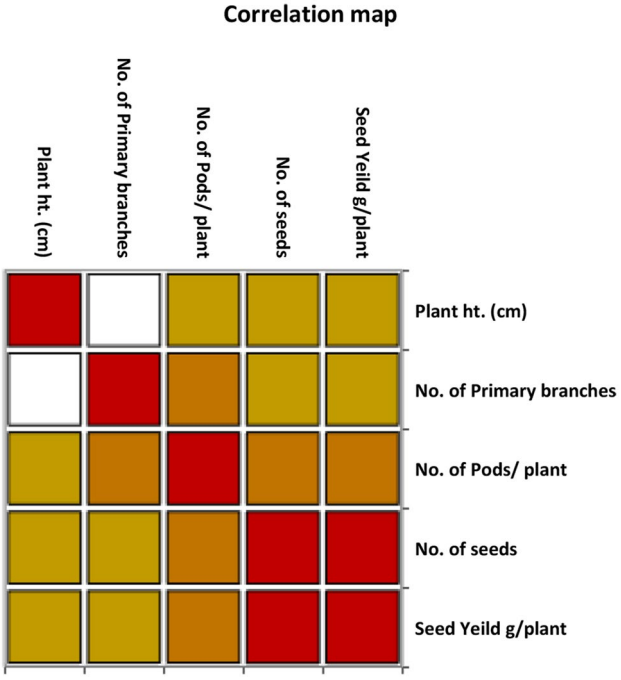

Fig. 25 Correlation map for all the morphological parameters of all mutants

significant positive correlation with no. of pods/plant, seed yield/plant and no. of seeds/plant (Fig. 25). Number of primary branches showed significant positive correlation with no. of pods/plant and no. of seeds/plant. Number of pods/plant showed significant positive correlation with number of primary branches and no. of seeds/plant. No. of seeds/plant highest significant positive correlation with no. of pods/plant.

Discussion

Currently, agriculture is bearing a dual pressure; escalating human population demands 25–70% more production of food to fulfill the nutritional requirements, and conversely weeds add to a considerable yield loss in every cropping system, which is an alarming situation for global food security. As weeds have developed resistance against herbicides, weed management has become complex to meet the food demand. Although, breeders from last two decades have adopted many of the laborious and time-consuming agronomic practices but herbicide weed management has remained the promising one [34]. An extensive study of the effects of glyphosate-based herbicide on morphological and biochemical processes was done. Plants evade the effects of herbicides they come across by different mechanisms. The basic herbicide resistance mechanisms can be catalogued as follows: (a) altered molecular/cellular/target-site resistance: typically represented by amino acid substitutions that affect herbicide interactions at the target enzyme. (b) enhanced metabolism: a chemical modification of the herbicide by either conjugation or degradation. (c) Compartmentalization or sequestration: exclusion of the herbicide from the target, either physically with enhanced cuticular, other structural barriers or physiologically with active transporters. (d) Over-expression of the target protein/ Avoidance: Pertaining to biochemical ability to handle the toxic agent produced by the herbicide and thereby avoid a toxic result [12]. The mechanisms of both target site resistance (TSR) and Non target site resistance NTSR are found to be involved in most of the herbicide classes e.g., in case of glyphosate [35]. The molecular site and inhibited process involved in glyphosate is EPSPS and shikimate pathway function respectively [35]. The gene responsible for herbicide tolerance has been reported in 1984. A single gene with a point mutation (C675 to T675) causing one amino acid (Ala 205 to Val 205) change has been reported in chickpea against herbicide tolerance [3]. This achievement is a key to success for the development of herbicide resistant variety in chickpea [22].

Variation is a backbone of plant breeding. In nature variation exist in the form of natural mutation or artificially induced mutations. In traditional plant breeding, breeders use different breeding techniques to develop herbicide resistant plants Since the 1930s induced mutagenesis is used to produce or modify the gene of interest in order to increase the yield and quality attributes in crops. Both chemical and physical mutagens are used in inducing mutations in seeds and other parts of a plant [36]. Physical mutagens have been widely used from past 80 years and 70% of the mutant varieties have been developed through physical mutagens [36]. Chemical mutagenesis has become a commonly adopted approach because it does not require special facilities and the resultant mutations are mainly SNPs. EMS (Ethyl methane sulfonate) is widely used chemical mutagen [37].

Faulkner adapted the mutation breeding technique in existing cultivars and then selected the mutations that depicted herbicide resistance or tolerance [4]. Galili et al. 2021 [38] developed a chickpea mutant line (M2033) using ethyl methane sulfonate (EMS) resistant to

imidazolinone. Toker et al.. 2012 [39] screened out three *Cicer* species including four desi, five kabuli and one accession of *C. bijugum* and two accessions of *C. reticulatum* gamma irradiated with 200, 300 and 400 Gy gamma rays from a ^{60}Co source. From *C. reticulatum*, one highly IMI resistant was isolated. Umavathi and Mullainathan 2016 [40] studied the effect of both gamma rays and EMS on chickpea mutants with special reference to the frequency and spectrum of chlorophyll mutations. They found EMS to be more effective in inducing chlorophyll mutations than gamma rays in chickpea. Many successful herbicide tolerant (HT) mutants have been identified and developed different HT crops by chemical mutagenesis e.g. sunflower tolerant to imidazolinines and sulfonylurea, soybean tolerant to sulfonylurea herbicides and wheat tolerant to sulfonylurea [34]. Thompson and Tara'n 2014 [22] identified a point mutation in AHAS1 (*acetohydroxyacid synthase)* gene resistant to imidazolinone in chickpea.

Herbicides usually have to face the plant at the soil-root, or leaf-air, interfaces. The first plant structure that encounters the herbicides are leaf in case of foliar application [41]. The most rapid and reliable measurement of tolerance in chickpea was reported on a 0–9 scale ratings based on plant injury [21]. We further simplified this scale and established a 1–5 scale that is quite convenient and reliable in measuring herbicide tolerance. To determine the herbicide resistance, the treatment plots were sprayed with the glyphosate, 30 days after sowing using a shoulder-mounted hand operated knapsack sprayer. The plots were observed under five visual injury ratings after an interval of 10 days. Out of 376 lines of mutants 72 mutant plants were selected on plant injury basis on a scale of 1–5. Leaf samples of the selected plants were collected and stored at -20°C for determination of biochemical activities. Out of 376 mutant lines, forty mutants were found to be highly tolerant with phenotypic scoring of five, thirty two mutants were found tolerant with phenotypic scoring of four, and twenty of the mutants were found highly susceptible with the phenotypic score of one. Glyphosate was showed to affect both crop growth and reproduction. The mutants of both the kabuli and desi genotypes showed various signs of phytotoxicity after the herbicide treatment. The first symptoms were seen in the meristematic tissue (growing tips and young leaves) (Figure. 4.1). Death of the plants was observed in highly sensitive genotypes (Figure. 4.2) with the meristemic tissues dying first followed by slow necrosis of the mature tissues. In tolerant genotypes, the death of epical meristem induced branching, similar to the effects of nipping practice followed in chickpea (Figure. 4.3 and Figure. 4.4). Other abnormalities observed were – stunted growth, elongation of branches similar to tendrils with very small or needle shaped leaves (Figure. 4.5), delaying of flowering, reddish leaves (Figure. 4. 6), deformed flowers (Figure. 4.7), and poor pod setting. Secondary growth was observed in several lines 20–25 days after herbicidal application leading to flowering and pod set [23]. As glyphosate slow down the synthesis of branched chain amino acids valine, leucine, and isoleucine, there will be rapid decrease in the pool sizes of these amino acids. This leads to decrease in protein synthesis. A lower rate of protein synthesis, in turn causes slowdown in the rate of cell division, and eventually death of the cells. Since mature tissues contain larger pools of amino acids as well as protein reserves which can be catabolized to amino acids upon protein starvation. Thus, mature leaves take longer to express the phytotoxic effects of the glyphosate. Apart from decrease in protein, factors such as accumulation of the cytotoxic EPSPS or derivatives, amino acid content imbalance, inhibition of DNA synthesis and cell division, and reduction of assimilate translocation will result in herbicide-induced growth [42].

The present study is reported for the first time and there is insufficient data in the literature related to this study. Therefore, making a comparison of the results obtained in the present studies was difficult. Nonetheless, a few papers reported some biochemical activities of *Cicer arietinum* and few other crops. The present investigation explores the presence of enzymatic constituents such as.

SOD, POD, APX, CAT, MDA, alpha amylase, non-enzymatic antioxidants, and other phytochemicals like AsA, TOS, TPC, TF, tannins and pigments. Different stress factors may cause overproduction of reactive oxygen species (ROS) and resultantly enhance the oxidative stress in crops. Lately, the association of the antioxidant system and herbicide treatment in crops is a matter of investigation. In this study, we observed plant defense mechanism, activation of antioxidant system against ROS production in chickpea due to herbicide exposure.

In this study, the effect of glyphosate was observed in enhancing oxidative degradation of lipids along with changes in antioxidants enzymes. Malondialdehyde (MDA) is normally exploited as an indicator of lipid peroxidation under various environmental stresses [43]. MDA reveals the amount of damage to the plant cells [44]. Here, The mutants of desi genotype D3009 (developed at 400 Gy) from highly tolerant category showed significantly higher MDA contents (370.684 µM/g f. wt.). Progressively, the highest MDA contents were associated with the increased concentration of herbicide as 3 times increased concentration of glyphosate produced the highest amount (13.8 µ mol g^{-1} f.w.) of MDA in leaves [43]. In a similar study, Curá et al. (2017) have showed progressive increase in MDA content of seedlings during stress condition as compared to control. The mutants of

Kabuli genotype K709 developed at 300GY showed highest (413.419 μ mol g^{-1} f. wt.) MDA contents.

Alpha amylase acts as a catalyst in the hydrolysis of the internal α-1,4-glycosidic linkages in starch, resultantly converts starch into low-molecular-weight products such as maltose glucose and maltotriose units [45, 46]. α-amylase activity was observed significantly higher in the mutants of desi genotype D3009 from highly tolerant category developed at 500 Gy while significantly lower in the same genotype in tolerant category. Some of the mutants of genotype D3009 (0.3%EMS) from tolerant category showed significantly lower amylase activity. Alpha amylase activity was highest (247.642 mg/g f. wt.) in the mutants of kabuli genotype K709 mutated at 200 Gy categorized as highly tolerant. The lowest amylase activity (162.736 mg/g f. wt.) was observed in the mutants of K709 (200 Gy) from tolerant category.

Total oxidant status (TOS) generally exhibits the overall oxidation state of the living organism [11]. Similarly, TAS (total antioxidant status) depicts the antioxidant defense system of the living body [47–49]. Lowest TOS (3760.000 μM/g f. wt.) was detected in the mutants of desi genotype D3009 (400 Gy) from highly tolerant category and highest value (9800.000 μM/g f. wt.) was observed in the same genotype mutated at 0.3% EMS from tolerant category. The mutants of kabuli genotype K709 developed by using 0.3% EMS showed higher TOS value (6716.667μM/g f. wt.) and lowest (1775.000 μM/g f. wt.) was observed in the mutants of K709 from tolerant category developed by using 300 Gy.

Large statistical data are increasingly widespread and are often hard to interpret. Principal component analysis (PCA) is one of the oldest and most reliable statistical techniques used. PCA is generally employed to reduce the dimensionality of a dataset, while preserving maximum possible variation in a dataset [50]. In the present study, genotypes were divided in to two categories i.e., Desi and Kabuli based on comparative values of different studied parameters. Data were subjected to principal component analysis. Out of the 17 principal components PC(s), five viz. PC-1, PC-II, PC-III, PC-IV, PC-V had Eigenvalues>1 and contributed for 83.77% in case of desi genotypes of total cumulative variability among the mutants of different genotypes (Table S1). The contribution of PC-I toward variability was highest (33.47%) followed by PC-II with a contribution of 18.78%, PC-III contributed 13.27%, PC-IV was 11.13% and PC-V had contributed 7.11% respectively. Similarly, data were subjected to principal component analysis for kabuli type. Out of the 17 principal components PC(s), six viz.PC-1, PC-II, PC-III, PC-IV, PC-V and PC-VI had Eigenvalues>1 and contributed 97% in case of Kabuli genotype of total cumulative variability among the mutants (Table S1). The contribution of PC-I toward variability was highest (29.63%) followed by PC-II with a contribution of 26.3%, PC-III contributed 13.79%, PC-IV was 11.26% and PC-V had contributed 8.89% and PC-VI had 7.11% respectively. The biplot represented overall association of chickpea genotypes treated at different mutagen doses for 17 traits (Fig. 1). To observe the association between different clusters the first two principal components which had contributed 52.26% (in case of desi genotypes) toward total variance were plotted as PC-I on x axis and PC-II on y-axis. Similarly, to observe the association in kabuli type the first two principal components which had contributed 55.94% (in case of kabuli genotypes) toward total variance were ploted as PC-I on x axis and PC-II on y-axis. The genotype by trait (G-T) biplot thus depicted 52.26% (desi genotypes) and 55.94% (kabuli genotype) respectively of the total variation. In the genotype by trait biplot a vector line was drawn from origin to every trait to describe the inter-relationship among characters.

Conclusion

We concluded that the mutants of both desi and Kabuli genotypes exhibited tolerance against glyphosate. These results were obtained on the basis of 2 years of field experiments. The mutants of desi genotypes showed more tolerance comparatively to kabuli on physical injury basis. Both desi and Kabuli genotypes showed antioxidant properties. Desi i.e. D1M1HT-2 and Kabuli i.e. KM3HT-2 type mutants with highest seed yield had maximum catalase activity (4200 Units/g f. wt and 540 Units/g f. wt.). The mutants of desi genotypes are more promising than Kabuli genotypes due to their antioxidant properties that played vital role in their herbicide tolerance. Identified mutants with highly tolerant properties can be utilized to introduce glyphosate tolerance trait in chickpea. Moreover, genotypes with high antioxidant properties can be directly utilized as development of diagnostic marker for herbicide tolerance.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05733-x.

Supplementary Material 1

Acknowledgements
we acknowledge all staf.

Author contributions
M.I. did the overall execution of the experiment, analytical work, collection of data, organization of resulting data, write up, and revision of manuscript. A.H. and T.M.S. performed the basic idea and planning, designed the experiments, helped the data analysis, and wrote, revised, and finalized the manuscript. All authors contributed to the article and approved the submitted version.

Funding
No funding was available for the work.

Data availability
Data is provided in the manuscript.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors agreed for publishing the paper in this journal.

Competing interests
The authors declare no competing interests.

Received: 15 August 2024 / Accepted: 21 October 2024
Published online: 30 October 2024

References

1. Nazir MJ, Hussain GUI, Jatoi SA. Abdul Aziz Khakwani, Ehtesham Ul Haq, Sami Ullah and Rashid Abbas, *Drought stress management in Chickpea at various growth stages through the foliar application of plant extracts and growth regulators*. Bioscience Res. 2024;21(3):432–41.
2. Kraus EC, Stout MJ. Direct and Indirect effects of herbicides on Insect herbivores in Rice, Oryza sativa. Sci Rep. 2019;9(1):1–13.
3. Iquebal MA, et al. Discovery of putative herbicide resistance genes and its regulatory network in chickpea using transcriptome sequencing. Front Plant Sci. 2017;8:958.
4. Rizwan M, Akhtar S. Development of herbicide resistant crops through induced mutations. Advancements Life Sci. 2015;3(1):01–8.
5. Khan I, et al. Managing weed–crop interactions enhances Chickpea (Cicer arietinum L.) Chemical Components. Plants. 2023;12(17):3073.
6. Xu J, et al. Glyphosate contamination in grains and foods: an overview. Food Control; 2019. p. 106710.
7. Kováčik J, et al. Glyphosate does not show higher phytotoxicity than cadmium: Cross talk and metabolic changes in common herb. J Hazard Mater. 2020;383:121250.
8. Yanniccari M, et al. Mechanism of resistance to glyphosate in Lolium perenne from Argentina. Front Ecol Evol. 2017;5:123.
9. Gomes MP, et al. Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid: an overview. J Exp Bot. 2014;65(17):4691–703.
10. Akpinar D et al. Protective effects of Thymoquinone on Doxorubicin-induced lipid peroxidation and antioxidant enzyme levels in Rat Peripheral tissues. Pakistan Veterinary J, 2023. 43(4).
11. Al-Saeed FA et al. Oxidative stress, antioxidant enzymes, Genotoxicity and Histopathological Profile in Oreochromis niloticus exposed to Lufenuron. Pakistan Veterinary J, 2023. 43(1).
12. Sammons RD, Gaines TA. Glyphosate resistance: state of knowledge. Pest Manag Sci. 2014;70(9):1367–77.
13. Caverzan A, et al. Defenses against ROS in crops and weeds: the effects of interference and herbicides. Int J Mol Sci. 2019;20(5):1086.
14. Yadav AS, et al. Pulses production and productivity: Status, potential and way forward for enhancing farmers income. Int J Curr Microbiol App Sci. 2019;8(4):2315–22.
15. Ullah A, Shah TM, Farooq M. Pulses production in Pakistan: status, constraints and opportunities. Int J Plant Prod, 2020: pp. 1–21.
16. Ghaffar A et al. *Correlation and Path Coefficient Analysis Studyfor Yield Improvement in Chickpea (Cicerarietinum L.)*. 2018.
17. Khan IA, et al. Evaluation of different techniques for Economical Control of Weeds Associated to Chickpea. J Plant Prot. 2017;12:115–22.
18. Gupta M, et al. Identifying new sources of tolerance to post emergence herbicides in chickpea (Cicer arietinum L). J Food Legumes. 2018;31(1):5–9.
19. Taran B, Holm F, Banniza S. Response of chickpea cultivars to pre-and post-emergence herbicide applications. Can J Plant Sci. 2013;93(2):279–86.
20. Chaturvedi S, et al. Genetic variations for herbicide tolerance (Imazethapyr) in chickpea (Cicer arietinum). Indian J Agric Sci. 2014;84(8):968–70.
21. Gaur P, et al. Large genetic variability in chickpea for tolerance to herbicides imazethapyr and metribuzin. Agronomy. 2013;3(3):524–36.
22. Thompson C, Tar'an B. Genetic characterization of the acetohydroxyacid synthase (AHAS) gene responsible for resistance to imidazolinone in chickpea (Cicer arietinum L). Theor Appl Genet. 2014;127(7):1583–91.
23. Teggi AM. Identification and characterization of herbicide tolerant mutant lines using SNP marker (s) in chickpea (Cicer arietinum L). Professor Jayashankar Telangana State Agriculture University; 2017.
24. Chance B, Maehly A. *[136] Assay of catalases and peroxidases*. 1955.
25. Dixit V, Pandey V, Shyam R. Differential antioxidative responses to cadmium in roots and leaves of pea (Pisum sativum L. Cv. Azad). J Exp Bot. 2001;52(358):1101–9.
26. Beers RF, Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem. 1952;195(1):133–40.
27. Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–ciocalteu reagent. Nat Protoc. 2007;2(4):875–7.
28. Lin J-Y, Tang C-Y. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. Food Chem. 2007;101(1):140–7.
29. Hameed A et al. Age and organ specific accumulation of ascorbate in wheat (Triticum aestivum L.) seedlings grown under etiolation alone and in combination with oxidative stress. 2005.
30. Varavinit S, Chaokasem N, Shobsngob S. Immobilization of a thermostable alpha-amylase. Sci Asia. 2002;28(3):247–51.
31. Lichtenthaler HK, Wellburn AR. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Portland Press Ltd; 1983.
32. DHINDSA RS, Plumb-Dhindsa P, THORPE TA. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot. 1981;32(1):93–101.
33. Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem. 2005;38(12):1103–11.
34. Hussain A, et al. Herbicide resistance: another hot agronomic trait for plant genome editing. Plants. 2021;10(4):621.
35. Gaines TA, et al. Mechanisms of evolved herbicide resistance. J Biol Chem. 2020;295(30):10307–30.
36. Oladosu Y, et al. Principle and application of plant mutagenesis in crop improvement: a review. Biotechnol Biotechnol Equip. 2016;30(1):1–16.
37. Zakir M. Mutation breeding and its application in crop improvement under current environmental situations for biotic and abiotic stresses. J Res Stud Agricultural Sci (IJRSAS). 2018;4(4):1–10.
38. Galili S, et al. Novel mutation in the Acetohydroxyacid synthase (AHAS), Gene confers Imidazolinone Resistance in Chickpea Cicer arietinum L. plants. Plants. 2021;10(12):2791.
39. Toker C, et al. Improvement in imidazolinone resistance in Cicer species by induced mutation. Plant Breeding. 2012;131(4):535–9.
40. Umavathi S, Mullainathan L. Induced mutagenesis in Chickpea (Cicer arietinum (L.) with special reference to the frequency and spectrum of chlorophyll mutations. J Appl Adv Res. 2016;1(1):49–53.
41. Dekker JH, Duke SO. Herbicide-resistant field crops. Adv Agron. 1995;54:69.
42. Duggleby RG, Pang SS. Acetohydroxyacid synthase. J Biochem Mol Biol. 2000;33(1):1–36.
43. Shahid M, Khan MS. Glyphosate induced toxicity to chickpea plants and stress alleviation by herbicide tolerant phosphate solubilizing Burkholderia cepacia PSBB1 carrying multifarious plant growth promoting activities. 3 Biotech. 2018;8(2):131.
44. Khalid A, Hameed A. Seed biochemical analysis based profiling of Diverse Wheat Genetic Resource from Pakistan. Front Plant Sci. 2017;8:1276.
45. Mikawlrawng K. *Aspergillus in biomedical research*, in *New and Future developments in Microbial Biotechnology and Bioengineering*. Elsevier; 2016. pp. 229–42.
46. Yue S et al. Impact of enzymatic hydrolyzed protein feeding on Rumen Microbial Population, blood metabolites and performance parameters of lactating dairy cows. Pakistan Veterinary J, 2023. 43(4).
47. Wu R, et al. Significance of serum total oxidant/antioxidant status in patients with colorectal cancer. PLoS ONE. 2017;12(1):e0170003.

48. Sindi RA et al. Investigations of Hemato-Biochemical, histopathological, oxidative stress and Reproductive effects of Thiram in albino rats. Pakistan Veterinary J, 2023. 43(2).
49. Çalışkan GÜ, Emin N. Protective efficacy of Fresh and Aged Macerated Garlic Oils in Safflower Oil against Intra-abdominal Adhesions in rats. Pakistan Veterinary J, 2023. 43(2).
50. Jolliffe IT, Cadima J. Principal component analysis: a review and recent developments. Philosophical Trans Royal Soc A: Math Phys Eng Sci. 2016;374(2065):p20150202.

Publisher's note