Shiraz University

Iran Agricultural Research

Journal homepage: https://iar.shirazu.ac.ir

Lentil (*lens culinaris* Medik.) genotype × PGR (Plant growth regulator) interaction and heritability of some traits under *in vitro* conditions

Hajar Ashoordan[a], Massoud Dehdari[a*], Asad Masoumiasl[a], Rahmatollah Karimizadeh[b]

[a] Department of Agronomy and Plant breeding, Faculty of Agriculture, Yasouj University, Yasouj, I. R. Iran.
[b] Dryland Agricultural Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Gachsaran, I. R. Iran.

ARTICLE INFO

Keywords:
Genetic parameters
Heritability
Plant growth regulators
Regeneration

ABSTRACT- Plant breeders are constantly evaluating genetic diversity resources to lay a foundation for crop improvement. Tissue culture techniques have been applied successfully in many species to generate genetic variation in plants. Researches on lentil genetic diversity and parameter estimation under tissue culture conditions are relatively limited. Therefore, this research was carried out in three separate experiments for genetic parameters estimation and to understand the response of 14 lentil genotypes to callus induction using kinetin (Kin) and 2,4-dichlorophenoxyacetic acid (2,4-D) growth regulators; shoot production using thidiazuron (TDZ), 2-Isopentyladenine (2iP), benzylamino purine (BAP), and Kin growth regulators; and rooting the plants using NAA growth regulator. Results showed that the best treatments were 1 mg/L Kin and 2 mg/L 2,4-D for callus induction, 3 mg/L BAP, 4 mg/L Kin, and 2 mg/L TDZ for shoot regeneration, and 3 mg/L NAA for rooting. The 09S 83259-14ILL6994/ILL5480 and FLIP2010-40L-10770-ILL8119/ILL7686 genotypes were the best in term of callus, shoot, and root induction. The broad sense heritability of most of the traits was high demonstrating lower contribution of environmental factors in phenotypic variances. Overall, large diversity identified among genotypes in vitro conditions and high heritability estimates indicated that selection based on the measured traits including callus, shoot, and root induction will be efficient in tissue culture conditions.

INTRODUCTION

Lentil (*Lens culinaris* Medik.) has a specific nutritional value due to its high percentage of protein, significant amounts of folic acid, fiber, iron, niacin, vitamin, and minerals. Moreover, it is considered to be a beneficial legume due to its low levels of fat and cholesterol (Ates, 2019). Lentil is suitable for areas with limited rainfall and drier growing season. These characteristics provide lentil as an important crop and ensured its survival to the present day (Kumar, 2019).

Successful plants regeneration system in tissue culture depends on use of appropriate explants (Chen and Chang, 2002). Bayrac (2004) examined regeneration of lentil in response to cotyledon petiole and epicotyl explants treated with different plant growth regulators (PGR) and the results indicated that the type of explant require different medium combination to callus and shoot induction. Moreover, the highest percentage of shoot induction were observed 40 and 60 percent for cotyledon petiole and epicotyl explants, respectively. Altaf et al. (2000) used cotyledon node explants for growing lentil seedlings in Murashige and Skoog (MS) culture medium containing 5 mg/L benzyladenine (BA) and 3% fructose. The results of Ghasemi Omran and Bagheri (2010) on Gachsaran and Philip lentil genotypes indicated that a modified MS culture medium is appropriate for in vitro culture of cotyledon. Embryo explant in 2 mg/L BAP generated the highest number of shoots. For induction of root in regenerated shoots, a culture medium containing 10 mg/L NAA as pretreatment for 3 days and an in vivo condition containing sponge-like peat, sand, and perlite was efficient for root development. The results of Zaker Tavallaie et al. (2009) indicated that the cotyledon explant of lentils along with a minor part of the embryonic axis was the best. Furthermore, MS culture medium fortified by 7.5 mmol/L 2-Isopentyladenine (2iP), 4 mmol/L Kin, and 2 mmol/L Thidiazuron (TDZ) was the best composition for shoot induction. So that more than 96% of shoot regeneration induction was obtained, and in some cases, each explant produced 40 elongated shoots. Williams and McHughen (1986) conducted a study on lentil shoot regeneration in a culture medium containing Kin. Only 11% rooting was obtained by transforming the regenerated shoots to sand. Khawar et al. (2004) claimed that an MS culture medium with 0.25 mg/L indole-3-butyric acid (IBA) was the best shoot regeneration culture medium for 10-day lentil callus. However, the rooting percentage in their study was so low (nearly 20%). Gulati et al. (2001)

* Corresponding author: Associate Professor, Faculty of Agriculture, University of Yasouj, Yasouj, I.R. Iran.
E-mail address: adehdari@yu.ac.ir
https://doi.org/ 10.22099/IAR.2024.48842.1563
Received 13 November 2023; Received in revised form 11 May 2024; Accepted 12 May 2024
Available online: 01 June 2024

used cotyledon node explants of lentil in an MS culture medium containing BAP. They produced mature plants, successfully.

The variation identified in tissue culture in the callus induction process is referred to somaclonal variation that might provide new variations for production of new genotype and expand genetic diversity in a plant (Kaya et al. 2015). Induction of somaclonal variation increases the success probability of plant selection in vitro conditions (Anil et al. 2018).

Despite reports on the optimization of in vitro regeneration of lentil, little information about genetic diversity, heritability of traits, somaclonal variation, and genotype × PGR interaction for selection of the best stress-tolerant plants in lentil is available. Therefore, the objective of this study was investigating the genetic diversity, genetic parameters estimation, and genotype × PGR interaction in lentil under in vitro culture condition.

MATERIALS AND METHODS

Healthy and uniform seeds of 14 lentil genotypes including 12 new breeding lines and two common cultivars were provided from Gachsaran Agricultural Research Station, Iran (Table 1). The seeds were immersed in a 2% sodium hypochlorite solution for two minutes. The sterilized seeds were cultured in the petri dish and explants were removed from healthy seedlings.

Table 1. The names and pedigree of studied genotypes

Genotype No.	Name and Pedigree
1	GACHSARAN
2	KIMIA
3	ACC 5588 ILL116 Sel
4	ACC 4605
5	ILL 7979
6	09S 83259-14 ILL6994 / ILL5480
7	FLIP2010-40L-10770-ILL 8119 / ILL768
8	FLIP2011-6L ILL 6434 / ILL6972-11
9	FLIP 2005-53L-7
10	FLIP 1996-15L(lbla 1) ILL 6209 / ILL5671-12
11	ILL 7547 / ILL 6002 2006-03-0G-0GA-0GA-11
12	ILL 4605 / ILL 6002 2066-02-0G-0GA-0GA-11
13	ILL 4605 /ADDA 2006-06-0G-0GA-0GA-11
14	ILL 6211/ILL 6002 2006-07-0G-0GA-0GA-11

The response of the lentil genotypes to PGR (containing 1 and 2 mg/L Kin and 1 and 2 mg/L 2,4-D) for callus induction was examined using embryo and a quarter of its cotyledon as explant in a modified MS culture medium (Ye et al. 2002). After 14 days, some important characteristics of the produced callus, such as length, height, volume, fresh weight, and callus induction percentage were measured.

For shoot production, the generated calluses of the above materials were placed on the MS culture medium containing two levels of PGR, including 4 mg/L Kin + 2 mg/L TDZ + 3 mg/L BAP and 4 mg/L Kin + 2 mg/L TDZ + 3 mg/L 2ip. For appropriate shoot production, the cultured samples were kept in a growth chamber (Arvin Tajhiz Espadana Company, Isfahan, Iran) under the following conditions: photon flux density of 400 ± 50 μmol/m^2/s, photoperiod of 16 h, and relative humidity of 65–70%. After 40 days, fresh shoot weight, shoot length, number of leaves, number of shoots, and shoot induction percentage were measured.

For root induction, the regenerated shoots of genotypes were cultured in a modified MS culture medium containing 2 and 3 mg/L NAA. Afterwards, these shoots were transferred into an in vivo environment containing sponge-like peat, sand, and perlite that were divided equally into covered small pots. Root fresh and dry weights, root length, number of roots, and root induction percentage were measured 14 days later. Finally, the seedlings were transferred into small pots containing sponge-like peat, sand, and perlite. To increase the adoptability of plants to the new conditions, the pots were covered with transparent plastics. Hoagland and Arnon's (1950) solution was used for irrigation and feeding of seedlings.

Statistical analysis of data in the above-mentioned stages was carried out separately in a factorial experiment based on a completely randomized design (CRD) with three replications. Mean values of significant main and interaction (PGR × Genotype) effects were compared based on least significant differences (LSD) and least square means (Ls means), respectively. Genetic parameters including, phenotype (Cvp) and genotype (Cvg) coefficients of variation, and broad sense heritability (hb2) were estimated using the expected mean of square (replication was considered as random and all other factors as fix effects) and following equations (Sharma, 2006):

$$Cv_p = \frac{\sqrt{\sigma_p^2}}{\bar{X}} \times 100 \quad \text{Eq. (1)}$$

$$Cv_g = \frac{\sqrt{\sigma_g^2}}{\bar{X}} \times 100 \quad \text{Eq. (2)}$$

$$h_b^2 = \frac{\sigma_g^2}{\sigma_p^2} \times 100 \quad \text{Eq. (3)}$$

In these equations, σ^2p, σ^2g, and σ^2e are phenotypic, genotypic, and environmental variances, respectively and calculated as bellow:

$$\sigma_p^2 = \sigma_e^2 + \sigma_g^2 \quad \text{Eq. (4)}$$

$$\sigma_g^2 = \frac{Ms_g - Ms_e}{r} \quad \text{Eq. (5)}$$

$$\sigma_e^2 = Ms_e \quad \text{Eq. (6)}$$

Ms_g and Ms_e are genotype and error mean squares, respectively.

Analyses were performed in SAS 9.1 (Goodnight and Sall (2003) software.

RESULTS AND DISCUSSION

Callus induction: Variance analysis results (Table 2) indicated that the effects of main factors, including 2,4-D, Kin, and genotype were significant for all the measured traits. The interaction of Kin × 2,4-D for dry and fresh weights, the interaction of Kin × genotype for callus width, and the interaction of genotype × 2,4-D for all the traits were significant except for callus width. The triple interaction of Kin × genotype × 2,4-D was not significant for the callus characters.

A comparison of means for the two Kin concentrations showed that the highest callus length (3.615 mm) and the highest callus volume (0.0259 mL^3) were observed in the 1 mg/L concentration of Kin. The highest callus width (0.422 mm) was observed in a 2 mg/L concentration of 2,4-D. As shown in Table 3, the highest callus volume (0.0471 mL3), callus length (6.012 mm), callus fresh weight (130.574 mg), callus dry weight (88.402 mg), and callus induction (83.33%), observed in 09S 83259-14 ILL6994 / ILL5480 genotype and MS medium supplemented with 2 mg/L 2,4-D (Fig. 1a). In addition, the highest callus width (3.363 mm) was observed in 1 mg/L Kin in 09S 83259-14 ILL6994 / ILL5480 genotype. The highest callus fresh weight (76.639 mg) and the highest callus dry weight (50.6544 mg) were observed in MS medium containing 2 mg/L 2,4-D, and 1 mg/L Kin. Although the reaction of genotypes to 2,4-D was different, at both levels of 2,4-D the highest percentage of callus formation was recorded to genotype 09S 83259-14 ILL6994 / ILL5480 and the lowest to genotype FLIP 2005-53L-7 (Table 3). It means that the initial potential of the genotype is important in callus formation, although increasing the concentration of 2,4-D leads to an increase in callus formation.

It is identified that auxin hormones such as 2,4-D, with high and medium concentrations, are the most common hormone which is used for callus induction. Callus induction of some plant species can be increased with high auxin and low cytokinin concentrations in the culture medium (Gordon, 2008). Saxena and King (1987) used embryo explants of lentil including embryo axis in MS culture medium containing 2,4-D. They concluded that 1 mg/L 2,4-D is the most efficient for callus induction which is lower than our experiment result (2 mg/L). They reported that higher than 5 mg/L of 2,4-D suppressed callus induction and growth.

Taleb Bidokhti et al. (2003) used shoot apex and cotyledon node explants of lentil for callus induction in an MS culture medium containing different concentrations of 2,4-D. Maximum callus growth was observed in 0.5 mg/L of 2,4-D. Callus growth decreased by increasing 2,4-D concentration. Callus induction of some plant species is only possible with one of the auxin or cytokinin regulators. But, some other species require both of these regulators (Chawla 2009). In our study, a combination of these two PGRs caused callus induction with more appropriate traits. Ghanem et al. (1989) used to leave, root, and epicotyl explants of lentil in an MS culture medium containing various concentrations of Kin and 2,4-D. The best callus induction in their research occurred in a culture medium containing 0.5 mg/L 2,4-D and 1 mg/L kinetin. Singh and Raghuwanshi (1989) reported that callus induction was achieved in a single node and apical shoot explants of lentil in an MS culture medium containing 1 mg/L Kin and 10 mg/L 2,4-D. In comparison to our results, these reports showed different concentrations of 2.4-D to callus induction. This is due to the difference in the type of explant and genotype.

Shoot regeneration: The effects of plant growth regulators (BAP, 2iP, Kin, and TDZ) and genotypes on all the measured traits were significant (Table 4). Moreover, genotype × PGR interaction for shoot fresh weight, shoot dry weight, shoot length, and shooting percentage was significant.

Analysis of the PGR effects indicated that the highest number of leaves (4.524) and shoots (1.4048) belonged to the culture medium containing 3 mg/L BAP. BAP is more effective in combination with two other growth regulators, namely Kin and TDZ. As shown in Table 5 09@ 83259-14 ILL6994 / ILL5480 genotype had the highest number of leaves (6 leaves) and the highest number of shoots (2 shoots). Comparison of means for genotype × PGR interaction (Table 6) indicated that the highest shoot length (48.36 mm), shoot fresh weight (26.70 mg), shoot dry weight (16.38 mg), and shoot induction (90.47%) observed in 3 mg/L BAP concentration for 09S 83259-14 ILL6994 / ILL5480 genotype (Fig. 1b). FLIP2010-40L-10770-ILL 8119 / ILL 7686 genotype also showed the same shoot induction (90.47%) at this BAP concentration.

To generate shoots from callus tissues, auxin concentration can be decreased and cytokinin concentration can be increased (Chawla 2009). For the first time, Bajaj and Danju (1979) investigated the regeneration of lentil from the meristem tip. They cultured meristem tip in an MS culture medium containing different PGR concentrations of Kin, 2,4-D, NAA, IAA, and casein hydrolysate. The most efficient amounts of growth and differentiation were observed in a culture medium containing 0.5 mg/L Kin and 2 mg/L IAA that are different from our results. Ye et al. (2002) used MS and B5 culture media containing different concentrations of Kin, BA, and TDZ, to regenerate lentil in vitro conditions. The results of their study indicated that BA and TDZ generated a similar number of shoot induction. While a lower number of shoots was produced in a culture medium supplemented with Kin treatment. Sarker et al. (2003) analyzed lentil regeneration from in vitro embryo culture, epicotyl, immature embryos, shoot nodes, and shoot tips explants on an MS culture medium containing different PGR concentrations. Eventually, cotyledon node explants on MS medium culture containing 0.5 mg/L BAP, 0.5 mg/L Kin, 0.1 mg/L GA3, and 5.5 mg/L tyrosine, produced 2 to 6 shoots per explant and selected as the best explant and PGR combination treatments which had lower concentrations than our experiment. This is due to the difference in the type of explant and genotype.

Root induction: Variance analysis (Table 7) indicated that NAA and genotype had significant effects on all the measured traits. However, their interaction was significant for root fresh and dry weights and rooting percentage.

The highest root length (5.92 mm) and the highest number of roots (2.12) were observed in 3 mg/L concentration of NAA. According to the comparison of genotype means (Table 8), it was observed that the highest root length (14.43 mm) and the highest number of roots (3.67) were related to 09S 83259-14 ILL6994 / ILL5480 genotype. Table 9 represents that the highest root fresh weight (28.45 mg), root dry weight (17.19 mg), and root induction (90.47%) are related to 3 mg/L NAA and 09S 83259-14 ILL6994 / ILL5480 genotype (Fig. 1c).

Lentil is one of the tough legumes regarding root induction and regeneration. There are inconsistent reports about the rooting of lentil in in vitro conditions. Williams and McHughen (1986) and Singh and Raghuwanshi (1989) reported on shoot regeneration of lentil in Kin and BAP-contained culture media but, in the first study, by transforming the regenerated shoots to sand, only 11% of rooting was obtained, also in the second one, by transforming the generated shoots to the hormone-less culture medium, few roots were generated. In these two studies, rooting percentage was higher in media containing BAP that is different from our study. Polanco et al. (1988) and Khawar et al. (2004) reported an MS culture medium containing 0.25 mg/L IBA as the best medium for rooting induction of shoots separated from 10 days' seedlings of lentil. However, the rooting percentage in their experiment was so low. In the study of Fratini and Ruiz (2001), the highest percentage of rooting was observed in the regenerated shoots in a culture medium containing 1.25-micromole zeatin. Taleb Bidokhti et al. (2003) reported root induction of lentil shoots in hydroponic culture containing NAA treatment (1 mg/L). Aasim (2012) conducted a study on lentil and reported that the best treatment for shoot regeneration was 10 mg/L BA, and for rooting was 0.25 to 1 mg/L IBA, or 1 mg/L IAA. He concluded that lentil regeneration in tissue culture is a complex process. Altaf et al. (2000) claimed that the best treatment for callus induction of lentil was 10 mg/L kinetin and 1 mg/L GA in dark conditions. Finally, they cut the shoots and placed them in soil conditions and generated roots and whole plants. Bagheri et al. (2012) reported a medium culture containing 1 mg/L NAA and 1 mg/L zeatin as the best treatment for lentil callus induction. They also introduced decapitated embryos with 1.4 cotyledons as the best explants. They concluded that the best condition for shoot regeneration and rooting was culture medium without PGR. They observed an expansive diversity among genotypes regarding embryonic callus production. Ye et al. (2002) introduced BA and TDZ as the best PGR combination for shoot regeneration. They also considered 1.5 mg/L NAA as the best treatment for rooting. In the investigation of Naqvi and Sultana (2010), maximum rooting was obtained at 48% in direct regeneration of lentil. They suggested that type of explant is important in root induction from shoot segments. Combinations of any concentration of IBA with any concentration of IAA in the rooting medium are inhibiting (Aasim, 2012). The root percentage induction in our study ranged from 14.28-90.47%. The highest rooting percentage was obtained in the current study for some genotypes (for example 09S 83259-14 ILL6994 / ILL5480 genotype treated with 3 mg/L NAA). But in other genotypes, as in other studies, root formation was low. The reason for the difference in the percentage of rooting in different studies can be related to the type of explant, the type and concentration of PGR, and the type of genotypes. Different approaches to plant regeneration from callus tissue culture have led to the formation of new plant forms with different phenotypic and genetic characteristics.

Genetic parameters: The phenotypic, genotypic, and broad-sense heritability of traits in the callus, shoot, and root induction stages are shown in Table 10. The highest phenotypic (46.62%) and genetic (45.72%) coefficients of variation were observed for callus volume and the highest value of broad sense heritability was related to callus fresh weight (96.46%). The lowest phenotypic (26.68%) and genetic (25.51%) coefficients and broad sense heritability (89.32%) were related to callus width. There was little difference between phenotypic and genetic coefficients, indicating of low effects of environmental factors.

According to Table 10 at the shooting induction stage, shoot dry weight had the highest values for phenotypic (58.43%) and genetic (57.82%) coefficients and broad sense heritability (97.94%). The lowest values of phenotypic (33.41%) and genetic (21.06%) coefficients and broad sense heritability (39.74%) were observed for the number of leaves. As the result of callus induction, the differences between phenotypic and genetic coefficients of variation was low (except for the number of shoot per plant), indicating low effects of environmental factors. It also indicates that phenotypic coefficients of variation are higher than genetic coefficients of variation. Similar to callus induction, most of the traits in this stage are heritable and can be used in the improvement of lentil in in vitro conditions. In this step, the number of shoots per plant had low heritability, which is indicative of the high effects of the environment on this trait.

Table 2. Mean square of source of variation for the measured traits at callus induction stage in lentil

Source of variation	df	Callus volume	Callus length	Callus width	Callus fresh weight	Callus dry weight	Callus induction
2,4-D	1	0.0003573**	644.814**	334.122**	4087.437**	1997.79**	16065.4**
Kin	1	0.0001366**	275.985**	107.355**	1815.038**	879.24**	4228.2**
Genotype (Gen)	13	0.0015701**	2432.673**	400.952**	9633.921**	4710.03**	6069.1**
2,4-D*Kin	1	0.0000008n.s	2.133n.s	0.1430**	173.740*	84.943*	30.4ns
Kin*Gen	13	0.000056n.s	13.215n.s	10.270*	19.020n.s	9.648n.s	110.4*
2,4-D*Gen	13	0.0000131*	30.976*	6.374n.s	122.087**	59.995**	455.7**

2,4-D *Kin*Gen	13	0.0000057$^{n.s}$	8.151$^{n.s}$	3.855$^{n.s}$	14.503$^{n.s}$	7.059$^{n.s}$	9.44^{ns}
Error	112	0.0000052	12.556	3.952	29.343	14.350	40.09
CV (%)	-	9.12	10.15	8.72	7.80	8.32	15.35

Table 3. Means comparison of 2,4-D × genotype intraction for callus length, volume, fresh and dry weight, and induction in lentil

Treatments		Traits				
2,4-D (mg/L)	Genotype	Callus volume (mL^3)	Callus length (mm)	Fresh weight (mg)	Dry weight (mg)	Callus induction (%)
1	1	0.0280^{fg}	4.0657^{f-h}	74.507^{g}	49.155^{g}	45.24^{d-g}
1	2	0.0346^{e}	4.4290^{e-g}	89.645^{de}	59.751^{de}	59.52^{b-e}
1	3	0.0232^{h}	3.6444^{h-i}	66.379^{h}	43.465^{h}	45.24^{d-g}
1	4	0.0331^{e}	4.4655^{ef}	83.597^{ef}	55.518^{ef}	45.24d-g
1	5	0.0286^{fg}	3.9891^{gh}	78.879^{fg}	52.215^{fg}	45.24^{d-g}
1	6	0.0419^{b}	5.3432^{bc}	112.722^{b}	75.905^{b}	73.81^{abc}
1	7	0.0387^{cd}	5.0713^{cd}	101.066^{c}	67.746^{c}	59.52^{b-e}
1	8	0.0209^{hi}	3.2485^{ij}	58.631^{ij}	38.092^{ij}	40.48^{e-h}
1	9	0.0112^{n}	1.7271^{n}	36.184^{p-r}	22.329^{pq}	14.29^{j}
1	10	0.0133^{l-n}	1.8800^{mn}	41.216^{n-p}	25.851^{n-q}	16.67^{ij}
1	11	0.0117^{n}	1.6275^{n}	33.955^{r}	20.964^{q}	16.67^{ij}
1	12	0.0179^{jk}	2.7705^{k}	47.584^{l-n}	30.309^{l-n}	21.43^{hij}
1	13	0.0109^{n}	1.6504^{n}	35.127^{qr}	21.639^{q}	14.29^{j}
1	14	0.0151^{k-m}	2.2216^{ln}	43.564^{l-o}	27.595^{l-o}	30.95^{g-j}
2	1	0.0304^{f}	4.4287^{e-g}	86.122^{de}	57.286^{de}	54.76^{c-f}
2	2	0.0411^{bc}	5.4219^{bc}	109.725^{b}	73.808^{b}	78.57^{ab}
2	3	0.0273^{g}	4.1387^{fg}	78.877^{fg}	52.264^{fg}	45.24^{d-g}
2	4	0.0373^{d}	5.0153^{cd}	93.003^{c}	65.602^{c}	64.29^{a-d}
2	5	0.0341^{e}	4.7139^{de}	90.616^{d}	60.431^{d}	64.29^{a-d}
2	6	0.0471^{a}	6.0119^{a}	130.574^{a}	88.402^{a}	83.33^{a}
2	7	0.0415^{b}	5.5212^{b}	112.664^{b}	75.865^{b}	78.57^{ab}
2	8	0.0226^{h}	3.4438^{ij}	62.721^{hi}	40.904^{hi}	40.48^{e-h}
2	9	0.0117^{n}	1.7126^{n}	39.451^{o-r}	24.849^{o-q}	14.29^{j}
2	10	0.0131^{l-n}	1.7850^{mn}	37.908^{o-r}	23.336^{o-q}	16.67^{ij}
2	11	0.0162^{k}	2.3354^{l}	49.398^{kl}	31.579^{kl}	14.29^{j}
2	12	0.0197^{ij}	3.0491^{jk}	54.619^{jk}	35.233^{jk}	21.43^{hij}
2	13	0.0124^{mn}	1.8126^{mn}	42.198^{m-p}	26.678^{m-p}	14.29^{j}
2	14	0.0156^{kl}	2.2292^{lm}	48.289^{k-m}	30.852^{k-m}	35.71^{f-i}
LSD 5%		0.0013	0.4250	6.2549	4.3742	7.31118

Means followed by the same letter in each column are not significantly different at $P < 0.05$. The name of genotypes can be found in Table 1.

Table 4. Mean square of source of variation for the measured traits at shoot production stage in lentil

Source of variation	df	Leaves number	Shoots number	Shoot length	Shoot fresh weight	Shoot dry weight	Shoot induction
PGR	1	3.857**	1.714**	639.21**	99.69**	34.73**	6047.78**
Gen	13	4.458**	0.531**	618.74**	280.38**	113.78**	4586.73**
PGR*Gen	13	0.319$^{n.s}$	0.125$^{n.s}$	24.81*	2.62*	0.84**	260.70**
Error	56	0.286	0.108	8.77	1.17	0.397	38.88
CV (%)		12.40	25.94	11.95	9.16	8.38	11.75

*, ** showed significant at 5% and 1% probability levels, respectively and ns showed non-significant.

Table 5. Means comparison of genotypes for number of shoots and leaves in lentil plants

	Traits	
Genotype No.	Leaves number	Shoots number
1	4.1667^{de}	1.3333^{b-d}
2	5.1667^{bc}	1.5000^{bc}
3	4.1667^{de}	1.1667^{cd}
4	5.0000^{bc}	1.3333^{b-d}
5	4.6667^{cd}	1.3333^{b-d}
6	6.0000^{a}	2.0000^{a}
7	5.50000^{ef}	1.6667^{ab}
8	3.8333^{ef}	1.0000^{d}
9	4.1667^{de}	1.0000^{d}
10	4.0000^{de}	1.0000^{d}
11	3.8333^{ef}	1.0000^{d}
12	3.0000^{g}	1.0000^{d}
13	3.1667^{fg}	1.1667^{cd}

14	3.6667^{ef}	1.1667^{cd}
LSD 5%	0.618	0.378

Table 6. Means comparison of PGR × genotype intraction for shoot length, fresh and dry weight, and induction in lentil

PGR			Traits			
Bap (mg/L)	2ip (mg/L)	Genotype No.	Shoot length (mm)	Shoot fresh weight (mg)	Shoot dry weight (mg)	Shoot induction (%)
3	-	1	27.283^{e-i}	14.1333^{e-g}	8.9747^{e-g}	76.18^{bcd}
3	-	2	38.333^{bc}	20.2333^{cd}	13.1815^{b}	85.71^{ab}
3	-	3	26.411^{f-i}	10.9500^{hi}	6.9533^{hi}	76.18^{bcd}
3	-	4	31.815^{d-f}	18.8667^{ef}	11.9803^{cd}	85.71^{ab}
3	-	5	29.653^{d-g}	15.0000^{ef}	9.5250^{ef}	85.71^{ab}
3	-	6	48.360^{a}	26.7000^{a}	16.3778^{a}	90.47^{a}
3	-	7	42.552^{b}	24.1333^{b}	15.3242^{a}	90.47^{a}
3	-	8	19.413^{kl}	8.5667^{jk}	5.4399^{kl}	66.66^{de}
3	-	9	23.337^{h-k}	5.6267^{l-n}	3.6946^{mn}	14.28^{j}
3	-	10	25.023^{g-j}	9.0000^{j}	5.7150^{jk}	61.90^{fe}
3	-	11	16.493^{l-n}	8.9000^{j}	5.6515^{jk}	52.38^{f}
3	-	12	19.733^{j-l}	5.9233^{l-n}	12.7613^{mn}	19.04^{ij}
3	-	13	19.067^{kl}	6.3600^{l-n}	3.8269^{mn}	28.57^{gh}
3	-	14	18.273^{k-m}	6.0733^{l-n}	3.8566^{mn}	28.57^{gh}
-	3	1	22.603^{i-k}	12.5667^{gh}	7.7998^{gh}	71.42^{cd}
-	3	2	32.347^{de}	18.4000^{d}	11.5940^{d}	71.42^{cd}
-	3	3	20.633^{j-l}	10.4000^{ij}	6.4060^{ij}	38.09^{g}
-	3	4	28.594^{e-h}	15.6333^{e}	9.9272^{e}	71.42^{cd}
-	3	5	27.833^{e-h}	13.7000^{gh}	8.6995^{fg}	71.42^{cd}
-	3	6	47.444^{b}	22.6300^{b}	15.3701^{a}	85.71^{ab}
-	3	7	34.880^{dc}	20.5000^{c}	12.6842^{bc}	80.95^{abc}
-	3	8	12.260^{no}	6.4300^{lm}	4.4164^{lm}	33.33^{gh}
-	3	9	11.880^{no}	5.6640^{l-n}	3.5967^{n}	14.28^{j}
-	3	10	10.600^{o}	7.0000^{kl}	4.4450^{lm}	33.81^{hij}
-	3	11	13.540^{m-o}	6.5333^{l}	4.1487^{m}	19.04^{ij}
-	3	12	11.337^{no}	4.3600^{n}	2.7686^{n}	4.28^{j}
-	3	13	16.433^{l-n}	1.6700^{o}	1.1826^{o}	4.28^{j}
-	3	14	18.123^{k-m}	4.4767^{mn}	2.8427^{n}	4.28^{j}
LSD 5%			4.836	1.7663	1.2145	10.1823

Means followed by the same letter in each column are not significantly different at $P < 0.05$. The name of genotypes can be found in the Table 1.

Table 7. Mean square of source of variation for the measured traits at rooting stage in lentil

Source of variation	df	Root number	Root length	Root fresh weight	Root dry weight	Root induction
NAA	1	8.048^{**}	47.485^{**}	297.215^{**}	143.25^{**}	6825.28^{**}
Genotype	13	3.842^{**}	102.483^{**}	270.766^{**}	89.588^{**}	4415.01^{**}
NAA× Genotype	13	$0.176^{n.s}$	$1.609^{n.s}$	6.283^{**}	3.960^{**}	320.17^{**}
Error	56	0.155	1.052	1.333	0.932	19.44
CV	-	21.740	19.848	10.696	14.624	9.03

** showed significant at 1% probability level, and ns showed non-significant.

Table 8. Means comparison of genotypes for umber of roots and root length in lentil

Genotype No.	Root number	Root length (mm)
1	1.8333^{ef}	5.1753^{ef}
2	2.5000^{c}	9.6447^{c}
3	1.5000^{fg}	4.3142^{fg}
4	2.3333^{cd}	6.9038^{d}
5	2.0000^{de}	5.8860^{de}
6	3.6667^{a}	14.4302^{a}
7	3.0000^{b}	11.2874^{b}
8	1.1667^{g}	3.5760^{gh}
9	1.5000^{fg}	3.0902^{g-i}
10	1.1667^{g}	1.9182^{ij}
11	1.1667^{g}	2.8124^{hi}
12	1.1667^{g}	1.0534^{jk}
13	1.1667^{g}	2.0903^{ij}
14	1.1667^{g}	0.1533^{k}
LSD 5%	**0.455**	**1.18**

Means followed by the same letter in each column are not significantly different at $P < 0.05$. The name of genotypes can be found in the Table 1.

Table 9. Means comparison of PGR × genotype intraction for root fresh and dry weight and root induction in lentil

NAA (mg/L)	Genotype No.	Traits		Root induction (%)
		Root dry weight (mg)	Root fresh weight (mg)	
3	1	7.3870^{e-g}	12.8686^{f}	71.43^{b}
3	2	12.4286^{c}	19.7083^{c}	85.71^{a}
3	3	7.9173^{e-g}	12.0667^{fg}	71.43^{b}
3	4	9.8953^{d}	15.5333^{de}	85.71^{a}
3	5	8.1142^{e-g}	14.9900^{e}	76.19^{a}
3	6	17.1915^{a}	28.4500^{a}	90.47^{a}
3	7	14.5308^{b}	23.1960^{b}	85.71^{a}
3	8	5.4486^{ij}	8.4067^{h-j}	52.38^{c}
3	9	7.2493^{e-h}	9.9333^{hi}	19.04^{fe}
3	10	6.4884^{g-i}	10.3603^{gh}	71.43^{b}
3	11	4.7041^{j-i}	7.4080^{j-l}	33.33^{d}
3	12	2.4500^{mn}	4.0000^{m-o}	14.28^{f}
3	13	5.5582^{h-j}	8.6207^{h-j}	38.09^{d}
3	14	1.2239^{no}	1.9273^{op}	14.29^{f}
2	1	6.3667^{g-j}	10.263^{gh}	57.14^{c}
2	2	8.6255^{de}	15.3333^{de}	71.43^{b}
2	3	5.1044^{i-k}	7.9147^{i-k}	33.33^{d}
2	4	8.3182^{d-f}	13.9000^{ef}	71.43^{b}
2	5	6.5684^{f-i}	10.4857^{gh}	57.14^{c}
2	6	11.9464^{c}	22.3382^{b}	85.71^{a}
2	7	9.9798^{d}	17.1727^{d}	71.43^{b}
2	8	3.5983^{k-m}	5.9333^{k-m}	14.28^{f}
2	9	3.2385^{ml}	5.3400^{mn}	14.28^{f}
2	10	1.8203^{m-o}	2.8667^{op}	23.81^{e}
2	11	3.5765^{k-l}	5.6323^{l-n}	14.28^{f}
2	12	1.9833^{m-o}	3.12333^{o}	14.28^{f}
2	13	2.3915^{mn}	3.7960^{no}	14.28^{f}
2	14	0.5944^{o}	0.9360^{p}	14.28^{f}
LSD 5%		1.2427	1.7773	7.2000

Means followed by the same letter in each column are not significantly different at $P < 0.05$. The name of genotypes can be found in the Table 1.

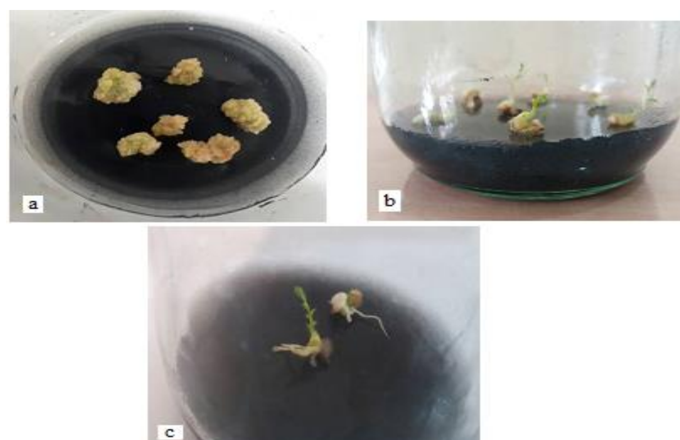

Fig. 1. Plants produced from embryo and a quarter of its cotyledon explant of 09S 83259-14ILL6994/ILL5480 lentil genotype in: (a) 1 mg/L Kin and 2 mg/L 2,4-D for callus induction, (b) 3 mg/L BAP and 4 mg/L Kin and 2 mg/L TDZ for shoot production, and (c) 3 mg/L NAA for root induction.

At the rooting stage (Table 10), the highest and the lowest values of the phenotypic coefficient of variation are related to root length (82.01%) and number of roots (48.47%), respectively. Also, the highest and the lowest values of genetic coefficient of variation were related to the root length (79.58%) and number of roots (43.32%), respectively. Root induction had the highest amount of

broad sense heritability (97.42%) and the lowest amount was related to the number of roots (79.88%).

There is little information about the study of the genetic diversity of lentil plants in in vitro conditions. Kaya et al. (2015) conducted a study on the somatic embryogenesis of 11 lentil in tissue culture. Similar to the current study, they reported high genetic diversity among the studied genotypes. Somaclonal variation can be attributed to the genetic heterogeneity of somatic cells of donor plants, induced genetic and epigenetic variation due to in vitro culture medium conditions, the composition of culture media, plant growth regulators, genotype, and type of mother plant (Kaya et al. 2015). Naqvi and Sultana (2010) reported the key role of genotype on the regeneration of lentil plants in in vitro conditions.

Table 10. Phenotypic and genotypic coefficient of variation and broad sense heritability of the measured characters on lentil genotypes in in vitro condition

	Callus induction stage					Shoot induction stage				Root induction stage			
Genetic Parameters	Callus fresh weight	Callus volume	Callus length	Callus width	Callus induction	Shoot dry weight	Shoot length	Shoot number	Shoot induction	Root dry weight	Root length	Root number	Root induction
Phenotypic coefficient of variation (%)	44.14	46.62	41.92	26.68	56.48	58.42	42.38	33.41	53.20	60.05	82.01	48.47	56.18
Genotypic coefficient of variation (%)	43.35	45.72	40.68	25.21	54.35	57.82	40.66	21.06	51.88	58.24	79.58	43.32	55.45
Broad sense heritability (%)	96.46	96.18	94.14	89.33	92.61	97.94	92.06	39.74	95.12	94.07	94.14	79.88	97.42

Generally, the broad sense heritability of most of the traits was so high which approves low effects of environmental factors. Therefore, selection based on these traits will be efficient. High broad sense heritability and genetic diversity were also reported for yield and morphological traits of lentil in the field conditions (Bakhsh et al. 1992; Gupta et al. 1996; Stoilova and Pereira 1999). Also, Ates (2019) for SNP markers, Chowdhury et al. (2020) for morphological and SSR markers, and Kumar (2019) for morphological traits reported wide genetic diversity among lentil genotypes.

CONCLUSION

One of the problems of tissue culture is the existence of epigenetic diversity, which causes misdirection and waste of time to breeders in selection programs. In this experiment, by using different genotypes and calculating genetic parameters, the contribution of genetic and non-genetic variances in tissue culture related traits was assessed. In general, the results of this study indicated a high diversity among different genotypes for callus induction, shoot production, and rooting stage for the measured traits which shows an efficiency of selection and breeding through lentil somaclonal variation. In this regard, 09S 83259-14 ILL6994 / ILL5480 and FLIP2010-40L-10770-ILL 8119 / ILL 7686 genotypes had the best response to tissue culture in all the three stages of callus induction, shoot regeneration, and root formation. The interaction of genotype and growth regulator was significant for several traits indicating that genotypes had different responses to plant growth regulators. Most of traits showed a high broad sense of heritability in in vitro culture indicating the selection based on these traits is efficient. Generally, the best PGR combination for callus induction, shoot regeneration, and rooting were 1 mg/L Kin and 2 mg/L 2,4-D, 3 mg/L BAP, 4 mg/L Kin, and 2 mg/L TDZ, and 3 mg/L NAA, respectively. The results of this study can be used in lentil breeding programs in in vitro conditions.

FUNDING

This article is part of the first author's Master's thesis, which is financially supported by the Vice-Chancellor Research of Yasouj University.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Massoud Dehdari (as supervisor) and Hajar Ashoordan (as graduate student) designed, outlined, and performed the experiment, interpreted and discussed the data, performed statistical analysis, and wrote the manuscript. Asad Masoumiasl (as advisor) advised on method of tissue culture and treatments application. Rahmatollah Karimizadeh (as advisor) provided seeds of the studied genotypes and advised on method of adaptation to in vivo condition.

DECLARATION of COMPETING INTEREST

The authors declare no conflicts of interest.

ETHICAL STATEMENT

All authors are aware on content of the manuscript and consented to submit it to Iran Agriculture Research Journal. We did not send this article to another journal. An ethics statement is not applicable because this study is based exclusively on published literature.

DATA AVAILABILITY

The raw data of this research are available at the request of the reviewers and editors.

REFERENCES

Aasim, M. (2012). Micropropagation of lentil using pulse treatment of immature plumular apices. *Pakistan Journal of Agricultural Sciences*, 49(2), 149-152.

Altaf, N., Iqbal, J., & Salih Ahmad, M. (2000). Somaclonal variation in microsperma Lentil (*Lens culinaris* Medik). *Pakistan Journal of Biotechnology*, 2, 697-699. https://doi.org/10.3923/pjbs.1999.697.699.

Anil, V. S., Bennur, S., & Lobo, S. (2018). Somaclonal variations for crop improvement: Selection for disease resistant variants *in vitro*. *Plant Science Today*, 5(2), 44-45. https://doi.org/10.14719/pst.2018.5.2.382.

Ates, D. (2019). Genetic diversity in lentil landraces revealed by diversity array technology (DArT). *Turkish Journal of Field Crops*, 24, 252-260. https://doi.org/10.17557/tjfc.656511.

Bagheri, A., Ghasemi Omran, V., & Hatefi, S. (2012). Indirect in vitro regeneration of lentil (*Lens culinaris* Medik.). *Journal of Plant Molecular Breeding*, 1(1), 43-50. https://doi.org/10.22058/jpmb.2013.1705.

Bajaj, Y. P. S., & Danju, M. S. (1979). Regeneration of plant from apical meristem tips of some legumes. *Current Science*, 48, 906-907. https://doi.org/10.1007/BF00043039.

Bakhsh, A., Ghafoor, A., & Malik, B. A. (1992). Evaluation of lentil germplasm. *Pakistan Journal of Scientific and Industrial Research*, 35, 48-49.

Bayrac, A. T. (2004). *Optimization of a regeneration and transformation system for lentil (Lens culinaris Medik., cv. Sultan-I) cotyledonary petioles and epicotyls*. (Master's thesis, Middle East Technical University, Ankara, Turkey).

Chawla, H. S. (2009). *Introduction to Plant Biotechnology* (3 rd.). Enfield, NH, USA: CRC Press.

Chen, J. T., & Chang, W. C. (2002). Effects of tissue culture conditions and explant characteristics on direct somatic embryogenesis in Oncidium `Gower Ramsey'. *Plant Cell, Tissue and Organ Culture*, 69, 41–44. https://doi.org/10.1023/A:1015004912408

Chowdhury, M. M., Haque, M. A., Malek, M. A., Rasel, M., Molla, M. R. & Ahamed. K. U. (2020). Morphological and SSR marker-based diversity analysis of lentil (*Lens esculenta*) genotypes using yield and yield contributing characters. *Indian Journal of Agricultural Research*, 54(4), 429-436. https://dx.doi.org/10.18805/IJARe.A-464.

Fratini, R., & Ruiz, M. L. (2001). Comparative study of different cytokinins in the induction of morphogenesis in lentil (*Lens culinaris* Medik.). *In Vitro Cellular & Developmental Biology*, 38, 46-51. https://doi.org/10.1079/IVP2001252.

Ghanem, S. A., Moursy, H. A., Reda, A. A., & Bahr, M. K. (1989). Effect of some growth regulators on the growth of *Lens culinaris* Medik., cultured in vitro. *African Journal of Agriculture*, 16, 155-161.

Ghasemi Omran, V., & Bagheri. A. (2010). Optimization of root regeneration of two lentil genotypes in vitro condition. Iranian Journal of *Field Crops Research*, 8(1), 90-97. https://doi.org/10.22067/gsc.v8i1.7397. (In Persian)

Goodnight, J., & Sall, J. (2003). SAS [computer program]. (Version 9.4). Cary, NC: SAS Institute Inc; https://www.sas.com/en_us/home.html

Gordon, J. S. (2008). The status of the in vitro embryo. *Bioethics*, 22(5), 296-298. https://doi.org/10.1111/j.1467-8519.2008.00635.x.

Gulati, A., Schryer, P., & McHughen. A. (2001). Regeneration and micro grafting of Lentil shoots. *In Vitro Cellular & Developmental Biology*, 37, 798-802. https://doi.org/10.1007/s11627-001-0132-9.

Gupta, A., M. Sinka, K., Mani, V. P., & Dube, S. D. (1996). Classification and genetic diversity in lentil germplasm. *Lens Newsletter*, 23, 19-14.

Hoagland, D. R., & Arnon, D. I. (1950). The water-culture method for growing plants without soil. *California Agricultural Experiment Station Circular* 347, 1-32.

Kaya, S., Turker, M., & Eray, N. (2015). Somatic embryogenesis on Turkish lentil *(Lens culinaris* Medik.) cultivars. *Journal of Applied Biological Sciences*, 9 (3), 49-58. https://www.jabsonline.org/index.php/jabs/article/view/469.

Khawar, K. M., Sancak, C., Uranbey, S., & Özcan, S. (2004). Effect of thidiazuron on shoot regeneration from different explants of lentil (*Lens culinaris* M.) via organogenesis. *Turkish Journal of Botany*, 28, 421-426.

Kumar, A. (2019). Genetic diversity of yield attributing components and seed yield in lentil (*Lens culinaris* Medik*.). Current Journal of Applied Science and Technology*, 33 (2), 1-6. https://doi.org/10.9734/cjast/2019/v33i230056.

Naqvi, S. M. S., & Sultana, T. (2010). Optimization of tissue culture and genetic transformation in Lentil (*Lens culinaris* Medik). Proceeding of 6th International Conference of Biological Science (Botn). Yogyakarta, Indonesia.

Polanco, M.C., Pelaez, M. I., & Ruiz, M. L. (1988). Factors affecting callus and shoot formation from in vitro cultures of *Lens culinaris* Medik. *Plant Cell, Tissue and Organ Culture*, 15, 175-182. https://doi.org/10.1007/BF00035759.

Sarker, R. H., Barkat, M. M., Ashappurno, B., Shirin, M., Mouful, N., Rehana, H., & Hoque, M. L. (2003). In vitro regeneration in lentil (*Lens culinaris*). *Plant Tissue Culture*, 13, 155-164.

Saxena, P. K., & King, J. (1987). Morphogenesis in lentil: Plant regeneration from callus cultures of *Lens culinaris* Medik. via somatic embryogenesis. *Plant Science*, 52, 223- 227.

Sharma, J. R. (2006). *Statistical and Biometrical Techniques in Plant Breeding*. New Delhi: New Age International (p) Limited Publishers.

Singh, R. K., & Raghuwanshi. S. S. (1989). Plantlet regeneration from nodal segment and shoot tip derived explants of lentil. *Lens Newsletters*, 16, 33–35.

Stoilova, T., & Pereira, M. G. (1999). Morphological characterization of 120 lentil accessions. *Lens Newsletter*, 26(2), 7-9.

Taleb Bidokhti, S., Safrinejad, A., Lahouti, M., & Bagheri, A. (2003). Effect of genotype, explants and

growth hormones in lentil tissue culture. In: Proceedings of the 3rd National Biotechnology Conference of the Islamic Republic of Iran. Mashhad, Ferdowsi University

Williams, D. J., & McHughen, A. (1986). Plant regeneration of legume *Lens culinaris* Medik. (lentil) In vitro. *Plant Cell, Tissue and Organ Culture*, 7, 149-15.
https://doi.org/10.1007/BF00043039.

Ye, G., Mcneil, A., Conner, J., & Hill, G. D. (2002). Multiple shoot formation in lentil (*Lens culinaris* Medik) seeds. *New Zealand Journal of Crop and Horticultural Science*, 30, 1-8.
https://dx.doi.org/10.1080/01140671.2002.9514193.

Zaker Tavallaie, F., Bagheri, A., Ghareyazie, B., & Sharma, K. K. (2009). Optimization of tissue culture condition in lentil to induce effective multiple shoot induction. *Iranian Journal of Field Crops Research*, 7(2), 411-419. (In Persian).
https://dorl.net/dor/20.1001.1.20081472.1388.7.2.8.8