High Heritability of Glycyrrhizic Acid, Glabridin, Liquiritin and Liquiritigenin in Licorice (Glycyrrhiza Glabra L.) Populations of Iran

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Research Article

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Abstract

Glycyrrhiza glabra L. is an herbaceous, perennial plant with high distribution in Iran. Genetic variability, heritability and correlation among characters in 22 populations of *G. glabra* L. were studied. The genetic parameters among the traits including phenotypic variances, genotypic variances, genotype by environment variances, broad-sense heritability and genotypic and phenotypic correlation coefficients were studied. Variance components analysis showed that the extent of phenotypic coefficient of variation (PCV) was fairly higher for all the examined traits compared with genotypic coefficient of variation (GCV). Glabridin (GLA) exhibited high GCV and PCV (156.07% and 156.68%, respectively). The broad sense heritability varied from 38.92% to 99.79% and narrow sense heritability ranged from 9.70 % to 24.94%. Heritability of GLA, glycyrrhizic acid (GLY), liquiritin (LI), liquiritigenin (LIQ), rutin (RU) and rosmarinic acid (RA) were very high, exhibiting more than 97% heritability. Therefore, these critical characteristics can efficiently be selected and inherited in breeding programs. In most traits, the genotypic correlations showed the same direction as the phenotypic correlations. The contents of GLA and LIQ showed a positive correlation with majority of morphological traits. Therefore, selecting individual plants having desired morphological traits can be correlated with high contents of bioactive compounds in the harvested root.

Keywords: Correlation, Diversity, Glycyrrhizic acid, Glabridin, Genotypic coefficient, Breeding

Abbreviations: PCV, phenotypic coefficient of variation; GCV, genotypic coefficient of variation; TPC, total phenolic content; TFC, total flavonoid content; GLY, glycyrhizic acid; LI, liquritin; LIQ, liquritigenin; RU, rutin; RA, rosmarinic acid; GLA, glabridin

Introduction

Glycyrrhiza glabra L. is considered as herbaceous perennial plant belonging to Fabaceae family (Asif et al., 2015). This plant is native to Mediterranean region, south of Russia and Asia, but today is cultivated throughout Europe, Asia, United Kingdom and USA (Parvaiz et al., 2014). Glycyrrhiza genus has 30 species in the world and 3 species in Iran, more importantly *G. glabra* L. (Karimi, 1996). This plant is one of the most important medicinal and industrial plants that its active ingredients, in different forms, are used namely in pharmaceutical, food and cosmetic industries (Mukhopadhyay and Panja, 2008). Pharmaceutical properties of the plant such as antimicrobial activity (Vlaisavljević et al., 2018), anti-inflammatory (Farag et al., 2015), antiviral (Wang et al., 2015), anti- *Helicobacter pylori* (Asha et al., 2013), antioxidant (Martins et al., 2015; Parvaiz et al., 2014) and antiproliferative activity (Dunlap et al., 2015; Huang et al., 2014) have been reported. Licorice root contains various sugars (up to 18%), flavonoids, sterols, amino acids, gum, starch, essential oils and saponins (Vlaisavljević et al., 2018). The most frequent saponin is known as glycyrrhizic acid (GLY) or glycyrrhizin (Hajimehdipoor et al., 2009). In addition, the licorice root contains acid-2-beta-glucuronosyl-glucuronic acid, glycyrrhetic acid, asparagine, glucose, sucrose, resin, volatile oils, flavonoids such as glabridin (GLA) isoliquiritigenine, liquiritigenine (LIQ) and liquiritin (LI) (Bode and Dong et al., 2015). Although, *G. glabra* L. inhabits all around Iran, plant harvest which is actually uprooting the herb, is confronting this species at risk of extinction (Hajimehdipoor et al., 2009). Therefore, special attention is needed to maintain sustainable reproduction of this plant. Efforts to protect the habitats and plant genetic resources through identification, permanent conservation, revitalization and replenishment of plant are effective steps towards the preservation and survival

of the species and its natural habitat. Access to indigenous populations of a plant species and genetic diversity among the populations are the fundamental requirements for breeding studies (Rao and Hodgkin, 2002). Nevertheless, vast potential of plant populations and their conservation is neglected in many areas. For breeding purposes, gaining insight about variation and genetic distances among populations or individuals, and understanding the kinship relations of the species concerned and effective sampling of genotypes is important (Guo et al., 2008). Therefore, access to populations possessing high genetic diversity and management of available genetic resources are considered as important components of plant improvement projects. Variation and selection are the two main pillars of each program and the selection is subject to the existence of an optimal variation (Sarbarze and Amini, 2011).

In the process of medicinal plants domestication, various factors such as ecology, reproduction, and fertility should be taken into consideration. Even though medicinal plants comprise a large number of species with different biological properties and invaluable applications, the number of cultivars obtained through the breeding of them is very small and insignificant (Kayser and Quax, 2007). Plant breeding is one of the most important methods for improving medicinal plants production to meet the specific demands of consumers (Kayser and Quax, 2007). Investigating the genetic diversity in existing genetic resources is primary stage of a breeding project that provides the possibility of categorization and accurate description of the plant samples (Mohammadi and Prarasana, 2003). To study variability, several attributes such as phenotypic and genotypic variances, broad sense heritability and genetic advance values should be evaluated quantitatively (Rao et al., 2013).

Knowing the nature and extent of genetic variation in any plant species plays an important role in designing a successful breeding program. The higher the genetic diversity, the greater the

likelihood of obtaining recombinant genes that show increased heterotrophic effects (Rao and Hodgkin, 2002). So far, some studies have been carried out to identify the distribution areas, the diversity of genetic traits in different regions and the conservation of genetic reserves of this plant in Iran (Hosseini et al., 2014; Esmaeili et al., 2019). However, introduction of these population into cultivation and evaluating them under cultivation environment have not been practiced extensively. Studies on licorice in the field have shown that active substances can be variable depending on geographical conditions and date of harvest throughout the year (Cheel et al., 2013; Hayashi et al., 1998). Cultivation of seed or vegetative propagation using segments of root can be practiced for field production of this crop. Mareshige et al., (2011) cultivated seeds of *G. uralensis* in the field and recorded high variability of GLY within the population ranging from 0.46 to 4.67%. Environmental conditions in the farm such as temperature and edaphic factors can greatly affect on bioactive constituents of licorice (Hosseini et al., 2014). The current study was aimed to reveal the genetic variability, phenotypic and genotypic relationship among different traits after introduction of licorice populations into cultivation and to calculate the genotypic and phenotypic parameters of the studied traits among *G. glabra* populations. Finding high variability, together with high heritability of traits particularly active substances can be considered as essential requirements for plant improvement projects.

Materials and methods

Plant materials

Licorice Plant species was identified in its natural habitats according to Flora of Iran (Ghahreman, 1999). Root samples of several individual plants were collected from a total of 22 populations in different regions of Iran in November 2016 (Fig. 1). Geographical information of studied population is mentioned previously (Esmaeili et al., 2019).

Fig. 1. The locations where the populations of *G. glabra* collected, and location of cultivation in Zanjan province.

Roots of each population (2 cm diameter and 15 cm length) were planted in a randomized complete block design (RCBD) with 5 replications with the spacing of 60×40 cm (5 plants per replication). The experiment was performed in University of Zanjan (Iran) research farm (Fig. 2).

Fig 2. Cultivated G. glabra populations in research farm located in University of Zanjan.

The geographic location of the experiment was 35° 25' N, 47 ° 1' E and altitude of 1663 m. Mean of morphological traits in 2018 and 2019 were measured. In November 2019, roots of all populations were harvested and root yield and other phytochemical traits were evaluated. The physical and chemical properties of the soil of the research farm are presented in Table 1.

Table 1

Agro-morphological traits

Means of plant height, plant width, leaf length, leaf width, number of leaflets, leaflet length, leaflet width, number of axillary branches, main stem diameter, shoot fresh weight, shoot dry

weight, shoot yield per m^2, root fresh weight, root dry weight, root yield, length and width of the limbs were measured by a ruler and a digital caliper. Aerial and root weights were weighed by digital scales. The shoot and root dry weight yield was calculated from the total plant weight per m^2.

Phytochemical traits

The total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity, GLY, LI, GLA, LIQ, rutin (RU) and rosmarinic acid (RS) were studied. For extraction, 10 mL solvent (methanol: water 80:20) was added to 500 mg dried well-ground root of each sample and incubated in an ultrasonic bath for half an hour followed by centrifugation at 4500 rpm for 15 min and filtered for later analysis (Hosseini et al., 2014). The TPC (Folin–Ciocalteu method) (Lister and Wilson, 2001), TFC (Aluminum Chloride method) (Chang et al., 2002) and antioxidant activity (DPPH method) (Geoffroy et al., 2017) were evaluated.

GLY, LIQ, GLA and LI contents of ground root of the samples were evaluated using Knauer HPLC instrument according to the method described previously (Esmaeili et al., 2019). The extracts were passed through a 0.22 nm filter and stored in a 2 ml tube at 4 °C until analysis by HPLC. To separate RS and RU from other compounds, C18 column (Waters, 15 cm × 4.6 mm) was used at room temperature. Mobile phase was water containing 0.02% trifluoroacetic acid (TFA) (solvent A), and phase B was methanol including 0.02% TFA (solvent B), in a linear gradient solution program as follows: 80% A for 10 min, fall out to 20% in 400 min. The flow rate of the mobile phase was kept at 0.5 mL min^{-1}. The detection wavelengths for monitoring RS and RU were 329 and 257 nm, respectively (Esmaeili et al., 2019). The injection rate of the

sample to the injector was 20 µL. To increase the accuracy, each sample was measured in three replications. Peak area was plotted against the concentrations to draw the calibration graphs of external standards. The acceptable R-squared value for all standards from linear regression was over 0.98.

Data analysis

Analysis of variance (ANOVA) for all measured characters was computed using SAS software (SAS Institute, 2008). The means were analyzed, and statistical significance recorded at two probability levels; $*P < 5\%$ and $**P < 1\%$ (SAS, 2008). The method described by Burton and DeVane (1953) was used for estimation of phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) according to the following formulas (Table 2):

Environmental variance (σ^2_e) = MSe

Phenotypic variance (σ^2_p) = ($\sigma^2_g + \sigma^2_e$)

Genotypic variance (σ^2_g) = ($MSe - MSt$)/r

Where, *Mse* and *Mst* are the mean square of error and mean square of treatment, respectively, and r is the number of replicates.

Table 2

The mean values were considered for genetic analyses to define coefficients of both PCV and GCV, according to procedure described by Singh and Chaudhary (1985).

$$GCV = \frac{\sqrt{\sigma^2 g}}{x} \times 100 \quad (1)$$

$$PCV = \frac{\sqrt{\sigma^2 p}}{x} \times 100 \quad (2)$$

159 Where, x = sample mean.

160 As the *G. glabra* populations were evaluated, the additive variance can be calculated from the

161 below equation.

162 $\sigma^2 g = \sigma^2 F_2 = Cov\ (HS) = \frac{1}{4}\sigma^2 A$ (3)

163 Where: $\sigma^2 A$ = Additive variance

164 Estimation of narrow sense and broad sense heritability (H^2n and H^2b) of each character was

165 performed according to the method of Falconer (1989):

166 $H^2b = \frac{\sigma^2 g}{\sigma^2 p}$ (4)

167 $H^2n = \frac{\sigma^2 A}{\sigma^2 p}$ (5)

168 Genetic advance (GA) was calculated according to the following formula proposed by Allard

169 (1960):

170 $GA = \sigma p \times i \times H^2\ b$ (6)

171 Where, σ_p = phenotypic standard deviation of F_2 population and i = constant value that shows

172 the selection intensity. The value used for i in this study at 5% selection intensity was 2.065.

173 The percentage of genetic advance of the mean (GAM) was assessed according to the method of

174 Johnson et al. (1955):

175 $GAM(\%) = \frac{GA}{x} \times 100$ (7)

176 Where, x is the symbol for grand mean of a character.

177 The characters associations represented by correlation coefficient between different pairs of

178 characters at the genotypic and phenotypic levels were calculated by Miller et al. (1958) as

179 follow:

$$rg_{xy} = \frac{\text{Cov } g_{xy}}{\sqrt{\sigma^2_{gx}} \times \sqrt{\sigma^2_{gy}}} \quad (8)$$

Where, $\text{Cov } g_{xy}$ is the genotypic covariance between two traits, σ^2_{gx} is the genotypic variance of the first trait, and σ^2_{gy} is the genotypic variance of the second trait.

$$rp_{xy} = \frac{\text{Cov } p_{xy}}{\sqrt{\sigma^2_{px}} \times \sqrt{\sigma^2_{py}}} \quad (9)$$

Where, $\text{Cov } p_{xy}$ is the phenotypic covariance of the progeny means between the two traits.

Results and discussion

High genotypic and phenotypic variability in a given species is critical to conduct a breeding program. The analysis of variance showed significant differences among populations for all studied traits. The significant difference among populations for both growth parameters as well as phytochemical characteristics provides a great opportunity for improvement of these characters through breeding. Estimated GCV and PCV, H^2b, H^2n and GAM of studied traits are presented in Table 3.

Phenotypic and genotypic coefficient of variations

Results of variance component analysis showed that PCV was higher in magnitude than the corresponding GCV for all the examined traits. This indicates that genetic factors are generally the sources of variability in these traits and environmental conditions were less influential (Table 3). The GCV values were ranging from 6.25% to 156.07% while PCV values ranged from 8.93% to 156.68% and the range of PCV was broader for all studied traits (Table 3). Glabridin showed

highest GCV and PCV estimates (156.07% and 156.68%, respectively) and the lowest was observed in antioxidant activity (6.25µg/mL and 8.93 µg/mL, respectively). Higher values of PCV and GCV exhibit the presence of considerable degree of variability among studied traits, providing abundant range for improvement of the traits via simple selection. According to Deshmukh et al. (1986), the values of GCV and PCV greater than 20% are considered as high, however, the values less than 10% are considered as low and values between 10 and 20% as intermediate. Accordingly, all the studied traits have high GCV and PCV.

Table 3

Heritability and genetic advance

In this study, the broad sense heritability ranged from 38.92% to 99.79% and narrow sense heritability ranged from 9.70% to 24.94%. Burton and DeVane (1953) suggested that GCV beside heritability estimates offer a better understanding of the amount of genetic gain through phenotypic selection. High heritability is a major advantage for efficient selection in a population improvement program. Furthermore, environment may also interact with the genotypic makeup to influence heritability (Raiz, 2003).

Heritability values greater than 80% are very high, values 60-79% are fairly high, values from 40 to 59% are intermediate and values less than 40% are low (Singh, 2015). According to this classification, heritability of the main stem diameter was low (38.83%). The heritability of GLY, GLA, LI, LIQ, RU and ROS was very high i.e. more than 97%, TPC was classified as high i.e. 70%, and the remaining traits were intermediate. In the present study, genetic advance revealed a wider gain ranging from 1.28% to 2.05% (Table 3). Johnson et al. (1955) categorized genetic advance as a percentage of the mean; values 0-10% are considered low, 10-20% are intermediate

and 20% and above are high. Based on this classification, genetic advance of the mean in studied traits exhibited high, intermediate and low heritability values ranging from 0.32% to 419.91% as shown in Table 3. The highest genetic advance was observed in RS (419.19%), LI (118.88%), GLA (72.51%) and RU (54.98%) indicating that these critical characteristics can efficiently be selected and inherited in breeding programs. Results from the combination of heritability and genetic advance revealed that the variation is imputable to a large degree of additive effect, so that selection procedure can be practiced for improvement of desired traits (Chauhan and Nanda, 1983). A very high variability was observed in terms of heritability and genetic advance among traits. The modifier, depending on the purpose and the degree of heritability and genetic advance, facilitates choosing the desired attribute for breeding program.

Assessment of correlation among studied traits

Phenotypic and genotypic correlation coefficients for all pairs of 22 studied traits are shown in Table 4. In most traits, the genotypic correlations showed the same direction as the phenotypic correlations. Significant correlations between GLY and LIQ, GLA with LI, LI with LIQ, TPC with TFC, are interesting to emphasis. However, correlations between leaf length with number of axillary branches, main stem diameter compared with shoot dry weight, GLY with RU, TFC with RU, RU with RS, are in the opposite direction. The highest negative genotypic correlation coefficient was observed between the root yield with plant height, plant width, main stem diameter, leaflet length, number of axillary branches, shoot fresh weight, shoot dry weight, shoot yield, root fresh weight and root dry weight. Also, GLY showed negative genotypic correlation

248 with TPC and RU. The negative genotypic correlation coefficient was observed between GLA
249 with plant height, plant width, main stem diameter, leaf length, shoot fresh weight and LI.
250 Considering crucial traits for plant improvement and breeding purposes, root yield showed a
251 positive correlation with vegetative traits including plant height, plant width, main stem
252 diameter, leaflet length and number of lateral branches. Therefore, selecting plants displaying
253 mentioned vegetative traits can lead to the selection of plants producing higher root yield. On the
254 other hand, the morphological traits did not show a positive correlation with the amount of GLY.
255 Therefore, morphological traits cannot be a good indicator for identifying plants with high GLY
256 content. However, the amount of GLA showed a positive correlation with some vegetative and
257 phytochemical traits such as plant height, plant width, main stem diameter, leaf length, No. of
258 leaflets, antioxidant activity and LI. Likewise, the amount of LIQ showed a positive correlation
259 with majority of morphological traits (Table 4). Thus, selecting the populations or individuals
260 based on desirable vegetative traits can result in improvement of licorice population having high
261 contents of GLA and LIQ.
262 Since the metabolites of licorice plants are mainly formed in the underground organs, therefore
263 any factor that increases root yield can be an effective factor in producing highest possible
264 metabolites. Therefore, traits that are positively correlated with root growth and root yield can be
265 chosen in breeding programs (Hosseini, et al., 2014).
266 Variance components analysis showed that PCV was higher than GCV for all the examined
267 traits. High genetic correlation indicates that the relationship between these traits is under genetic
268 control and genetic variance playing a greater role in controlling these traits. The lower value of
269 phenotypic correlation could be due to the modification of environmental effects on the
270 characters at the genetic level (Sabit et al., 2017; Kumar et al., 2005).

271 Table 4

272 In order to plan an efficient breeding program, the emphasis has been placed on correlations
273 between traits. If the trait has a low heritability, other correlated traits with high heritability value
274 can be chosen as indirect criteria in the selection. Genetic correlation is due to pleiotropic effects
275 of the genes or linkage of the genes or possibly both. The most important cause of genetic
276 correlation is pleiotropic which is due to the multiplicity of the effects of a gene. Pleiotropic or
277 linkage may exist between two desirable characters or between a desirable and an undesirable
278 attribute. In the former case, it causes genetic progress and in the latter case, it prevents genetic
279 progress. The existence of these types of correlations has also been reported by other researchers
280 (Sabit et al., 2017; Vollman et al., 2000).

281 Cluster analysis grouped the 22 population of *G. glabra* L. into four distinct classes (Table 5).
282 Cluster 1 was the largest group, constituting 36.36% of the total genotypes. Clusters 2 and 3, and
283 4 constituted 13.64%, 18.81% and 31.81% of the total populations, respectively. Although
284 several genotypes of nearby regions are divided in the same clusters, some other genotypes of the
285 same area were not grouped into a single cluster. This indicates that proximity of populations in
286 natural habitats does not necessarily reflect genotype similarity. Therefore, populations that
287 sampled from nearby areas may have various genetic make-up and other factors besides
288 geographical diversity can be responsible for genetic diversity. The results are in a good
289 agreement with Khatun et al. (2010), Mahbubur Rahman et al., (2017) and Rabbani et al. (2012).

290 Table 5

291 Mean comparison of measured traits (Table 6) within the 4 clusters showed that cluster 1
292 populations were superior to shoot fresh weight, shoot dry weight, shoot yield, LIQ and RU. The
293 highest plant height, plant width, main stem diameter, leaf length, leaf width, number of leaflets,

leaflet length, leaflet width, number of axillary branches, root fresh weight, root dry weight, GLA, LI, antioxidant activity and RS traits was observed in cluster 2 populations. Highest root yield was observed in cluster 1 and 2 populations. The cluster 3 populations had the highest values for TPC and TFC. The cluster 4 populations were superior in GLY content. Highest coefficient of variation (CV) was observed for GLA and LI (about 96 and 68% respectively) indicating that populations are highly variable in these characters. Traits that have a high coefficient of variation have a wider range of attribute quantities, providing wider range of selection for that trait in breeding programs.

Table 6

In the previous study (Esmaeili et al., 2019), where analysis performed on populations collected from natural habitats, very high variability on bioactive compounds was reported. Comparing CV% of the two studies reveals that variability is reduced due to introduction of the populations into cultivation. Since the populations are cultivated in the field in current study, the environmental conditions were less variable and difference between populations was identified to be more related to genotypic factors (Table 3).

The superior populations of licorice in this study displayed high contents of bioactive compounds (Table 6) including 9.66% GLY, 0.28% GLA, 0.17% LI and 0.67% LIQ. High variability of these phytochemicals were reported in previous studied (Hayashi et al., 1998, 2003; Esmaeili et al., 2019). Variable contents of GLY in Italy (1.6–3%), Spain (0.7–4.4%), Uzbekistan (4.76%–6.13%) and Iran (1.34– 7.40) and variable contents of GLA in Italy (0.07–0.27%), Spain (0.21–0.80%) and Uzbekistan (0.08–0.35%) and Iran (trace– 1.28%) have been reported.

316 The pairwise generalized squared distance among the four clusters is presented in Table 7. The
317 genetic divergence within all possible pairs of clusters were highly significant (P<0.01). The
318 highest value of inter-cluster D^2 (87.17) was obtained between clusters 2 and 3 and the lowest
319 value was found between clusters 1 and 4 (12.55). The lower values recorded from intra-cluster
320 and higher inter-cluster values indicate that the studied populations were homogeneous between
321 the clusters and heterogeneous within the cluster (Rabbani et al., 2012; Mahbubur Rahman et al.,
322 2017). Therefore, the classified populations in clusters 2 and 3 are predicted to offer high
323 heterosis after hybridization and will possibly display high variability to support breeding
324 programs.

325 Table 7

326 **Conclusions**

327

328 There is a great variation among the studied populations of licorice on the basis of different
329 characteristics indicating high genetic potential among different populations. Results of variance
330 component analysis showed that genetic factors are generally the sources of variability in these
331 traits and environmental factors were less influential. Heritability of GLA, GLY, LI, LIQ, RU
332 and RA determined to be very high. Therefore, these critical characteristics can efficiently be
333 selected and inherited in breeding programs.

334

335 **Author's contribution** GE: Investigation, Writing original draft, Validation, Formal analysis.
336 MS: Writing-reviewing & editing, Resources, Conceptualization, Supervision, Project
337 administration. AK: Conceptualization, Editing, Resources, Supervision. JH: Methodology,
338 Editing, Resources. All authors read and approved the submitted version.

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Data availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Declaration

Conflict of interest The authors declare that they have no conflicts of interest.

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Figures

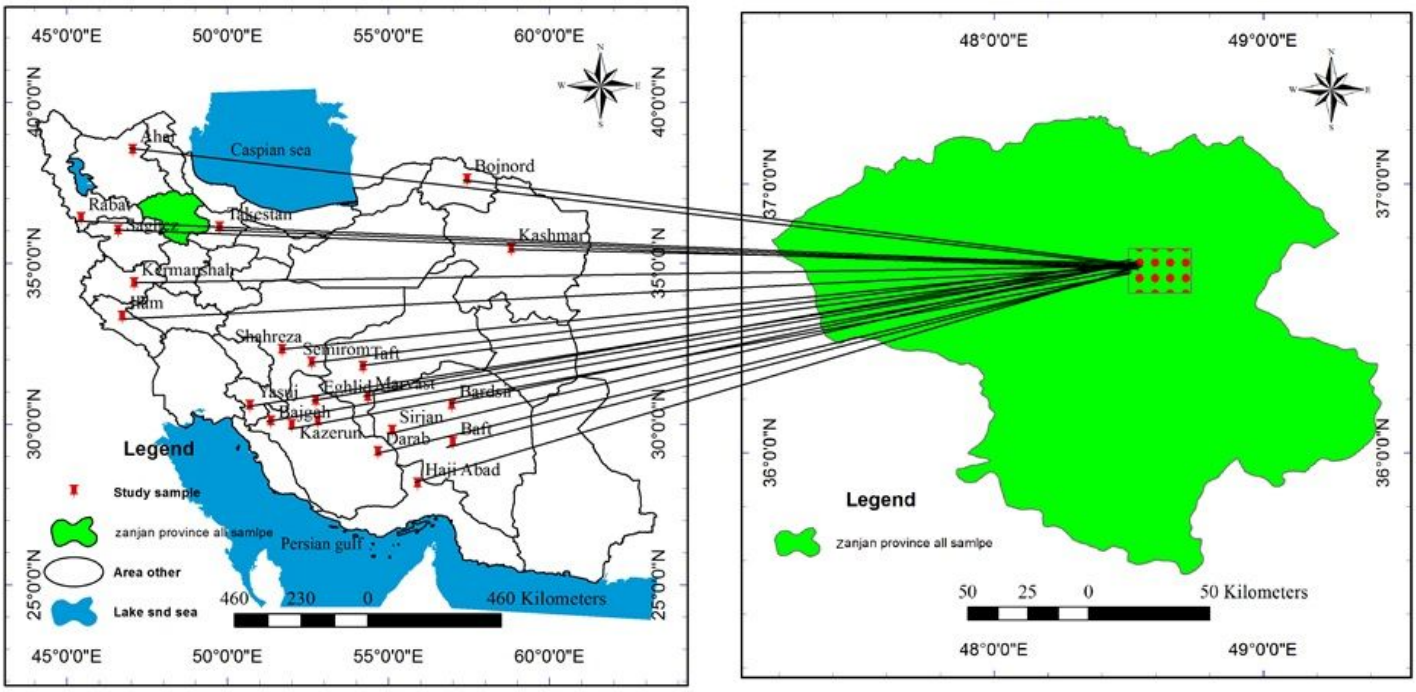

Figure 1

The locations where the populations of G. glabra collected, and location of cultivation in Zanjan province.

Figure 2

Cultivated G. glabra populations in research farm located in University of Zanjan.