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Research Article

Morphological evaluation of regenerated plantlets of the medicinal plant Jujube (*Ziziphus jujuba* Mill.) with emphasis on somaclonal variation

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Abstract

Jujube (*Ziziphus jujuba* Mill.) is a medicinally and agriculturally significant fruit tree with a long history of cultivation. *In vitro* propagation techniques offer an alternative to traditional vegetative propagation, often leading to genetic and phenotypic changes known as somaclonal variation. This study aimed to investigate the impact of long-term *in vitro* subculturing on the morphological traits of regenerated Jujube plantlets. Single-node explants from three cultivars, Taghab, Siojan and Shahzileh, were cultured on Murashige and Skoog (1962) (MS) medium supplemented with different concentrations of benzylaminopurine (BA) and indole-3-butyric acid (IBA). The three Jujube cultivars showed the most marked increase in the mean number of shoots when grown on MS medium with amounts of BA (2 mgL^{-1}) and IBA (0.2 mgL^{-1}). With regards to morphological traits, the Shahzileh cultivar demonstrated the greatest branching among all cultivars, suggesting superior regenerative capabilities. Several morphological features were evaluated under five subcultures of the Shahzileh cultivar. The Shahzileh cultivar showed the highest branching in the third subculture, and longer shoot length was also recorded at the fifth subculture. With each subsequent subculture, there was a gradual decrease in leaf and node number per branch, suggesting that somaclonal variation may be occurring in these cultivars through regeneration in plantlets. In this study, 10-year-old Shahzileh Jujube trees were used for explants. Only one tree was used as a source of explants, so variations observed in the regenerated plantlets were likely due to several factors, particularly the explant source. The single nodes from the Shahzileh Jujube cultivar were used as explants, which contain meristematic tissues. The use of meristematic tissues such as axillary buds exhibits the chances of somaclonal variation. Plantlets regenerated from nodes of Jujube showed optimal results with 0.2 mgL^{-1} IBA and 2 mgL^{-1} BA. The combination of auxins and cytokinins has been shown to play a key role in causing somaclonal variation. The volatile chemical compounds present in fruit extracts of three jujube cultivars were identified using Gas Chromatography-Mass Spectrometry (GC-MS). This indicates that the chemical composition of jujube fruits varies significantly among different cultivars. This study investigates the correlation between phenotypic variation induced by *in vitro* plantlet regeneration across different cultivars and the nutritional quality as well as bioactive compound profiles of their fruits. The outcomes of this research provide valuable insights for cultivar improvement, genetic resource conservation, and the enhancement of production strategies for economically and medicinally important plants such as jujube (*Ziziphus jujuba* Mill.).

Keywords: Fruit, *In vitro* culture, GC-MS analysis, Jujube, Regeneration, Somaclonal variation

Introduction

Jujube (*Ziziphus jujuba* Mill.) is the most renowned species within the Rhamnaceae family, which includes a diverse group of plants. It is among the oldest cultivated fruit trees, with a recorded history of more than 3,000 years in Asia, primarily valued for its medicinal

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properties. The *Ziziphus* genus comprises approximately 40 species of thorny shrubs and small trees found in tropical and subtropical regions worldwide. Jujube is a highly valuable fruit tree in Asia and is currently cultivated in at least 48 countries across all continents, except Antarctica. Its significance continues to grow, particularly in arid and semi-arid marginal lands (Wang *et al.*, 2009). Research evaluating jujube as a potential superfruit has led scholars to propose its classification as such (Liu *et al.*, 2020).

In Iran, jujube is distributed across most provinces, with particularly high concentrations in Khorasan, Isfahan, Golestan, Mazandaran, Fars, Yazd, Hamedan, and Qazvin. It thrives in mountainous regions at elevations of up to 2,000 meters. The greatest abundance is found in the Khorasan provinces, including Razavi, Northern, and Southern, while in Khorasan Province the highest number of cultivars is observed in Kashmar. With improvements in cultivation practices, efforts have also been made to promote the growth of other species (Ghows, 2008; Safarnejad, 2015).

Jujube demonstrates remarkable resistance to soil salinity and drought, making it an excellent resource for soil conservation in Iran. Recently, in South Khorasan Province, jujube cultivation has expanded significantly, with a recorded 98% increase in cultivated area, establishing Iran as the leading global producer.

Jujube is a versatile medicinal plant valued for its nutrient-rich fruit, traditional therapeutic uses, and role in food, beverage, and honey production. Historically, its fruit, seeds, leaves, roots, and gum have been used to treat various ailments, reflecting its long-standing significance in traditional medicine.

The in vitro technology of plant cultivation for medicinal purposes serves two primary objectives: (1) to preserve the genetic integrity of valuable genotypes and (2) to develop new somclones with enhanced levels of bioactive constituents (Sidhu, 2011; Efferth, 2019; Sagharyan *et al.*, 2020).

A critical challenge in achieving these objectives is the proper genetic and epigenetic monitoring of regenerated plants. This ensures that propagated plants retain the same genetic constitution as the mother plant while effectively exploiting somaclonal variation that enhances adaptive ability without additional costs (Duta-Cornescu *et al.*, 2023).

The ubiquitous jujube (*Z. jujuba* Mill.) is well-suited for rapid propagation through plant tissue culture, a widely used asexual reproduction technique for perennial trees. Tissue culture, also referred to as *in vitro* culture, enables large-scale propagation of jujube plants using various explants. Several tissue culture protocols have been established for different Chinese jujube cultivars, utilizing plant parts such as leaves (Qing Rong *et al.*, 2011; ChunHua *et al.*, 2012), embryos (Wang *et al.*, 2009), cotyledons (Wang *et al.*, 2011), and nodal segments (Goyal *et al.*, 2006).

Numerous studies have investigated the key factors influencing *in vitro* culture in Chinese jujube using various explant sources (Cao and Du, 2009; Dai *et al.*, 2009).

Research on tissue culture-induced variation has primarily focused on traits such as morphology, disease resistance, and maturity time (Hammerschlag, 1992). The extent of these variations is influenced by both the conditions of tissue culture and the genetic background of the species and cultivars involved (Karp, 1982). Somaclonal variation was first documented in sugarcane plants regenerated from cell cultures in 1969 by researchers at the Hawaiian Sugar Planters' Association Experiment Station. Their findings revealed widespread variation in chromosome numbers, morphology, and enzymatic activity among regenerated plants (Sarmah *et al.*, 2017). Some of these plants exhibited increased tillering, slower growth rates, and more upright growth habits. Ahloowalia (1983) further reported extensive morphological variations, such as albino phenotypes, leaf shape distortions, and differences in vigor, as well as chromosomal variations, including polyploidy, aneuploidy, and structural rearrangements in ryegrass plants regenerated via callus culture (Sarmah *et al.*, 2017).

Genetic diversity can be evaluated using various methods, with morphological traits being among the most significant for agronomic assessment and taxonomic classification (Jannatabadi *et al.*, 2013). Morphological characterization remains a simple, cost-effective, and widely used approach for assessing genetic diversity and assisting plant breeders in selecting superior breeding materials. While extensive literature has reported morphological and genetic changes in many plant species, primarily herbaceous plants, research on perennial fruit crops remains limited, highlighting the need for further studies on somaclonal variation in these species (Rani and Raina, 2000). To ensure genetic integrity in perennial fruit species, various molecular, physiological, and morphological markers are now available for validation (Leva *et al.*, 2014). Assessing genetic diversity among different jujube cultivars is crucial for the successful conservation and utilization of this valuable plant species. In Iran, elite jujube cultivars are rare, and the conservation and multiplication of known varieties are essential.

The South Khorasan Agriculture Research Center has identified three locally known jujube cultivars: Taghab, Siojan, and Shahzileh (Sour Jujube). The present study aimed to establish an efficient *in vitro* propagation system for the large-scale multiplication of these valuable jujube cultivars. Additionally, somaclonal variation in *Z. jujuba* (Shahzileh cultivar) was evaluated based on morphological characteristics across multiple subcultures.

The extraction of fruit extracts from jujube (*Ziziphus jujuba*), recognized as a medicinal plant, from the studied cultivars constitutes another important component of this research. Gas Chromatography-Mass

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Spectrometry (GC-MS) analyses were conducted to identify and compare the levels of volatile biochemical compounds in the fruit, including fatty acids, esters, alcohols, aldehydes, ketones, carbohydrates, and terpenoids, across the different cultivars under investigation.

Materials and methods

In vitro Propagation of Jujube

Plant Specimens and Sterilization Protocols: Nodal segments of *Ziziphus jujuba* Mill. were collected from fully mature trees of three cultivars grown at the South Khorasan Agriculture Research Center, located in South Khorasan Province, Iran. The cultivars, locally known as Taghab (Longitude: 58º 55' E, Latitude: 32º 50' N), Siojan (Longitude: 58º 57' E, Latitude: 32º 52' N), and Shahzileh (Sour Jujube) (Longitude: 58º 53' E, Latitude: 32º 50' N), exhibit distinct morphological characteristics, as shown in Figure 1. For *in vitro* propagation, the explants used were nodal segments (Nabavi *et al.*, 2019).

The explants were initially washed for one hour in running tap water, followed by a five-minute exposure to a detergent solution. Surface sterilization was performed under aseptic conditions using an antioxidant solution containing 1 g of ascorbic acid and 5 g of citric acid, dissolved in 200 ml of sterilized distilled water. After this step, the explants were immersed in commercially available bleach solutions of 1% sodium hypochlorite at varying concentrations (5%, 10%, 15%, and 20%) for durations of 5, 10, and 15 minutes. Additionally, a disinfection procedure involving Dettol (2 ml/200 ml of sterilized distilled water) was applied for one minute. After sterilization, the explants were thoroughly rinsed with six consecutive washes of sterile distilled water (Farahani and Majd, 2012). The sterilized explants were then sliced into single-node segments and cultured vertically on sterile nutrient medium.

Culture medium and growth conditions: The explants were placed on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962), supplemented with 3% (w/v) sucrose and solidified with 5 g L^{-1} agar (Duchefa, Haarlem, Netherlands). For inducing the hormonal state that promotes bud sprouting and multiple shoot induction, various concentrations of benzyl adenine (BA) and indole-3-butyric acid (IBA) (Sigma Cell Culture, St. Louis, USA; purity ≥ 90%) were added to the medium. The pH of the medium was adjusted to 5.7–5.8 prior to autoclaving at 121°C and 1.2 kgcm-² pressure for 15 min. Cultures were incubated in a growth chamber at 26±2°C with a 16-h photoperiod of cool white fluorescent light (Nabavi *et al.*, 2020).

Establishment of culture and shoot multiplication: To induce shoot formation and growth, various concentrations and combinations of BA (0.5, 1, and 2 mg L^{-1}) and IBA (0.05, 0.1, and 0.2 mg L^{-1}) were added to the MS medium (refer to Table 1). A total of seven treatments were tested. Morphological characteristics, including the number of shoots, nodes, and branches, as well as shoot length (cm), were measured after five weeks of culture.

The morphological characteristics of the plantlets regenerated from the three cultivars (Taghab, Siojan, and Shahzileh) were compared across the seven treatments. The effect of tissue culture on plantlet growth was evaluated through multiple subcultures. Since morphological characteristics are key indicators of genetic change, agronomic performance, and taxonomic classification, they were used to assess somaclonal variation between the regenerated plantlets. The Shahzileh cultivar was selected for further investigation due to its higher pulp-to-seed ratio compared to the other cultivars.

Among the tested treatments, the seventh treatment (MS medium supplemented with 2 mg L^{-1} BA and 0.2 mg L^{-1} IBA) was chosen for an in-depth study of somaclonal variation in morphological characteristics. Various parameters, including shoot elongation (cm), number of nodes, leaves, and shoots, branch length (cm), and the number of nodes and leaves per branch, were recorded over five subcultures.

Biochemical analyses: For biochemical studies, jujube fruits from the cultivars Taghab, Siojan, and Shahzileh were used. The extraction of jujube fruit extract was performed using a Soxhlet apparatus following the method of Elaloui *et al.* (2014) and Rassem *et al.* (2016).

Biochemical analyses were conducted to identify the volatile chemical compounds of jujube fruit extract, including esters, alcohols, aldehydes, ketones, terpenoids, organic acids, fatty acids, etc., using a GC-MS device with 3 repetitions (Wang, 2017) .

The Agilent 6890 gas chromatograph coupled with a 5973 MSD mass spectrometer (manufactured in the USA) was used for the analyses. The jujube extract was injected into the GC-MS system in splitless mode. The column dimensions were HP 30 m × 0.25 μm × 0.25 μm, and the stationary phase was a non-polar poly dimethyl siloxane (PDMS) column. Helium gas was used as the carrier gas (mobile phase) with a flow rate of 1 milliliter per minute. The oven temperature program started at 40°C and was increased to 280°C with a rate of 8°C/min over a 50-minute period. The injector temperature was 210°C, and the injection volume was 3 microliters (EL-Hefny *et al.*, 2018) .

Interpretation of the GC-MS spectra was performed using the NIST (National Institute of Standards and Technology) database to predict the names of the identified compounds. The spectrum of the unknown component was compared with the known components stored in the NIST library to identify the substance. The purity factor (p) in the results indicates the probability percentage of the correctness of the compounds from the library, with values above 80% being considered acceptable (Wang, 2017) .

The quantity of the identified compounds was calculated based on the peak area percentage from the GC-MS analysis .

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Figure 1. Forms of fruits of the three Jujube cultivars: a) Taghab, b) Siojan, and c) Shahzileh.

Table 1. Effect of different concentrations of BA and IBA (Treatments) on regeneration plantlets

Treatment (No.)	1	2	3	4	5	6	7
BA (mgL^{-1})	0.5	1	1	1	2	2	2
IBA (mgL^{-1})	0.05	0.05	0.1	0.2	0.05	0.1	0.2

Determination of peak area: The peak area of the graphs obtained from the device was used to determine the percentage of each compound (quantification of the compound amount) identified by the device. The relative percentage (peak area %) of each compound was calculated by comparing the average peak value of that compound to the total peak area. The lower the mobile phase flow rate, the greater the separation between the peaks, leading to better resolution.

Statistical analysis: The results from the *in vitro* propagation trials were subjected to statistical analysis using analysis of variance (ANOVA), Duncan's multiple range test (Duncan, 1955), and the Least Significant Difference (LSD) test. Each experimental treatment was repeated in at least 15 cultures. The means of treatments were tested for significance at a 5% confidence level ($P \leq 0.05$). Means with the same letter were considered not significantly different.

Results and discussion

In vitro propagation of *Ziziphus jujuba*

Multiplicative and branching processes: The data collected after a 5-week culture period indicated that shoot induction was successfully achieved in all media formulations used, with no statistical differences observed between the different MS media formulations that contained plant growth regulators (seven treatments). A maximum shoot induction rate of 90% was observed in the Taghab and Siojan cultivars, while the Shahzileh cultivar demonstrated a 100% shoot induction rate when cultured on MS medium containing IBA, either with or without BA (Figure 2). Table 2 shows that explants grown on MS medium supplemented with higher concentrations of BA (2 mg L^{-1}) and IBA (0.2 mg L^{-1}) exhibited the most significant enhancement in the mean number of shoots across the three Jujube cultivars examined.

Shoot multiplication rates were significantly influenced by the concentration of BA. The Shahzileh cultivar yielded an average of 9 shoots per explant, whereas the Taghab and Siojan cultivars produced only 3 shoots each when cultured under 2 mg L^{-1} BA and 0.2 mg L^{-1} IBA (Figure 3). A reduction in the concentration of BA led to a considerable decrease in the mean number of shoots per explant. These results align with findings by Goyal *et al.* (2006), who observed an improved shoot regeneration capacity of *Z. jujuba* when cultured on MS medium supplemented with varying levels of BA, with 1 mg L^{-1} BA being the most effective for shoot growth. Additionally, previous studies on Chinese jujube, such as Wang *et al.* (2011), reported successful shoot regeneration and proliferation on MS medium supplemented with 1 mg L^{-1} BA and 0.5 mg L^{-1} IBA, resulting in a multiplication coefficient of 3.2. This is lower compared to the results of the current study.

Interestingly, lower concentrations of cytokinin, such as BA, have been shown to stimulate the growth of lateral buds more effectively than higher concentrations, as noted by AI-Sulaiman and Barakat (2010). reported

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Table 2. Effect of MS medium and hormones (BA and IBA) on the *in vitro* shoot regeneration of the three Jujube cultivars (T: Taghab Cultivar, S: Siojan Cultivar, SH: Shahzileh Cultivar). The survival and growth percentages reached 100% on all tested treatments for the three cultivars.

NO medium culture	BAP (mgL^{-1})	IBA (mgL^{-1})	Mean of number shoot			Mean of length shoot (cm)			Mean of number node			Mean of number branch		
			TC	SC	SHC	TC	SC	SHC	TC	SC	SHC	TC	SC	SHC
1	0.5	0.05	1.0	2.0	2.0	2.70	1.63	4.22	2.00	1.33	3.60	1.00	.33	1.60
2	1	0.05	2.00	3.00	5.00	3.16	3.23	4.56	3.3	3.33	4.40	.33	.66	1.60
3	1	0.1	3.00	3.00	4.00	4.00	4.43	5.46	3.00	3.33	4.40	.66	.66	1.20
4	1	0.2	1.00	2.00	3.00	3.30	4.50	5.44	2.00	3.66	3.60	1.00	.66	1.20
5	2	0.05	3.00	3.00	9.00	3.80	3.70	5.12	4.33	4.00	6.00	1.66	1.00	1.80
6	2	0.1	3.00	2.00	6.00	3.66	2.73	5.62	3.33	2.33	4.80	.66	1.00	2.00
7	2	0.2	2.00	2.00	4.40	4.70	4.13	5.74	3.00	3.00	4.40	1.66	1.33	2.20

Figure 2. *In vitro* establishment of stem node sections of the three Jujube cultivars: a) Taghab, b) Siojan and c) Shahzileh.

Figure 3. Shoot multiplication of the three Jujube cultivars: Taghab, Siojan, and Shahzileh

Contrastingly, Quiroz-Figueroa and Zeel (2001) reported that relatively low levels of BA in culture medium enhanced proliferation efficiency in *Z. spina-christi* tissue cultures. The findings of this study, however, are consistent with other research, including Soliman and El-Moneim Hegazi (2013), who observed successful shoot regeneration in various jujube cultivars (Comethry, Balahy, and Balady) when cultured on MS medium supplemented with 2 mg L^{-1} BA and 0.05 mg L^{-1} NAA, achieving a multiplication coefficient of 1.2.

Regarding morphological characteristics, the Shahzileh cultivar exhibited the highest stem length, number of nodes, and branch development, indicating its superior regenerative capacity compared to the other cultivars. This greater regenerative capacity was observed when compared under a range of basal media conditions (MS medium) at various concentrations of BA and IBA. In contrast, the Taghab cultivar exhibited the lowest regenerative capacity under the same culture media conditions.

Shoot length, shoot number, and node character: The nodal segments of the three Jujube cultivars (Taghab, Siojan, and Shahzileh) survived and formed axillary shoots across all the media used, including MS basal medium supplemented with varying concentrations of BA and IBA. This finding supports previous research by Soliman and El-Moneim Hegazi (2013), who recommended the use of MS medium for the micropropagation of *Z. jujuba* via nodal explants.

In terms of the average number of shoots and nodes, data ranged from 2 to 9 shoots and 1.33 to 6.00 nodes. No significant differences were detected among the media for the three cultivars. However, shoot length was significantly influenced by the combination and concentration of hormones. The longest average shoot length was observed on MS medium with 0.2 mg L^{-1}

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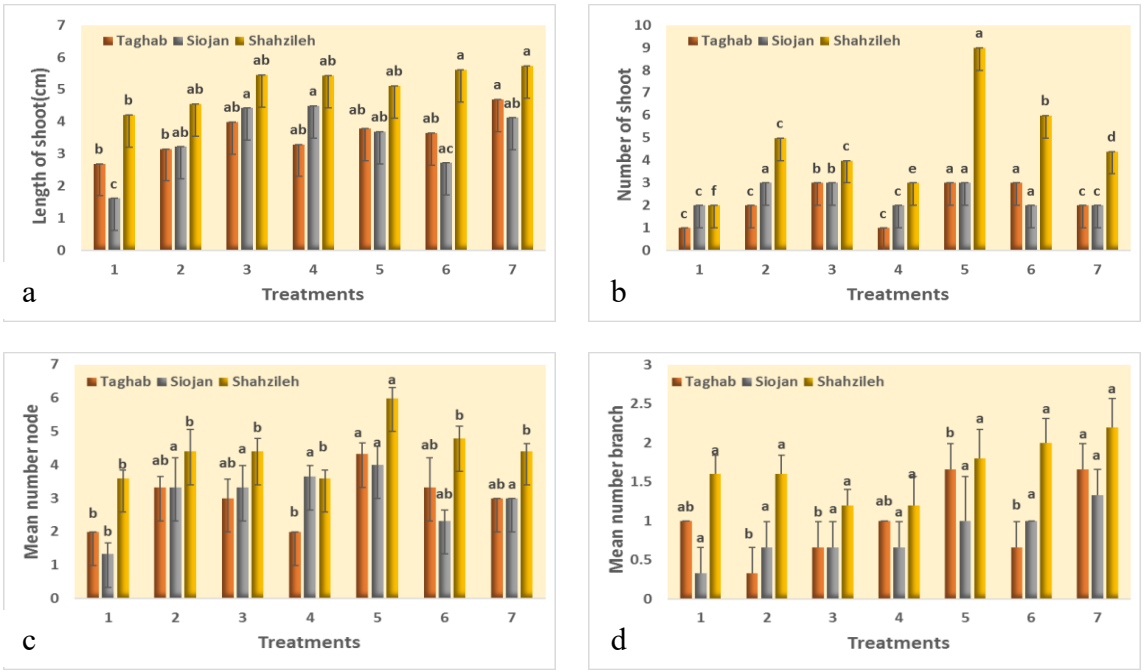

Figure 4. Effect basal medium (MS) and hormonal different concentrations on a) mean number of shoot, b) length of shoot, (cm) c) number node and d) number of branch in Jujube (Taghab, Siojan and Shahzileh cultivars)

IBA and 2 mg L^{-1} BA. This combination resulted in a mean shoot length of 4.70 cm for Taghab, 4.13 cm for Siojan, and 5.74 cm for Shahzileh, with a sequential reduction in shoot length when the concentration of BA was reduced to 0.5 mg L^{-1} (Figure 4a, b).

The highest mean number of nodes was achieved on MS medium enriched with 0.05 mg L^{-1} IBA and 2 mg L^{-1} BA, with 4.33, 4.00, and 6.00 nodes for Taghab, Siojan, and Shahzileh, respectively. Among the IBA concentrations tested, 0.2 mg L^{-1} IBA was optimal for shoot length, whereas significant variations were observed for other growth characteristics. Regarding the number of branches, MS medium enriched with 0.2 mg L^{-1} IBA and 2 mg L^{-1} BA resulted in the highest mean number of branches: 1.66 for Taghab, 1.33 for Siojan, and 2.2 for Shahzileh (Figure 4c, d).

MS medium supplemented with 0.2 mg L^{-1} IBA and 2 mg L^{-1} BA was the best for nodal segment establishment across all three Jujube cultivars. However, for the Shahzileh cultivar, this medium performed best across all parameters. The combination of hormones, their concentrations, and the basal medium composition are critical factors influencing adventitious shoot induction, as previously suggested by Feng *et al*. (2010).

In most studies of the *Ziziphus* genus, a mix of BA and IBA is preferred for *In vitro* shoot induction. Khazaei *et al.* (2015) found the highest shoot inductions (4.25 shoots/explant) and longest shoots (5.12 cm) on a medium containing 0.2 mg L^{-1} IBA and 2 mg L^{-1} BA. Furthermore, they observed that increasing the BA to IBA ratio beyond 10:1 led to a decrease in shoot production. The concentration and ratio of auxins and cytokinins play a crucial role in shoot proliferation, as evidenced by the decline in shoot production when the BA to IBA ratio exceeded 10:1. The relationship between the cytokinin/auxin ratio and adventitious shoot proliferation in *In vitro* organogenesis, both indirect and direct, has been well-documented (Ibrahim *et al.*, 2012). This study also examined the influence of morphological traits such as shoot length, node number, leaves, branches, and branch length on the three cultivars (Taghab, Siojan, and Shahzileh). The results from the orthogonal test and variance analysis indicated that the frequency of plantlet regeneration was greatly affected by hormone combinations (Table 3). Specifically, shoot number in Taghab and Shahzileh cultivars and shoot length in Siojan had the highest values among the morphological traits. Variations in mean square for some morphological traits in Taghab, Siojan, and Shahzileh cultivars were significant at the 0.05 level of significance. However, the mean square of shoot length was non-significant in both Shahzileh and Taghab cultivars, and the number of branches was non-significant in the Shahzileh cultivar.

Morphological characteristics under subcultures: Several morphological features, including shoot length (cm), number of nodes and leaves, number of branches, branch length (cm), and number of nodes and leaves per branch, were evaluated under five subcultures of the Shahzileh cultivar.

Shoot length: The effects of prolonged subculturing on the morphological characteristics of Jujube plantlets are shown in Figure 5. Explant growth and shoot regeneration were induced by the cytokinin benzyl adenine (BA). The Shahzileh cultivar showed an increase in shoot length through the subcultures, with the highest shoot length recorded in the third subculture at 9.3 cm. However, longer shoot lengths were also recorded at the end of the subcultures. Statistical

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Table 3. Results of analysis of variance to regeneration plantlets frequency in different concentrations and combinations of plant growth regulators

Variable (Taghab Cultivar)	df	Mean of Square			
		Number of stem	Length of stem (cm)	Number of node	Number of branch
Treatments	6	2.429*	1.250ns	2.00*	0.778*
Error	20	.000	0.558	0.571	0.238
Variable (Siojan cultivar)					
Treatments	6	0.857*	3.227*	2.00*	0.778*
Error	20	.000	0.720	0.810	0.381
Variable (Shahzileh cultivar)					
Treatments	6	25.829*	1.634ns	3.314*	0.714ns
Error	20	0.114	0.765	0.743	0.486

Data was recorded after 30 days of culture. Mean separation was determined using Duncan's multiple range test. *: significant at P< 0.05; ns: non-significant

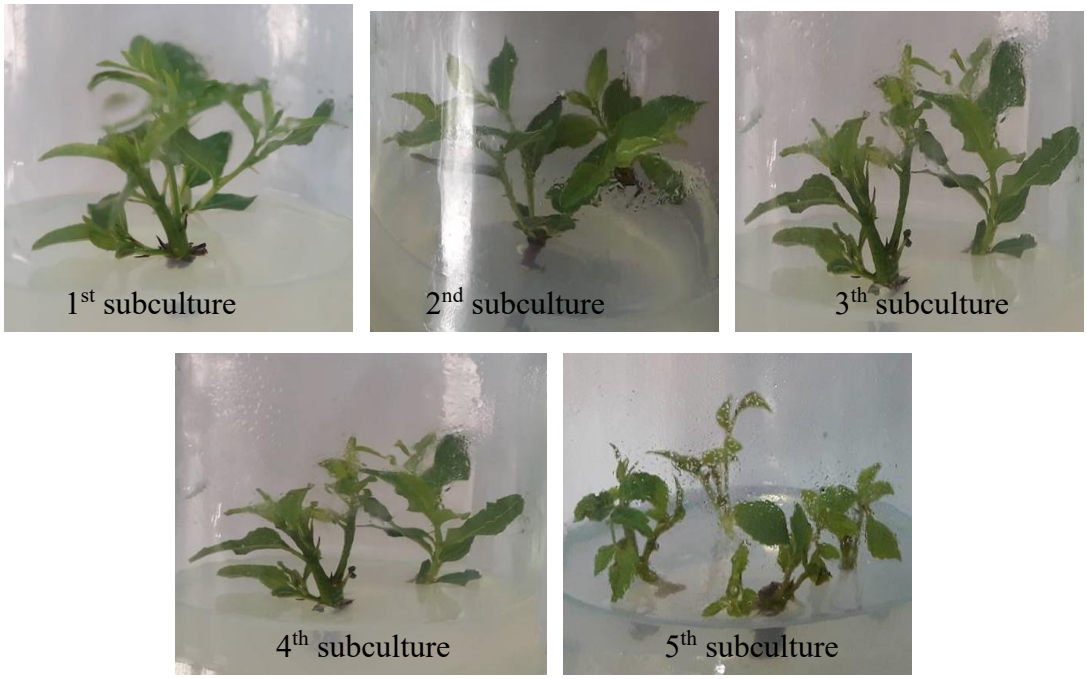

Figure 5. Shoot elongation and multiplication of the Jujube Shahzileh cultivar during five subcultures

analysis confirmed a significant increase in shoot length during the subcultures, as indicated by the Least Significant Difference (LSD) test (Table 4).

Number of nodes, leaves, and branches: Although the number of nodes, leaves, and branches increased during subculturing, statistical analysis indicated that the number of nodes, but not the number of leaves and branches, showed a significant difference in the Shahzileh cultivar. The highest increases in the number of nodes, leaves, and branches (7.26, 2.13, and 2.13, respectively) were observed in the third subculture (Figures 6a, b, c, and d).

Branch length, number of nodes, and number of leaves per branch: Statistical analysis revealed remarkable differences in the morphological changes of branching (Table 5). The Shahzileh cultivar exhibited the most favorable response regarding branch length, with an average of 3.75 cm in the first subculture (Figure 6e). The maximum number of nodes per branch (3.61) and the maximum number of leaves per branch (4.33) were observed in the second subculture. Interestingly, the first and last subcultures were the most ideal for studying variations in the number of nodes and leaves per branch (Figure 6f).

With each subsequent subculture, there was a gradual decrease in leaf and node number per branch, suggesting that somaclonal variation may be occurring in these cultivars through auxiliary budding cultures. The measurements of the morphological traits and the statistical analysis results, as presented in Table 5, indicated that the third subculture exhibited the most striking changes with increased shoot length, nodes, and branches (Figures 6a-d and Table 4). The initial subculture led to the elongation of branches, whereas the second subculture promoted the multiplication of nodes and leaves on each branch (Figures 6e-g and Table 4).

The most significant morphological alterations in the Shahzileh cultivar were observed during the fifth subculture, including reduced shoot length, branch

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Table 4. Representative Least Significant Difference Test (LSD) for morphological characters between subcultures. The mean differences are significant at the 0.05 level. (T1: the first subculture, T2: the second subculture, T3: the third subculture, T4: the fourth subculture, T5: the fifth subculture)

Morphological characteristic		Parameters	
Length of shoot (I)	Length of shoot (J)	Mean Difference (I-J)	Significant
T 1	T3	-1.00000*	.006
	T4	.86667*	.016
	T5	1.93333*	.000
T2	T4	1.53333*	.000
	T5	2.60000*	.000
T3	T1	1.00000*	.006
	T4	1.86667*	.000
	T5	2.93333*	.000
T4	T1	-.86667*	.016
	T2	-1.53333*	.000
	T3	-1.86667*	.000
	T5	1.06667*	.003
T5	T1	-1.93333*	.000
	T2	-2.60000*	.000
	T3	-2.93333*	.000
	T4	-1.06667*	.003
Number of node (I)	Number of node (J)	Mean Difference (I-J)	Significant
T1	T3	-1.00000*	.035
	T4	1.26667*	.008
	T5	2.06667*	.000
T2	T4	1.66667*	.001
	T5	2.46667*	.000
T3	T1	1.00000*	.035
	T4	2.26667*	.000
	T5	3.06667*	.000
T4	T1	-1.26667*	.008
	T2	-1.66667*	.001
	T3	-2.26667*	.000
T5	T1	-2.06667*	.000
	T2	-2.46667*	.000
	T3	-3.06667*	.000
Length of branch(I)	Length of branch(J)	Mean Difference (I-J)	Significant
T1	T4	1.30533*	.002
	T5	1.50000*	.000
T2	T4	1.08400*	.008
	T5	1.27867*	.002
T3	T5	.97167*	.018
T4	T1	-1.30533*	.002
	T2	-1.08400*	.008
T5	T1	-1.50000*	.000
	T2	-1.27867*	.002
	T3	-.97167*	.018
Number of node/branch(I)	Number of node/ branch (J)	Mean Difference (I-J)	Significant
T2	T4	1.51067*	.000
	T5	1.83067*	.000
T3	T4	1.30000*	.002
	T5	1.62000*	.000
T4	T1	-1.18333*	.005
	T2	-1.51067*	.000
	T3	-1.30000*	.002
T5	T1	-1.50333*	.001
	T2	-1.83067*	.000
	T3	-1.62000*	.000
Number of leaf /branch (I)	Number of leaf/ branch (J)	Mean Difference (I-J)	Significant
T2	T4	1.33333*	.017
	T5	1.71067*	.002
T3	T4	1.20573*	.030
	T5	1.58307*	.005
T4	T2	-1.33333*	.017
	T3	-1.20573*	.030
T5	T1	-1.40467*	.012
	T2	-1.71067*	.002
	T3	-1.58307*	.005

***is significant at P< 0.05**

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Table 5. Results of analysis of variance to regeneration plantlets frequency

Source of variance	df	Mean square						
		Length of shoot (cm)	Number of node	Number of leaves	Number of branch	Length of branch (cm)	Number of node per branch	Number of leaves per branch
Subcultures	4	21.287*	23.580*	0.820ns	0.653ns	6.579*	10.407*	8.983*
Error	74	0.920	1.623	0.884	0.882	1.199	1.273	2.210

Data was recorded after 30 days of culture. Mean separation was determined using Duncan's multiple range test. *: significant at P< 0.05; ns: non-significant

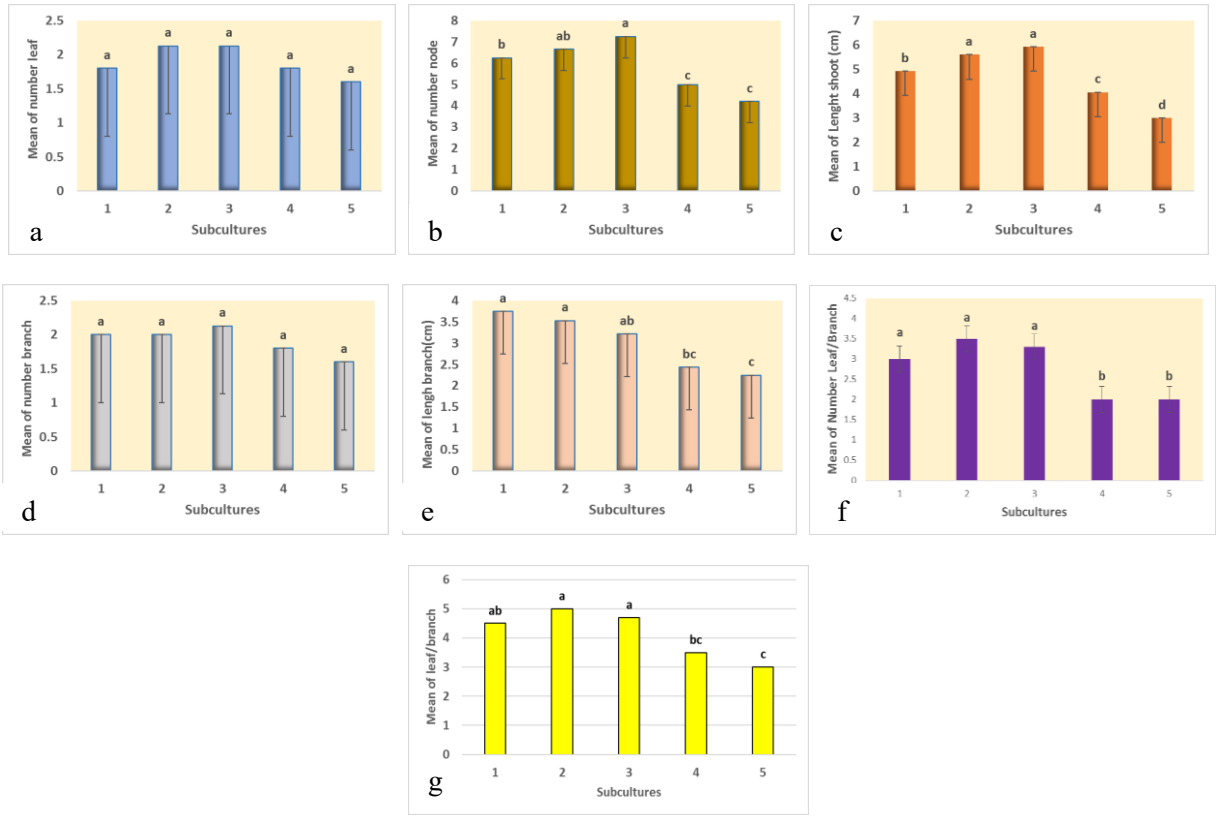

Figure 6. (a) Mean length of shoot; (b) mean number of nodes; (c) mean number of leaves; (d) mean number of branches; (e) mean length of branch; (f) mean number of nodes per branch; (g) mean number of leaves per branch during five subcultures in the Shahzileh cultivar.

length, and the number of nodes, leaves, and branches. Overall, the Jujube tree serves as a useful model for investigating the effects of prolonged vegetative propagation on morphological characteristics. These findings support the likelihood of stable and desirable somaclonal variation in Jujube explants through extended subculture periods, as indicated by Leva (2009).

Somaclonal variation in plantlets of jujube *in vitro* regenerated: Somaclonal variation can arise from both pre-existing genetic variation in the somatic cells of the explants as well as from changes occurring during tissue culture (epigenetic changes). Several factors induce in vitro variability, including explant source and age, culture duration, number of subcultures, culture environment, chemical additives or growth regulators, media composition, ploidy level, and genetic mosaicism (Nwauzoma and Jaja, 2013; Mohajer Kaboli *et al.*, 2023).

In this study, 10-year-old Shahzileh Jujube trees were used for explants. These trees were fertilized annually and bore fruit. Only one tree was used as the source of explants, so the variations observed in the regenerated plantlets were likely due to several factors, particularly the explant source. Explants derived from different plants of the same cultivar or from different shoots of the same plant can exhibit variation.

Other researchers explored the regeneration of cashew plants from cotyledon nodes and emphasized the influence of explant type on somaclonal variation. Shoots derived through an intermediary callus phase tend to be more morphologically and genetically variable, while those formed directly from explants without callus formation tend to be more homogeneous (Nivas and D'Souza, 2012). Additionally, wounding or mechanical injury can lead to the release of biomolecules that interfere with plant development, resulting in somaclonal variation.

In the present study, single nodes from the Shahzileh Jujube cultivar were used as explants, which contain

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Table 6. Volatile Chemical Compounds Identified in *Ziziphus jujuba* Fruit Extracts by GC-MS, Categorized by Population and Retention Time

		Cultivars	Taghab	Siojan	Shahzileh		
No.	Compound Name	* Compound Type	RT	RT	RT	Molecular Formula	Molecular Weight (g/mol)
1	Oleic acid (C18:1)	UFA(ω−9)	20.901	16.643	17.232	$C_{18}H_{34}O_2$	282.5
2	Linoleic acid (C18:2)	UFA(ω−6)	33.555	21.025	16.893	$C_{18}H_{32}O_2$	280.4
3	Linolenic acid (C18:3)	UFA(ω−3)	29.435	19.569	23.854	$C_{18}H_{30}O_2$	278.4
4	Cis-vaccenic acid (C18:1)	UFA(ω−7)	26.862	46.475		$C_{18}H_{32}O_2$	282.5
5	Myristoleic acid (C14:1)	UFA(ω−5)		46.805	23.793	$C_{14}H_{26}O_2$	226.35
6	Palmitoleic acid (C16:1)	UFA(ω−7)	32.254			$C_{16}H_{30}O_2$	254.41
7	Arachidonic acid (C20:4)	UFA(ω−6)	42.722	47.244	22.326	$C_{20}H_{32}O_2$	304.5
8	Paullinic acid (C20:1)	UFA(ω−7)	19.278	22.971	24.963	$C_{20}H_{38}O_2$	310.5
9	Arachidic acid (C20:0)	SFA		19.028	21.121	$C_{20}H_{40}O_2$	312.5
10	Myristic acid (C14:0)	SFA			19.789	$C_{14}H_{28}O_2$	228.37
11	Palmitic acid (C16:0)	SFA	47.016	25.331	20.573	$C_{16}H_{32}O_2$	256.42
12	Stearic acid (C18:0)	SFA	35.963			$C_{18}H_{36}O_2$	284.5
13	Furfural	Aldehyde	4.528	5.099	14.873	$C_5H_4O_2$	96.08
14	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	Aldehyde	8.529	4.419	14.939	$C_6H_6O_3$	126.11
15	Formaldehyde, methyl(2-propynyl)hydrazine	Aldehyde				$C_5H_8N_2$	96.13
16	Benzaldehyde	Aldehyde	7.637	8.033	16.122	C_7H_6O	106.12
17	9,12-Octadecadiynoic acid, methyl ester	Ester	16.196		13.483	$C_{19}H_{30}O_2$	290.4
18	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	Ester		9.782		$C_{21}H_{38}O_4$	354.5
19	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl este	Ester	13.094			$C_{21}H_{40}O_4$	356.5
20	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	Ester		12.802	20.119	$C_{35}H_{68}O_5$	568.9
21	Acetic acid, oxo-, methyl ester	Ester	24.945	34.473	15.378	$C_3H_4O_3$	88.06
22	Stigmastan-3,5-diene	Terpenoid			26.01	$C_{29}H_{48}$	396.7
23	β-Sitosterol	Terpenoid	46.955	40.586	27.325	$C_{29}H_{50}O$	414.7
24	Ethyl iso-allocholate	Terpenoid		47.997		$C_{26}H_{44}O_5$	436.6
25	Pregn-5-en-20-one, 3-(acetyloxy)-17-hydroxy-, (3β)-	Terpenoid		48.871		$C_{25}H_{36}O_6$	432.5
26	2-Furanmethanol	Alchohol	4.354	8.428	17.353	$C_5H_6O_2$	98.1
27	benzyl alcohol	Alchohol	9.7	4.331		C_7H_8O	108.14
28	6-Acetyl-β-d-mannose	Carbohydrate	5.281	18.552	17.112	$C_8H_{14}O_7$	222.19
29	L-Arbinose	carbohydrate	12.853	12.652	15.774	$C_5H_{10}O_5$	150.13
30	α-D-Glucopyranoside, O-α-D-glucopyranosyl-(1.fwdarw.3)-β-D-fructofuranosyl	carbohydrate	10.863	13.57		$C_{18}H_{32}O_{16}$	504.4
31	Cyclohexan-1,4,5-triol-3-one-1-carboxylic acid	Ketone	3.628	3.19		$C_7H_{10}O_6$	190.15
32	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Ketone		7.206	18.141	$C_6H_8O_4$	144.12
33	Undecadien-2-one,6.10-dimethyl	Ketone				$C_{14}H_{24}OS$	240.41
34	4-Methyl-5-nonanone	Ketone	14.623	15.625	13.894	$C_{10}H_{20}O$	156.26
35		Number of Identified Compounds	23	26	22		

RT: Retention time (min); UFA: Unsaturated Fatty Acyl; SFA: Saturated Fatty Acyl
***The compound types were identified based on the comparison of retention times (RT) and mass spectra with the reference data from the NIST library**.

meristematic tissues. The use of meristematic tissues such as axillary buds reduces the chances of somaclonal variation (Shenoy and Vasil, 1992). In contrast, highly differentiated tissues, which require a callus phase, produce more variants (Shepard *et al.*, 1980). Plantlets regenerated from solitary nodes of Jujube showed optimal results with 0.2 mg L^{-1} IBA and 2 mg L^{-1} BA, which was the most effective combination for shoot regeneration. However, very high and imbalanced growth hormone levels in the culture medium can also induce variation (Thorpe, 1987).

The combination of auxins and cytokinins has been shown to play a key role in causing somaclonal variation. Auxins, in particular, promote the formation of shoots and branches, while cytokinins are involved in cell division and differentiation.

Biochemical analysis of Jujube (*Ziziphus jujube* Mill.) fruit extracts: The volatile chemical compounds present in the fruit extracts of three jujube cultivars, Taghab, Siojan, and Shahzileh, were identified using Gas Chromatography-Mass Spectrometry (GC-MS). Identification was performed by comparing the retention

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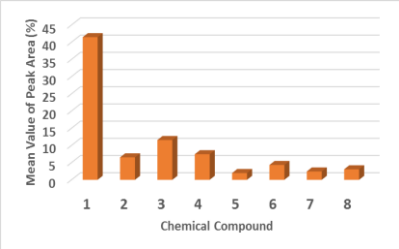

Figure 7. The average content of the chemical compounds identified in the jujube fruit across the studied cultivars Unsaturated Fatty Acyl (1), Saturated Fatty Acyl (2), Aldehyde (3), Ester (4), Terpenoid (5), Alcohol (6), Carbohydrate (7), Ketone (8)

times (RT) and mass spectra of the detected compounds with those in the NIST mass spectral library, as shown in Table 6.

The qualitative and quantitative analysis of the volatile constituents revealed notable variations among the cultivars. According to the averaged data summarized in Figure 7, unsaturated fatty acids were the predominant class of compounds, accounting for an average of 41.463% of the total volatile content. This was followed by aldehydes (11.563%), esters (7.437%), and saturated fatty acids (6.535%), respectively. These findings highlight the richness of unsaturated fatty acids in the jujube fruit extracts, suggesting potential nutritional and industrial applications.

Among the fatty acids identified in the jujube (*Ziziphus jujuba*) fruit extract, oleic acid, linoleic acid, and cis-vaccenic acid classified as unsaturated fatty acids were the most abundant, accounting for 15.934%, 15.137%, and 3.109% of the total fatty acid content, respectively. In addition, palmitic acid, a saturated fatty acid, exhibited the highest average concentration among saturated fatty acids, comprising 3.553% of the total, as shown in Figure 8.

The average quantity of chemical compounds identified, based on the peak area percentage (peak area %) in the GC-MS chromatogram, is presented by population in Table 7. Given the nutritional significance of fatty acids, particularly omega fatty acids (unsaturated fatty acids), all saturated and unsaturated fatty acids identified via GC-MS, along with the ratio of unsaturated to saturated fatty acids (UFAs/SFAs), were subjected to statistical analysis, along with other identified chemical compounds.

The results of ANOVA revealed a statistically significant difference ($P < 0.01$) among the studied cultivars with respect to all analyzed compounds. This indicates that the chemical composition of jujube fruits varies significantly among different cultivars. According to the mean comparisons, the Siojan cultivar (South Khorasan) exhibited the highest content of cis-vaccenic acid (5.812%), myristoleic acid (6.752%), and palmitoleic acid (3.88%) among the evaluated cultivars. The Taghab cultivar (South Khorasan) had the highest levels of saturated fatty acids such as arachidic, myristic, and palmitic acids.

Furthermore, the highest ratio of unsaturated to saturated fatty acids (UFAs/SFAs), 10.424%, was observed in the Siojan cultivar. Overall, the highest total content of unsaturated fatty acids was also recorded in the Siojan cultivar (51.11%) (Table 7).

The order of separation of these compounds, based on their volatility in the jujube (*Ziziphus jujuba*) fruit extract, is as follows: aldehydes, ethers, esters, carbohydrates, and finally fatty acids and terpenoids. These compounds are typically extracted using organic solvents such as hexane, methanol, or a mixture of these solvents (Wang, 2017). Jujube fruit is rich in biologically important compounds. In the present study, the highest identified compounds were, in order, fatty acids and aldehydes; terpenoids, carbohydrates, and ketones had the lowest concentrations among the identified compounds. Furthermore, no ether compounds were detected by GC-MS in this study, as shown in Table 4. The aldehyde content ranged from 11.53% in the cultivars, which was low (Table 7).

These findings align with those of Wang (2017), who compared and studied the chemical composition of 15 jujube cultivars. According to his results, jujube fruit extract had low levels of alcohols, esters, and ketones, while aldehydes and fatty acids were present in large quantities.

Our results also correspond with the findings of Widad *et al.* (2017), who reported a fatty acid content of 78.9% in jujube fruit extract. In our study, unsaturated fatty acids, with an average of 41.463%, represented the highest concentration among the identified compounds. The average content of saturated fatty acids was 6.535%, and the ratio of saturated to unsaturated fatty acids (SFAs/UFSs) was 7.052%. On the other hand, the ratio of unsaturated to saturated fatty acids (UFAs/SFAs) varied between 10.424% and 3.45% across the Sivan cultivar populations. These findings are consistent with those of Allalout *et al*. (2011) and Yorulmaz et al. (2010). In Allalout *et al.* (2011) study, unsaturated fatty acids ranged from 62.63% to 72.40%, and the unsaturated/saturated fatty acid ratio (U/S) varied from 1.68 to 2.37 across the studied cultivars. In their research, unsaturated fatty acids were more prevalent across all ecotypes. In the present study, carbohydrate and terpenoid contents were 2.435% and 1.97%, respectively, while Widad *et al.* (2017) reported higher levels of hydrocarbons (10.10%).

In studies conducted by Yang *et al.* (2013) on the chemical composition of jujube fruit, oleic acid and linoleic acid were the predominant unsaturated fatty acids, a result that aligns with the current study. In

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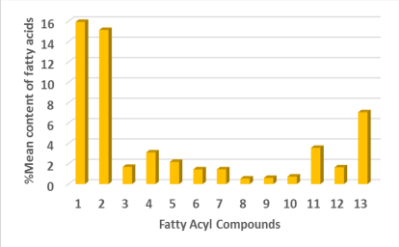

Figure 8. The average content of fatty acids identified in the jujube fruit across the studied cultivars
Oleic Acid (1), Linoleic Acid (2), Linolenic Acid (3), Cis-Vaccenic Acid (4), Myristoleic Acid (5), Palmitoleic Acid (6), Arachidonic Acid (7), Paullinic Acid (8), Arachidic Acid (9), Myristic Acid (10), Palmitic Acid (11), Stearic Acid (12), UFAs/SFAs: Ratio (Unsaturated Fatty Acids/Saturated Fatty Acids) (13)

Table 7. Percentage of Identified Chemical Compounds in *Ziziphus jujuba* Fruit Extracts Across the Studied Cultivars

Cultivar	1	2	3	4	5	6	7	8	9
Taghab	17.176±0.21	15.452±0.25	2.013±0.02	4.275±0.05	n.d	2.559±0.21	3.923±0.08	1.103±0.05	n.d
Siojan	17.12±0.23	16.21±0.2	2.472±0.31	5.679±0.09	6.752±0.21	n.d	2.125±0.12	0.751±0.012	1.112±0.42
Shahzileh	17.197±0.2	17.481±0.5	1.459±0.02	n.d	5.227±0.11	n.d	3.854±0.27	0.693±0.74	0.715±0.21

Oleic Acid(1), Linoleic Acid(2), Linolenic Acid(3), Cis-Vaccenic Acid(4), Myristoleic Acid(5), Palmitoleic Acid(6), Arachidonic Acid(7), Paullinic Acid(8), Arachidic Acid(9), Myristic Acid(10), Palmitic Acid(11), Stearic Acid (12), UFAs/SFAs: (Unsaturated Fatty Acids / Saturated Fatty Acids)(13), Aldehyde(14), Ester (15), Terpenoid(16), Alcohol(17), Carbohydrate18), Ketone (19), Unidentified compounds; data are presented as mean ± standard error.**

Continu of table 7.

Cultivar	10	11	12	13	14	15	16	17	18	19
Taghab	n.d	3.291±0.03	3.017±0.01	7.372±0.23	12.51±0.08	9.704±0.14	1.315±0.02	5.52±0.18	4.107±0.25	1.153±0.05
Siojan	n.d	3.791±0.06	n.d	10.424±0.15	11.82±0.54	7.333±0.08	2.907±0.1	5.307±0.12	3.182±0.38	2.322±0.00
Shahzileh	1.591±0.09	3.472±0.05	n.d	7.946±0.06	9.63±0.25	6.058±0.25	1.604±0.05	3.238±0.29	2.484±0.37	3.723±0.38

Oleic Acid(1), Linoleic Acid(2), Linolenic Acid(3), Cis-Vaccenic Acid(4), Myristoleic Acid(5), Palmitoleic Acid(6), Arachidonic Acid(7), Paullinic Acid(8), Arachidic Acid(9), Myristic Acid(10), Palmitic Acid(11), Stearic Acid (12), UFAs/SFAs: (Unsaturated Fatty Acids / Saturated Fatty Acids)(13), Aldehyde(14), Ester (15), Terpenoid(16), Alcohol(17), Carbohydrate18), Ketone (19), Unidentified compounds; data are presented as mean ± standard error.**

contrast, Gu *et al.* (2009) identified linoleic acid, oleic acid, and linolenic acid as the major fatty acids in Ziziphus species, although their findings on the saturated fatty acid profile in Indian jujube were consistent with our results.

All tested samples showed similar phytochemical profiles, but the levels of chemical compounds varied. These results suggest that jujube ecotypes differ mainly in the quantity of compounds, while they possess similar qualitative profiles (Yang *et al.*, 2013).

Carbohydrates are one of the primary components of fruits, playing an important role in fruit quality and flavor. L-arabinose, a monosaccharide, was identified as one of the most significant carbohydrates in the fruit of various jujube populations. This aldopentose is an important compound in biotechnology and bioengineering and serves as a key intermediate in the synthesis of organic pharmaceutical compounds. L-arabinose is a precursor in the structures of many bioactive substances, such as antibiotics (Xiong *et al.*, 2005).

Conclusion

The experiment successfully demonstrated that regenerated Jujube plantlets exhibited appropriate shoot proliferation and growth characteristics, with optimal branching achieved using 2 mg L^{-1} BA and 0.2 mg L^{-1} IBA. The study confirmed that prolonged *in vitro* subculturing induces somaclonal variation, affecting morphological traits such as shoot length, node count, and leaf production. These variations became more pronounced in later subcultures, particularly the fifth, where reduced shoot elongation and branching were observed. The findings suggest that subculturing can be strategically employed to achieve stable and desirable somaclonal variations for effective propagation and genetic improvement of Jujube cultivars. This research underscores the importance of monitoring morphological changes during micropropagation and highlights its potential applications in plant breeding and large-scale commercial propagation. The GC-MS analysis showed a significant difference in the chemical composition of the fruit across different cultivars.

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Conflicts of interest statement

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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