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RESEARCH ARTICLE

Induced mutagenesis through gamma radiation in chickpea (*Cicer arietinum* L.): developmental changes and improved resistance to the parasitic weed *Orobanche foetida* Poir.

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Abstract

Induced mutagenesis trough gamma radiation was tested in chickpea to improve resistance to the parasitic weed *Orobanche foetida*. Seeds cv. Amdoun were exposed to increased doses up to 750 Gy. Germination rates and plant survival were scored 7 and 90 days after sowing, respectively. The 150 Gy dose was determined as the optimum dose limit causing 50% reduction in survival. The effects of doses ranging from 50 to 200 Gy were determined on various developmental traits. Fifty Gy treatment improved plant growth while doses of over 100 Gy, especially 200 Gy, reduced all the analyzed parameters of growth. Multi-shoots and delayed flowering were clearly observed in 150 and 200 Gy mutants. Gain in resistance to *O. foetida* and seed yield in 150 Gy mutants was demonstrated in field experiments. Genetic similarity coefficient among selected M2 plants and unirradiated plants ranged from 0.02 to 0.94, attesting of the high genetic variability induced. In conclusion, seed irradiation (LD_{50}) was efficient in chickpea to create variable initial material in mutation breeding of new lines resistant to *O. foetida*.

Introduction

Chickpea (**Cicer arietinum** L.) is one of the most abundant crops consumed in the Mediterranean areas and also in western world, then representing the third important food legume of the world (AICRP, 2010). A large range of fungal and viral diseases cause significant economic losses. Low and stagnant productivity results also from susceptibility to parasitic weeds including different species of broomrape, namely crenate broomrape (*Orobanche crenata* Forsk.), fetid broomrape (*O. foetida* Poir.), Egyptian broomrape (*Phelipanche aegyptiaca* Pers.) and *O. minor* (Rubiales and Fernandez-Aparicio, 2011). Broomrapes are parasitic plants devoid of chlorophyll and typical root system. Seed germination is induced by stimulating molecules exudated from roots of neighboring plants. Following germination, the radicle invades host root cortex and connects to vascular tissues through a haustorium. Hence, the parasitic plant develops thanks to nutrient spoliation from the host plants. Once attached, it develops a tubercle then a shoot which flowers after emergence from the soil, producing several hundreds of minuscule seeds (Musselman, 1980).

While *O. foetida* infects primarily wild legumes in western Mediterranean countries, it is adapted to cultivated legumes in Tunisia, especially in Beja Region (Kharrat et al., 1992), and more recently in Morocco (Rubiales et al., 2005). Cultural practices, chemical and genetic means have been proposed to farmers for broomrape control, without improving significantly the situation, notably for chickpea (Yenish, 2007). Success in stopping increased infestations need a reliable strategy of integrated management (Rubiales and Fernández-Aparicio, 2011; Goldwasser and Rodenburg, 2013), where breeding resistant crops should be one of the key elements (Pérez-Vich et al., 2013). In general, most of the desired genetic variation explored in breeding programs has occurred naturally and is preserved in germplasm collections. When collections fail to provide a source for a particular trait, i.e. broomrape resistance in legumes (Fernandez-Aparicio et al., 2009; Rubiales, 2014), induced mutation is highly effective for a quick production of this trait (Parry et al., 2009). Gamma radiation is the most widely used in plant mutation breeding (Maluszynski et al., 2009; Pushparajan et al., 2014; Ravichandran and Jayakumar, 2014). A number of studies report the negative effects of high radiation doses on metabolic alterations, growth and reproduction capacity, as well as the hormetic effects of low radiation doses such as an increase in germination rate, improved growth and gain in carbon assimilation and yield (Esnault et al., 2010; Marcu et al., 2013). Surprisingly, very few data are available on the interest of gamma radiation for improving plant resistance to broomrape. Interest was reported only in sunflower and faba bean challenged with *O. cumana* and *O. crenata*, respectively (Abdel-Hady et al., 2008; Encheva et al., 2003, 2012). The present study brings to light new information on this topic in chickpea by demonstrating the benefit of the pre-exposure of seeds to gamma radiation to create novel genetic variation which could be used in selection of resistance to *O. foetida*. The effects of increased doses on plant development and susceptibility to *O. foetida* were investigated. Induced genetic variation was assessed using ISSR strategy.

Materials and methods

Uniform and healthy dry seeds (M0 generation) of local chickpea variety (cv. Amdoun) were exposed to gamma radiation doses ranging from 50 to 750 Gy (50 Gy frequency) with a dose rate of 10,606 Gy min^{-1} using the Co^{60} gamma cell irradiator facility of the National Center for Nuclear Sciences and Technologies, Sidi Thabet, Tunisia. Germination rates were scored after 7 days in Petri dishes on a sterile filter paper imbibed with distilled water (n=100).

A new set of seed treatments (n=20) were conducted with increased doses ranging from 20 to 200 Gy. Unirradiated control and irradiated seeds were sown in pots filled with loam sandy soil free of broomrape seeds (1L). Pots were transferred into greenhouse (14h photoperiod, 25°C /18°C day/night temperature, 200 µmol m^{-2} s^{-1} photosynthetic active radiation PAR) and watered daily. After 90 days after sowing (das), the limit dose of gamma radiation (LD_{50}) corresponding to the dose causing 50% reduction in plant survival was determined and various developmental traits, including leaf morphology, shoot branching (number of primary and secondary branches) and growth (length and FW), root growth (length and FW) and whole plant architecture (shoot/root FW ratio), were scored. Chlorophyll contents of control and treated plants were estimated spectrophotometrically as described by Arnon (1949).

A new treatment with the specific dose of 150 Gy was conducted. Unirradiated control and irradiated seeds were sown fairly in 3 broomrape-infested fields in the Beja Region (Tunisia). Susceptibility to *O. foetida* was scored by counting the number of emerged broomrapes at the bottom of chickpea plants. Seed yields were determined and expressed as the number of seeds per plant and 1,000 seed weight (M2 progeny). Number of pods per plant was also scored.

M2 individuals for ISSR analysis corresponded to progenies of M1 plants selected following field experiments. Total genomic DNA was extracted from young leaves of 35 day old plants (Doyle and Doyle, 1987). Fifteen ISSR primers (Table 1), used in previous studies of genetic variability in chickpea (Tahir and Karim, 2011; Singh et al., 2014) were checked for the initial testing of variability and reproducibility. PCR was performed as previously described by Williams et al., (1990).

All ISSR analyses were carried out using appropriate programs of the Felsenstein's PHYLIP software (Felsenstein, 1995) and the TreeView software of Page (1996).

Data were subjected to analysis of variance for comparison between treated and control plants (ANOVA, $P \leq 0.05$, Bonferroni t-test) using Sigma-Plot 10.0 (Systat Software, Inc., San Jose, CA).

Results

Seed germination

As expected germination rate of unirradiated seeds was high (80%). It remained unchanged for seeds (M1 generation) irradiated with doses below 100 Gy, but fall strongly with higher doses (100-250 Gy; Fig 1). Hence, 200 Gy treatment caused 50% reduction in seed germination and 250 Gy treated seeds germinated only by 30%. Beyond 250 Gy, increasing doses caused a slow decline in germination rate, reaching values lower than 10% for treatments of over 650 Gy.

Plant survival and development in greenhouse conditions

Unirradiated control and irradiated M1 seeds were sown in pots for plant survival assessment in greenhouse (Fig 2). Doses below 100 Gy did not affect plant survival at 90 das. On the other hand, plant survival decreased significantly and proportionally following treatment beyond 100 Gy. Hence, plant mortality reached almost 90% in 200 Gy mutants. The LD_{50} value causing 50% reduction in plant survival was then assessed at 150 Gy.

Seed treatment with the 50 Gy dose stimulated plant growth (Table 3), as shown by the significant increase in root FW and shoot length in 50 Gy mutants. In addition, shoot branching was also modified due to the increase in the number of primary shoots. On the contrary, no significant change in leaf chlorophyll contents was noted (Table 3, Fig 3).

By contrast, radiation doses including 100, 150 and 200 Gy had a negative impact on plant growth and chlorophyll content of leaves (Table 3, Fig 3). Length and FW of roots and shoots declined in M1 plants, without impacting shoot/root FW ratio. Dwarfism was evident in surviving 200 Gy mutants which displayed a 2.5-fold reduced length of shoots at 90 das (Table 3). Reduced chlorophyll contents mainly resulted from alterations in chlorophyll a accumulation (Fig 3). This range of radiation doses impacted also leaf development since abnormalities including reduction in leaf area and even absence of leaves were observed in 200 Gy mutants (data not shown). In addition, it increased the number of primary and secondary branches. Hence, shoot branching increased by twice in 200 Gy mutants when compared to unirradiated control plants (Table 3). Flowering onset was also influenced since 150 and 200 Gy mutants flowered two or three day later than unirradiated control plants (Table 3).

Selection of M2 plants sharing resistance to broomrape and reliable grain yield

Unirradiated control and M1 seeds from 150 Gy treatment (LD_{50}) were sown in three naturally broomrape-infested fields. Susceptibility to *O. foetida* was high in unirradiated control plants and similar in the three fields (8.4 ± 1.2 emerged broomrapes per host plant). In contrast, a large variability in susceptibility to *O. foetida* was observed in mutants, ranging from 0 (M10, M62) to 9 emerged broomrapes per host plant (data not shown).

Data concerning twenty eight mutants of interest are presented in Figures 4 and 5. They shared low number of emerged broomrapes at their bottom in addition to number of seeds per plant at least equal to the unirradiated control plant one. These mutants can be categorized according to seed productivity (Fig 5): individuals showing similar levels of seed number and 1,000 seed weight than unirradiated control plants (M14, M37, M63, M81, M83, M92); individuals showing a similar number of seeds per plant than unirradiated control plants but a lower 1,000 seed weight (M4, M6, M9, M12, M39, M52); and individuals exhibiting enhanced grain yields since seed number per plant doubled without impacting 1,000 seed weight (M11, M21, M36, M62, M72), then contrasting with the individual M51 which exhibited a spectacularly high increase in seed number per plant to the disadvantage of seed weight. The number of seeds per pod was 1.00 ± 0.31 in unirradiated control plants and did not change significantly in mutants (data not shown).

Induced genetic variability in M2 mutants of interest

Genetic variability was analyzed in unirradiated control plants and M2 lines of interest of which parental lines were selected in infested fields. All the M2 lines developed well excepting plants belonging to the line M13 which died after 3 weeks.

Out of the 15 primers tested initially, five (ISSR-1, ISSR-2, ISSR-3, ISSR-4, ISSR-5; Table 2) gave clear banding pattern (data not shown) and were then selected for further genetic analysis. Amplified bands had a molecular size in the range of 200-800 bp. ISSR primers generated 6 (ISSR-2) to 11 (ISSR-4) bands with an average of 8.2 bands per primer in a total of 41 bands (Table 2). M2 mutants shared 100% polymorphic bands with ISSR markers. Genetic similarity matrix varied in M2 lines from 0.02 (M72 vs M21) to 0.94 (M93 vs M37). Within unirradiated control plants, the lowest and highest values of similarity was shared with the lines M83 (0.24) and M39 (0.76), respectively. The neighbor-joining method generated a dendrogram showing two main clusters of 18 and 11 mutants (Fig 6). Unirradiated control plants and the M2 line M89 had a basal position considering the upper branch.

Table 1. ISSR primers used for initial testing of variability and reproducibility

Primer	Sequence (5'- 3')	Annealing Temperature (°C)
ISSR-1	$(AC)_7T$	52
ISSR-2	$(AC)_8C$	50
ISSR-3	$(GA)_8T$	52
ISSR-4	$(TG)_8GA$	52
ISSR-5	$(GAA)_6$	50
ISSR-6	$(GA)_8A$	50
ISSR-7	$(CT)_7T$	50
ISSR-8	$(CA)_8A$	52
ISSR-9	$(TG)_8A$	52
ISSR-10	$(CT)_8C$	50
ISSR-11	$(CAC)_5$	50
ISSR-12	$(GACA)_4$	54
ISSR-13	$(ACTG)_4$	52
ISSR-14	$(AG)_8YT$	52
ISSR-15	$(GT)_8YC$	50

Table 2. Genetic polymorphism detected by using 5 random primers within 30 mutant lines and unirradiated control plants of chickpea (cv. Amdoun)

Mutant lines corresponded to selected lines showing low susceptibility to broomrape and reliable seed yield in naturally-infested fields.

Primer	Total Nb of bands	Nb. polymorphic bands	% polymorphism
ISSR-1	7	7	100
ISSR-2	11	11	100
ISSR-3	7	7	100
ISSR-4	6	6	100
ISSR-5	10	10	100
Total	41	41	100
Average	8.2	8.2	100

* ISSR sequences and conditions of use are described in Table 1.
Nb: number.

Table 3. Impact of increased gamma radiation doses on various developmental parameters in chickpea (cv. Amdoun)

Seeds were irradiated and sown in pots. Plants were cultivated in greenhouse. Parameters were scored 90 days after sowing. Data are means ± confidence intervals (n=20). Means accompanied by (*) are significantly different to control at P≤ 0.05 (Bonferroni t-test). Nb: Number

	Root length (cm)	Root FW (g)	Shoot length (cm)	Shoot FW (g)	Ratio FW shoot / roots	Nb. primary branches	Nb. secondary branches	Days flow
0 Gy	23.20 ± 0.76	14.72 ± 0.53	37.00 ± 1.36	18.00 ± 1.06	1.23 ± 0.10	1.16 ± 0.21	2.50 ± 0.26	65.16
50 Gy	25.20 ± 1.16	16.21 ± 0.67*	41.30 ± 1.20*	18.90 ± 1.57	1.17 ± 0.09	1.83 ± 0.15*	2.66 ± 0.21	66.00
100 Gy	20.40 ± 1.41*	13.80 ± 0.86*	31.10 ± 0.65*	16.00 ± 0.78	1.17 ± 0.10	1.83 ± 0.32*	2.83 ± 0.17	64.00
150 Gy	21.00 ± 1.11	13.60 ± 0.52	30.90 ± 1.34*	15.52 ± 1.01*	1.14 ± 0.08	2.00 ± 0.28*	2.84 ± 0.16	67.30
200 Gy	16.20 ± 1.42*	9.41 ± 0.80*	14.80 ± 1.45*	11.06 ± 1.33*	1.25 ± 0.18	3.33 ± 0.21*	4.58 ± 0.33*	68.00

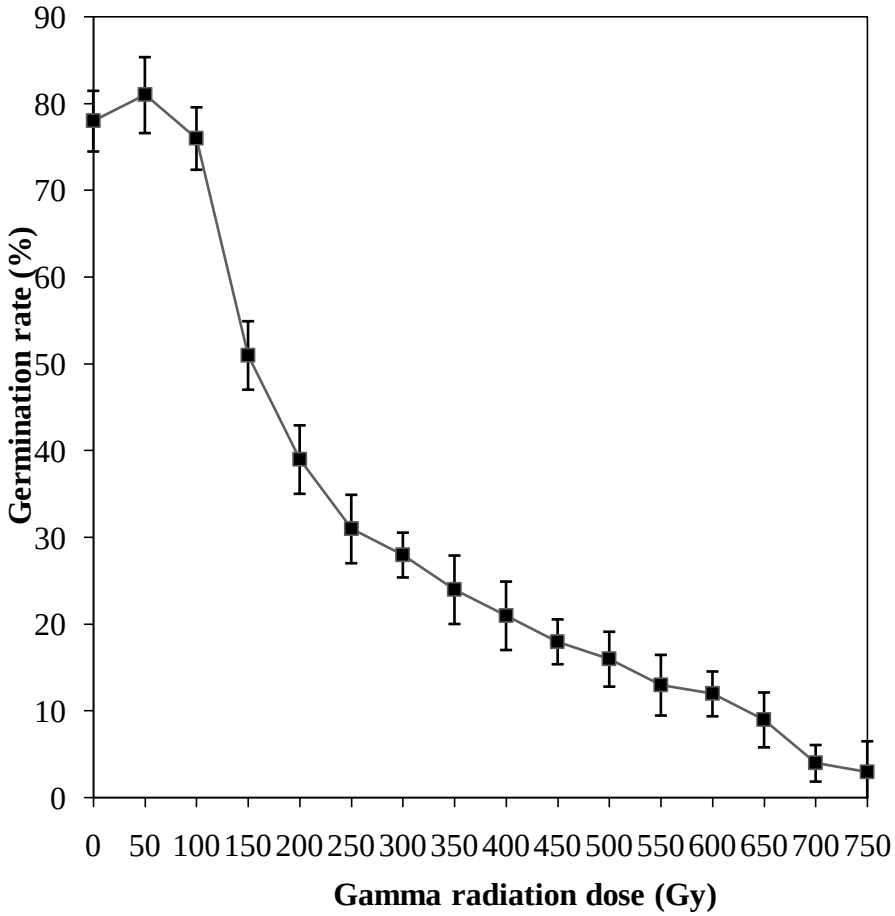

Figure 1. Impact of increased gamma radiation doses on germination rate of chickpea seeds (cv. Amdoun) Germination rates were scored 7 days after seed treatment and transfer into Petri dish. Data are means ± confidence intervals (n=100).

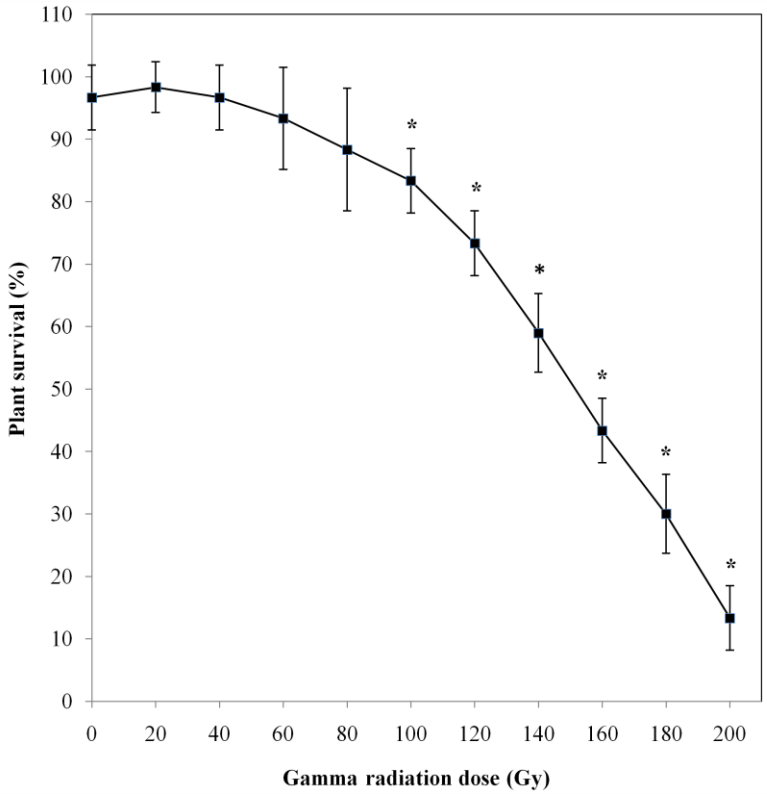

Figure 2. Impact of increased gamma radiation doses on plant survival in chickpea (cv. Amdoun)
Following treatment, seeds were sown in pots and plant survival was scored 90 days after sowing. Data are means ± confidence intervals (n=20). * indicates significant difference with the control ($p \leq 0.05$; Bonferroni t-test).

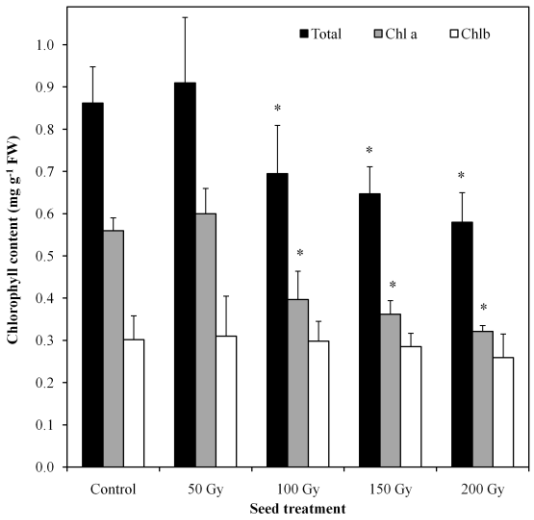

Figure 3. Impact of seed treatment using increased gamma radiation doses on chlorophyll contents of young leaves in chickpea plants (cv. Amdoun).
Control and irradiated seeds were sown in pots. Plants were cultivated in greenhouse. All leaves were collected from 90d old plants. Data are means ± confidence intervals (n=6). * indicates significant difference with the control ($p \leq 0.05$; Bonferroni t- test).

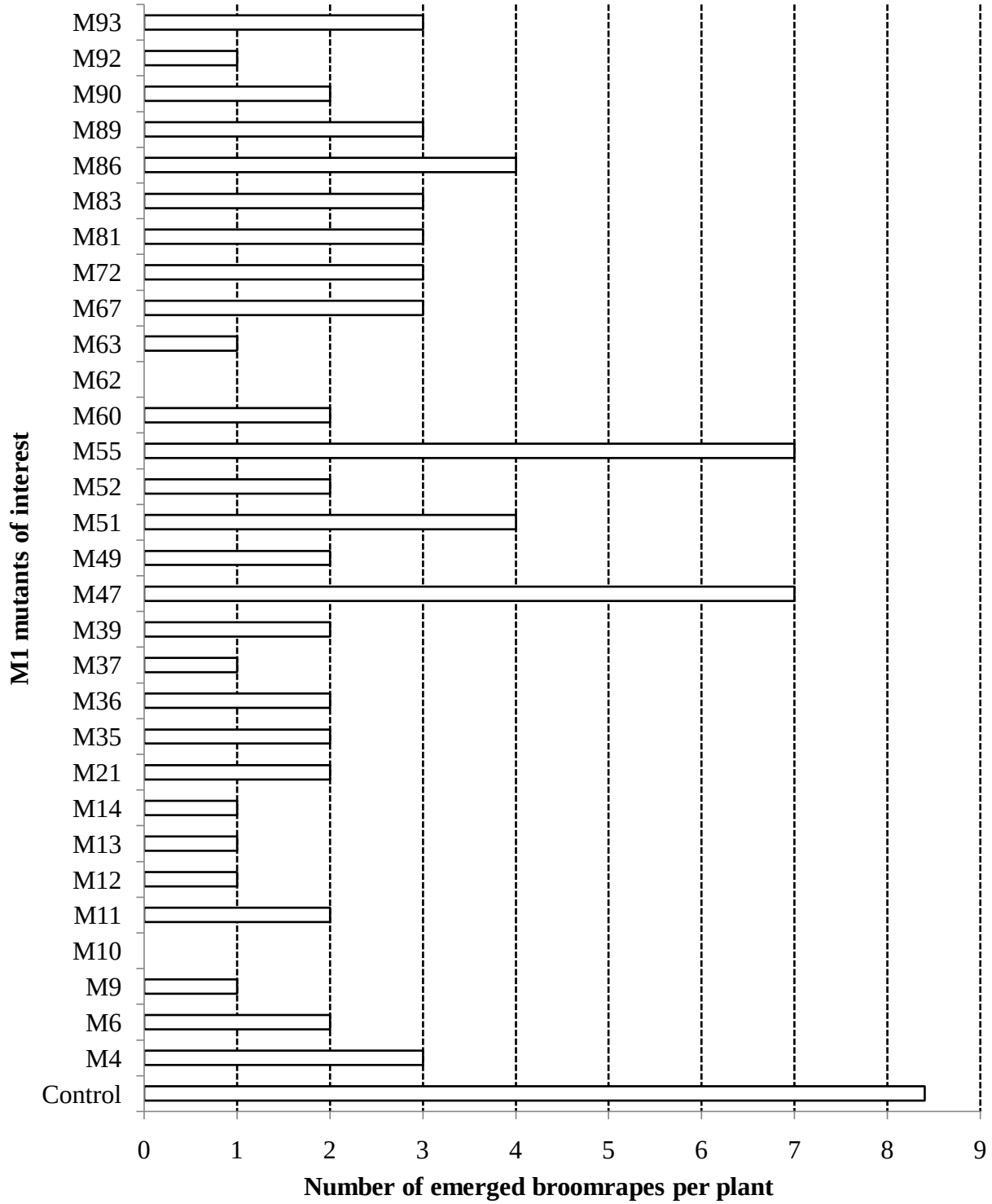

Figure 4. Levels in susceptibility to *O. foetida* of control chickpea plants (cv. Amdoun) and 150 Gy treated plants of interest in broomrape infested fields

Unirradiated control seeds and 150 Gy treated seeds were sown in naturally infested fields in Beja region (Tunisia). Levels in susceptibility to broomrape were scored at the harvest period (June 2011). Data for the control is the average value from 75 plants (8.4±1.2).

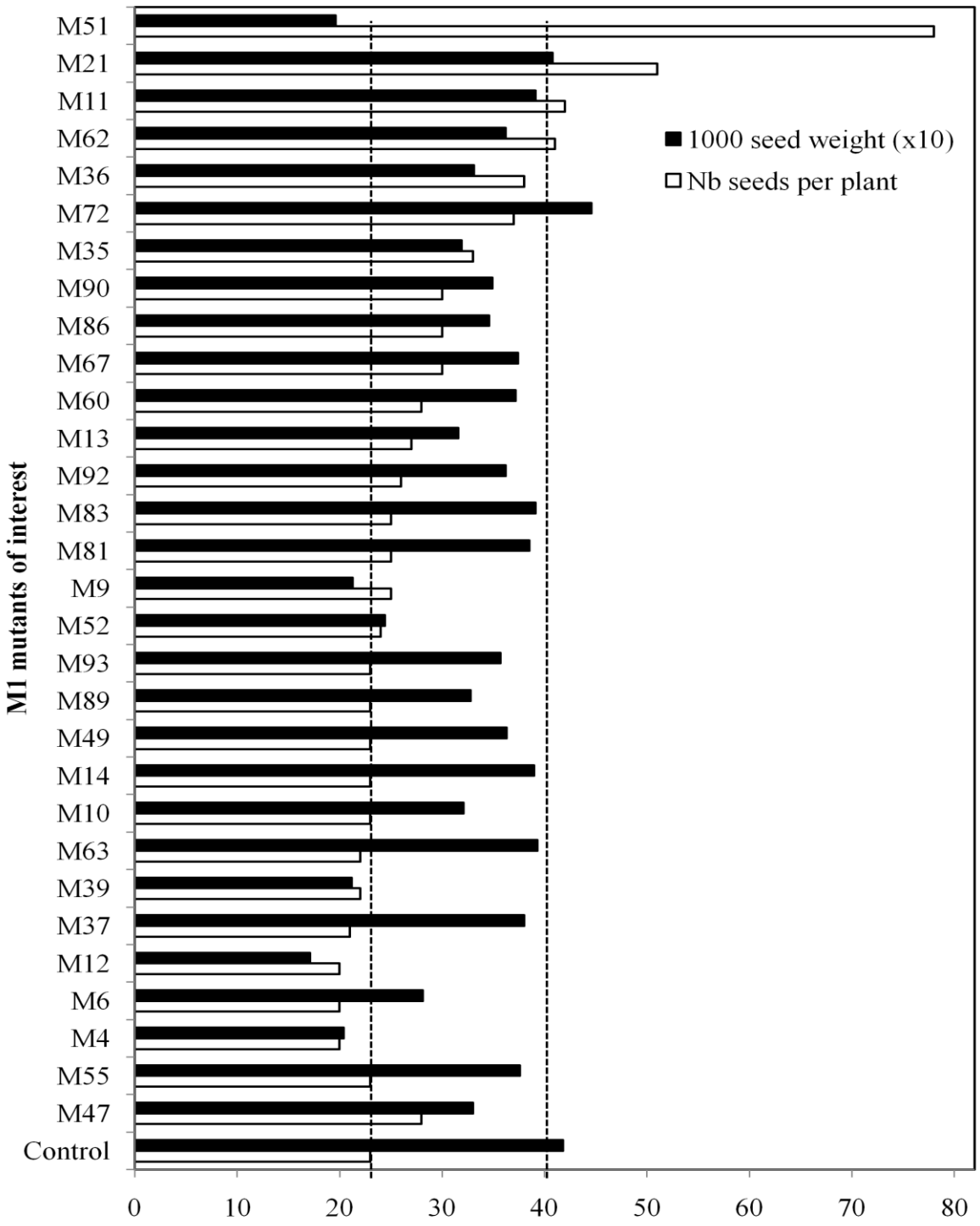

Figure 5. Yield components of control chickpea plants (cv. Amdoun) and 150 Gy treated plants of interest in broomrape infested fields

Unirradiated control seeds and 150 Gy treated seeds were sown in naturally infested fields in Beja region (Tunisia). Numbers of seeds per plant and seed weight were scored at the harvest period (June 2011). These M1 mutants of interest shared low susceptibility to broomrape (*O. foetida*) in comparison with unirradiated control plants (Fig 4). Data for the control are average values from 75 plants. Nb: number.

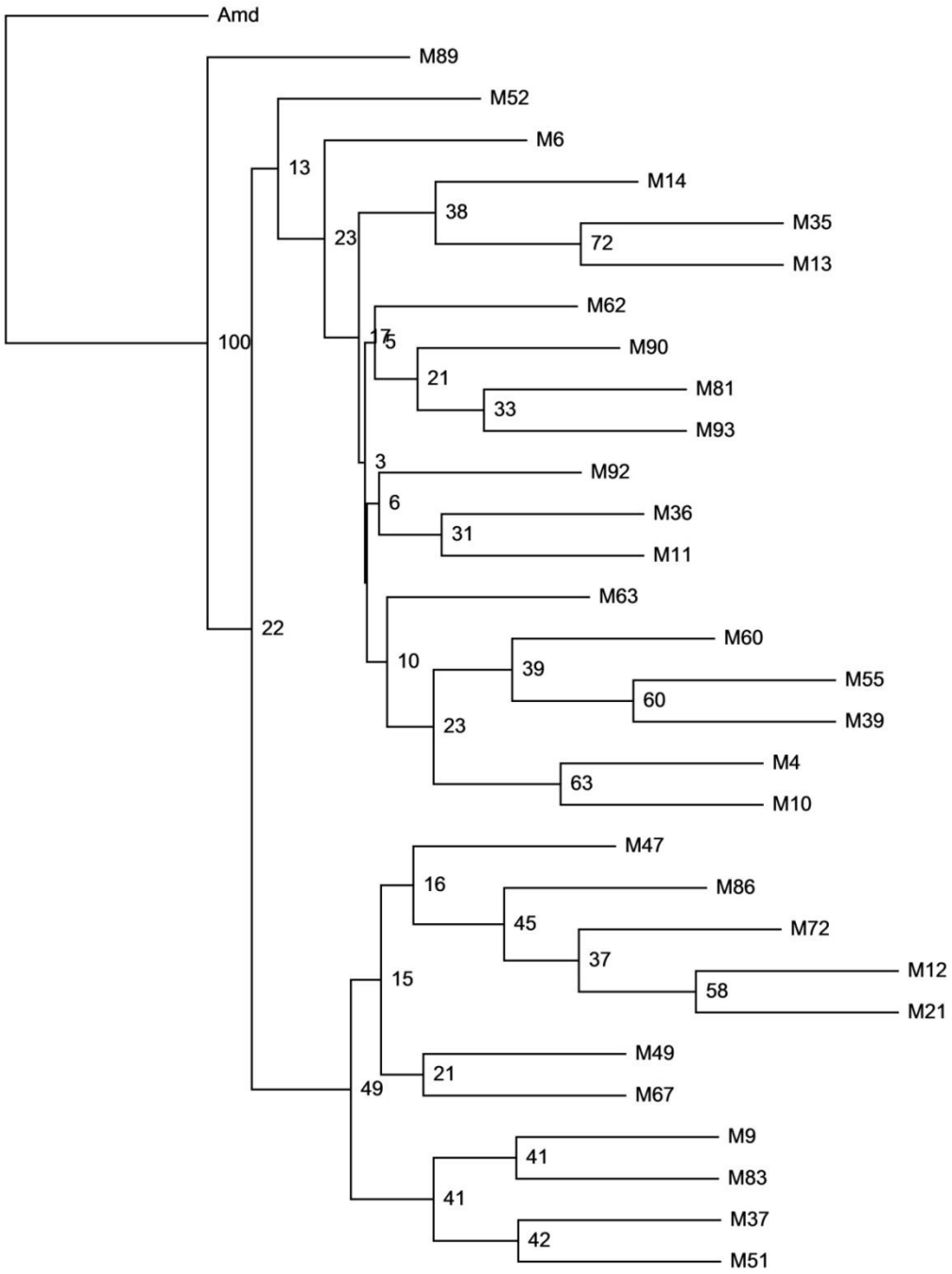

Figure 6. Dendrogram representing the genetic variation among control and 150 Gy mutant lines of chickpea (cv. Amdoun)

DNA was extracted from unirradiated control plants and progenies of M1 lines of interest showing improved resistance to *O. foetida* in infested fields (Fig 4). Genetic variability was assessed using five ISSR markers (Table 2).

Discussion

The LD_{50} index is commonly employed as an indicator of plant sensitivity to gamma radiation. In present study, LD_{50} was estimated at 150 Gy in chickpea cv. Amdoun which reduced plant survival by half in greenhouse experiments (Fig 2). Sensitivity to gamma radiation of this chickpea cv. might be considered as moderate when compared to higher sensitive pea seeds for which doses ranging from 25 to 100 Gy reduced viability (Veselovskii et al., 2006) and to lower sensitive rice seeds for which doses until 200 Gy stimulated germination (Macovei and Tuteja, 2013). In present study, doses below 100 Gy did not affect seed germination or plant survival. Consistent data were reported previously using another chickpea cv. exposed to moderate doses ranging from 5 to 25 Gy (Melki and Sallami, 2008). On the other hand, our findings show that doses of over 100 Gy induced increasingly harmful effects, especially on plant development following germination. Indeed high rate of plant mortality (90%) were scored in 200 Gy treatment at 90 das (Fig 2) while germination rate was only reduced by twice (Fig 1), revealing strong limitations in fixing the radiation-related damages when seeds were treated with this range of radiation doses. While minor modifications in leaf development were observed following 100 to 200 Gy treatments, limited growth, multi-shoots and delayed flowering characterized the surviving mutants (Table 2). Reduced growth is commonly observed in irradiated plants due to alterations in cell cycle in meristematic tissues, breakdown of photosynthetic pigments and concomitant loss of photosynthetic capacity (Sengupta et al., 2013; Macovei et al., 2014). Chlorophyll breakdown is consistent with high sensitivity of mesophyll and chloroplasts to gamma radiation in comparison with other tissues and cell organelles, respectively (Kim et al., 2011). Consistently chickpea mutants exhibited low leaf chlorophyll contents in 100 to 200 Gy treatments in comparison with unirradiated plants (Fig 3**)**, suggesting some radiation-induced perturbations in photosynthetic performance. Effects of radiation on phenotypical and physiological traits can vary according to the chickpea cv. Indeed no changes in first days of flowering and shoot branching (primary branches) were observed in 200 Gy mutants of two another genotypes Binasola-2 and CPM-834 (Karim et al., 2008).

Very low doses of gamma radiation in 5 Gy to 20 Gy range improved seed germination in soybean and groundnut (Moussa, 2011; Ahuja et al., 2014). While such dose ranges were not tested in present study, the latter gives evidence of growth stimulation in the chickpea cv. Amdoun by irradiating seeds with moderate doses. Indeed, most of the growth parameters analyzed at 90 das including root length and FW, shoot length and shoot branching (primary branches) were enhanced in 50 Gy mutants in comparison with unirradiated plants (Table 3). Similar observations were reported in chickpea exposed to 15 Gy (Melki and Salami, 2008). Such hormetic effects of low radiation were well documented in plants, assuming that low dose may induce growth stimulation by changing hormonal signaling network, increasing efficiency of PSII photochemistry, and activating antioxidant defense to overcome daily stress factors (Esnault et al., 2010; Marcu et al., 2013).

Host plant resistance to broomrape is well documented and includes various mechanisms occurring before or after attachment to the host roots (Pérez-de-Luque et al., 2008). While resistance to broomrapes is scarce in legumes, it is common in chickpea against the species O. crenata and is based on low production of germination stimulants and blocking of parasite penetration into host roots (Rubiales et al., 2003). By contrast, no data is available to date on resistance to *O. foetida* in chickpea. Considering the LD_{50} value for chickpea cv. Amdoun (Fig 2) and the genetic divergence in Tunisian *O. foetida* populations (Roman et al., 2007), resistance was searched among the 150 Gy mutants in three naturally-infested fields in Beja region. We demonstrate that induced mutagenesis through seed exposition to LD_{50} was efficient to improve resistance in a number of chickpea mutants, especially in mutants M10 and M62 of which no parasitic plants emerged (Fig 4). Even though the mechanisms involved in resistance could not be determined in field experiments, escape due to low root biomass is unlikely in 150 Gy mutants. Indeed treatment did not impact severely root development in pot experiments (Table 3). Globally, twenty eight individuals of interest shared a reliable or enhanced seed yield in addition to a significant reduction in susceptibility to broomrape (Fig 5). Interestingly, induced resistance was accompanied by enhanced seed yield (g $plant^{-1}$) in some chickpea mutants, especially in the individual M21 (Fig 5). In present study, enhanced yield could result from only the improved resistance considering the negative impact of infestation in crop productivity. Thus seed yield of chickpea mutants should be also assessed further in orobanche-free fields. M1 plant testing in fields has allowed a rapid screening of a high number of mutants for both susceptibility to *O. foetida* and seed yield.

The cluster analysis based on ISSR data confirmed the important genetic variation induced by gamma rays (Fig 6). Indeed the genetic similarity coefficient among the M2 individuals and the unirradiated control plants ranged from 0.02 to 0.94, indicating high genetic variability among the mutants. In mutagenesis experiments, the treated material has to be advanced through few seed generations for stability analysis and selection of the recessive treated plants in the second (M2) or third (M3) generation after the treatment. Resistance should be checked further under artificial infestation in pots or mini-rhizotrons where broomrape seed innoculum is easily controlled. Mechanisms involved in resistance could be also clarified.

Conclusion

The present study demonstrates that induced mutagenesis through seed treatment by gamma radiation is an efficient tool to develop variable initial material in selection of new lines resistant to *O. foetida* in chickpea.

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