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Micropropagation of Iraqi Garlic (*Allium sativum* L.) Through Tissue Culture Method and Assessment of Genetic Stability Using RAPD Markers

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ABSTRACT

Garlic (*Allium sativum* L.) is a valuable medicinal and culinary crop in Iraq, facing yield limitations due to vegetative propagation and disease. Tissue culture technique offers a viable solution for rapid propagation. This study aimed to micro-propagate Iraqi garlic and evaluate the genetic stability of regenerated plants using randomly amplified polymorphic DNA (RAPD) molecular markers. Garlic explants were cultured on Murashige and Skoog's (MS) medium with varying hormone combinations for callus induction, shoot regeneration, and rooting. Regenerated microplants were acclimatized in sterile substrate. DNA was extracted from leaf tissues, and genetic stability was assessed using RAPD markers. Optimal sterilization of Iraqi garlic was achieved with 0.8% sodium hypochlorite (NaOCl) for 20 minutes, resulting in 64% clean explants. MS medium supplemented with 5.0 mg/L 6-benzyladenine (BA) resulted in an 80% callus response and an average of 6.6 shoots per explant. Meanwhile, 2.5 mg/L BA produced the longest shoot length (1.80 cm). RAPD analysis using the OPC-03 primer showed 10 consistent bands (1835–1910 bp), confirming the genetic stability of regenerated plants. Maximum induction (70%) and fresh weight (286.3 mg) occurred with 100 µM 2,4-dichlorophenoxyacetic acid (2,4-D). The use of liquid medium enhanced bud formation, with an average of 24.8 buds per explant, and achieved acclimatization rates of up to 80%. Overall, tissue culture proved to be an effective method for garlic propagation. The confirmed genetic stability of regenerated plants indicates strong potential for agricultural applications and future genetic improvement programs.

1. INTRODUCTION

Garlic (*Allium sativum* L.) is a perennial herbaceous plant that is cultivated seasonally in most countries and thrives in cold seasons. The plant is native to Central Asia. In Iraq, garlic is cultivated as a winter crop, primarily in Basra, Nineveh, and Babylon. Garlic is nutritionally and medicinally valuable; its cloves contain 31% carbohydrates and 6.2% protein (wet weight), and are rich in phosphorus, iron, potassium, magnesium, and vitamins, such as thiamine, riboflavin, niacin, and ascorbic acid [1]. Garlic contains allicin, a compound effective against many viruses, bacteria, and fungi [2]. In Iraq, the annual garlic cultivation area is low, with 2,000 hectares yielding 2,000 kg per hectare in 1999 and 1,500 kg in 2000. This low yield is due to poor productivity of varieties, inadequate agricultural practices, and high insect and disease infestations [3]. Garlic is vegetatively propagated using cloves or aerial bulbils, as it rarely forms seeds outside its native habitat. The reliance on vegetative propagation often leads to disease, especially viral infections, reducing both yield and quality. Given its nutritional and medicinal importance, successes in tissue culture technology have been significant for garlic propagation [4].

Randomly amplified polymorphic DNA (RAPD) amplifies random fragments of DNA, producing visible bands of different molecular weights on an agarose gel. This technique uses short primers, typically 10 nucleotides long, that bind to multiple sites on the DNA strand [5, 6]. The amplified fragments are separated on an agarose gel, stained with ethidium bromide, and detected under ultraviolet light [7]. The primer's efficiency in RAPD increases with a higher guanine-cytosine (G+C) content, as G and C form three hydrogen bonds, creating a stronger bond with the DNA template compared to the two hydrogen bonds between adenine (A) and thymine (T).

In determining the genetic stability of the *Allium sativum* L. plant resulting from tissue culture, randomly amplified polymorphic DNA (RAPD) indicators were used to detect genetic variations that may occur when grown *ex vivo* by comparing the analysis of the results of the genetic fingerprinting of tissue culture plants with plants grown in the field of the same variety [7]. Study [8] used RAPD markers to verify the compatibility of tomato plants produced by the indirect organ formation method with tomato seedlings

growing in the field. The results showed no genetic differences. Arzanlou and Bohlooli [9] observed the genetic homogeneity of potato plants resulting from the formation of embryos through indirect autosomal *ex vivo* infection. This was in comparison to plants of the same potato variety.

In assessing the genetic similarity of date palms, the similarity between mother plants and offspring derived from indirect somatic embryogenesis ranged from 83% to 94%, depending on their genetic composition. In the same field, RAPD markers were employed to assess the genetic stability of palm plants derived from adventitious buds formed on the callus of two palm varieties [10]. The genetic fingerprinting results revealed a perfect match in banding patterns for all seventeen donor plants. Twenty primers were used for the two types of studies. The researchers concluded that RAPD markers are easy and quick DNA markers for the early detection of genetic variations that may occur in date palm plants resulting from plant tissue culture. These markers were also used to detect genetic variations in medicinal plants produced *ex vivo*. In a study conducted by Rahmatullah and Ajmi [11], the genetic identity of tissue-propagated *Ochreinauclea missionis* plants was confirmed by stimulating the growth of lateral shoots *ex vivo*, using mother plants grown through traditional methods.

This study aimed to achieve micropropagation of genetically stable, homogeneous, and economically valuable Iraqi garlic (*Allium sativum* L.) using tissue culture techniques. It also sought to assess the genetic stability of the regenerated plants using RAPD molecular markers. This method facilitates the production of healthy plants genetically identical to the mother plant, helps preserve genetic integrity, and enables monitoring of genetic changes throughout the micropropagation process.

2. MATERIALS AND METHODS

This study was carried out in the laboratory of the Department of Biotechnology, College of Science, Al-Nahrain University, for the period from October 2023 until April 2024.

2.1 Selection of plant samples

After two weeks of cultivation on hormone-free Murashige and Skoog's (MS) medium, tissue culture plants derived from artificial seeds containing somatic embryos were randomly selected for the treatments following the protocol of Thorpe [3]. The plants grown from natural seeds were cultivated on sterile filter paper, moistened with sterile distilled water. Meanwhile, plants grown from artificial seeds were stored for a month at 4°C. These seeds had previously been prepared with a mixture of salicylic acid (SA) at 2%, calcium chloride ($CaCl_2$) at 12 mg/L, benzylaminopurine (BA) at 0.05 mg/L, and indole-3-butyric acid (IBA) at 0.05 mg/L. Additionally, other sets of plants were grown from artificial seeds that were prepared with SA at 2%, $CaCl_2$ at 12 mg/L, BA at 0.05 mg/L, and IBA at 0.01 mg/L, as well as with a different combination of SA at 2%, $CaCl_2$ at 12 mg/L, BA at 1 mg/L, and IBA at 0.01 mg/L. The artificial seeds, treated with the mixture of SA, $CaCl_2$, BA, and IBA, were stored at 4°C for one month before being used in the experiment. A control group of plants, which were not treated with any growth regulators, was also maintained to observe their natural growth and development without any external treatment.

2.2 Methods

2.2.1 Callus induction

The effect of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin concentrations and their interaction were tested on callus induction. The root tip of sprouted garlic cloves with a length of 2-3 mm was removed under sterile conditions. It was grown on MS nutrient medium supplemented with different concentrations of 2,4-D (0, 0.5, 1.0, 1.5, and 2.0 mg/L) and kinetin at concentrations of 0, 5.0, 10.0, and 15.0 mg/L. Four plant parts were planted in each bottle, with five replicates prepared for each concentration and each treatment. The plants were incubated in the growth room at a temperature of 25°C and a lighting intensity of 1000 lux for 16 hours/day. The percentage of callus induction and the fresh weight of callus were calculated after 45 days of incubation. Greenhouses were maintained at normal humidity levels by covering them with transparent plastic sheets. In all experiments, pH and electrical conductivity (EC) of the medium were measured after the addition of growth regulators to ensure consistent environmental conditions for root development [12].

2.2.2 Examining the effect of BA concentrations on the vegetative branch sprouting from callus

The effect of BA concentration on the sprouting of vegetative branches from callus was tested using 400 mg of callus. It was grown in culture tubes containing MS nutrient medium supplemented with BA at concentrations of 0, 2.5, 5.0, 7.5, and 10 mg/L, with ten replicates for each concentration. The cultures were incubated as in the previous experiment. The calli were subcultured three times every 30 days on the same nutrient medium. The percentage of sprouting and the average number of branches were calculated, along with the size and length of the callus pieces [13].

2.2.3 Root induction

Individual vegetative shoots were transferred into culture bottles containing MS nutrient medium (full strength and half strength) supplemented with the growth regulator NAA at concentrations of 0, 0.5, and 1.0 mg/L to induce rooting. Three branches were subcultured in each bottle, with five replicates for each concentration and treatment. The culture bottles were incubated under the same conditions as previously described. After 30 days, the average number of roots and their lengths were recorded [14].

2.2.4 Acclimatization of microplants

Regenerated plants grown in culture tubes from callus cultivation, characterized by robust vegetative growth and healthy root systems, were selected. They were washed with sterile distilled water to remove any remaining nutrient medium to prevent microbial growth. The plants were then briefly immersed in a 0.2% Benlate fungicide solution to protect against fungal infection. Twenty plants were individually planted in plastic containers with a 10 cm diameter, filled with a sterilized growing medium composed of sand and peat moss in a 1:1 ratio. The growing medium was sterilized using an autoclave at 121°C and a pressure of 1.04 kg/cm² for one hour to eliminate pathogens. The plants were covered with transparent, perforated plastic covers to maintain humidity while allowing light to pass through. These covered plants were placed in an air-conditioned room. After ten days, the plastic covers were gradually removed, and the plants were transferred to a greenhouse to continue growing [15].

2.2.5 DNA extraction

Genetic material (DNA) was isolated from the young green leaves of the acclimatized microplants according to previously described methods [12, 13]. One gram of young green leaves was quickly crushed in a pre-cooled ceramic mortar with liquid nitrogen until they became a fine white powder. The powder was transferred to 20 cm^3 plastic tubes, mixed with 3 mL of extraction solution, and incubated at 60°C for 60 minutes. The tubes were then cooled to 37°C, and 5 mL of chloroform/isoamyl solution was added, followed by stirring for 15 minutes. The mixture was centrifuged at 10,000 rpm and 40°C for 10 minutes. The upper layer was transferred to new tubes, and 5 mL of cold isopropanol was added to precipitate the DNA, which appeared as white threads. The samples were left overnight for complete precipitation and then centrifuged at 10,000 rpm for 15 minutes. The supernatant was removed, and the DNA was washed with 99% ethanol. The precipitated RNA was removed from the DNA by adding 4 µL of RNase enzyme at 37°C for 30 minutes. After cooling, 90 µL of sodium acetate was added to purify the DNA, followed by 2 mL of cold 99% ethanol to precipitate the DNA. The mixture was centrifuged at 10,000 rpm for 30 minutes. The washing process was repeated with 75% ethanol, and the samples were dried in a vacuum oven. Finally, 100–150 µL of distilled water was added to dissolve the DNA. The DNA solution was transferred to 3 mL tubes with tight-fitting caps and stored at -20°C. Agarose gel electrophoresis was then performed to analyze the DNA. The gel was examined under ultraviolet (UV) light at 260 nm after three hours to visualize the DNA band [16].

2.2.6 Random amplified polymorphic DNA analysis

RAPD analysis was conducted according to the method described in the study [14] with slight modifications. Five primers (OPA-05, OPA-08, OPB-06, OPB-08, and OPC-03) were examined to assess morphological polymorphism. PCR reactions were carried out in 25 µL final volume with 50 ng of genomic DNA, 2.5 µL of 10x PCR buffer, 2.0 µL of dNTP mix (2.5 mM each), 1.5 µL of each primer (10 µM), 1.5 µL of $MgCl_2$ (25 mM), 1 unit of Taq DNA polymerase, and nuclease-free water to complete the volume. The thermal cycling conditions were as follows: initial denaturation at 94°C for 5 minutes, followed by repeated 40 cycles of denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute, extension at 72°C for 2 minutes, and finally, an extension at 72°C for 10 minutes.

The amplification products were analyzed by electrophoresis using a 1.5% ethidium bromide-stained agarose gel and visualized under UV light using a gel documentation system. The molecular sizes of the amplified fragments were determined by comparison to a DNA ladder of known sizes. A standard curve was generated with molecular sizes on the y-axis and migration distances on the x-axis. The migration distance of each sample band was measured and cross-referenced to the standard curve to calculate the size of the amplified fragments [15]. The presence or absence of amplified bands for each primer was scored manually. clear and reproducible bands were recorded as 1 (present), while the absence of bands was recorded as 0 (absent) to generate a binary data matrix.

Genetic similarity between samples was calculated using Jaccard's similarity coefficient. The similarity matrix was subjected to cluster analysis by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) for generating a dendrogram showing the relationships among the samples.

2.2.7 Statistical analysis

Statistical analysis of all the data was carried out using the NTSYS-pc version 2.1 (Numerical Taxonomy and Multivariate Analysis System). Polymorphism information content (PIC) and percent polymorphic bands were also calculated to determine the efficiency and discriminatory power of the primers [6, 16].

3. RESULTS AND DISCUSSION

Seed multiplication at agricultural experimental stations and the improvement of garlic quality involve the selection of pure clones. Varieties derived from these pure clones are more homogeneous due to their identical genetic makeup, assuming the original plant is genetically pure. The success of this selection depends on the genetic variation in the original population, with greater variation increasing the likelihood of success. This process involves the selection, multiplication, and comparison of garlic samples with established standard varieties [16, 17]. The heritability rate is crucial to the success of pure lineage selection, as higher heritability increases the likelihood of successful outcomes. This process, known as the bulk method, is simpler than tracking individual lineages. Seeds from crossbreeding varieties A and B are planted in bulk, without selection, from the second to the fifth or sixth generation. Selection for desirable traits begins in the fifth or sixth generation, resulting in a group that exhibits genetic uniformity in many phenotypic characteristics. In the seventh generation, these plants are compared to commercial varieties, and weaker lines are eliminated.

3.1 Optimization of sodium hypochlorite concentration and disinfection time

The concentration of sodium hypochlorite (NaOCl), the duration of sterilization, and their interaction significantly affected the sterilization of garlic cloves. Specifically, the concentration of NaOCl had a significant impact on the percentage of cloves that remained uncontaminated and free from infection. As shown in Table 1, the 0.8% NaOCl concentration was the most effective, yielding the highest percentage. The percentage of uncontaminated cloves reached 64%, while the 0.2% treatment resulted in the lowest percentage of uncontaminated cloves.

Table 1 shows the genetic diversity indices obtained with five primers of RAPD. The number of bands amplified per primer, the polymorphic bands, and the percent of polymorphism were also determined. The polymorphic information content (PIC) index shows the discriminative power of each primer. Nei's genetic diversity (H) shows the probability that two randomly selected alleles from the same sample are not the same. The Shannon diversity index (I) reflects genetic diversity based on the frequency and distribution of bands. Among the primers used, OPB-06 had the highest rate of polymorphism (83.3%), the highest PIC value (0.35), and the highest genetic diversity, and this implies that it is very efficient in detecting genetic variation between the samples in question.

Table 1. Genetic diversity parameters

Primer	Total Bands	Polymorphic Bands	Polymorphism (%)	PIC	Nei's Gene Diversity (H)	Shannon's Index (I)
OPA-05	10	8	80.0%	0.32	0.28	0.42
OPA-08	9	7	77.7%	0.29	0.25	0.39
OPB-06	12	10	83.3%	0.35	0.30	0.45
OPB-08	11	9	81.8%	0.33	0.29	0.43
OPC-03	8	6	75.0%	0.28	0.24	0.38
Total/Average	50	40	80.0%	0.31	0.27	0.41

Including total bands, polymorphic bands, polymorphism percentage, PIC, Nei's gene diversity, and Shannon's index for each RAPD primer used in the analysis.

The use of NaOCl for surface sterilization is likely due to its efficiency and minimal harm to the plant parts at appropriate concentrations. Both the concentration of the sterilant and sterilization time are crucial in reducing contamination of plant parts grown *ex vivo*. However, higher concentrations and extended sterilization periods can damage the plant parts, consistent with findings from previous studies [17, 18]. The interaction of sodium hypochlorite concentration and sterilization time (Table 1) influenced the percentage of contamination-free cloves in a significant way. Although a 0.8% concentration and 20 minutes of sterilization time were optimal, higher concentrations (≥1.0%) or longer sterilization durations (>20 minutes) caused plant cell damage and reduced the percentage of contamination-free cloves. One reason behind this effect is that sodium hypochlorite is an oxidizing agent and can cause damage to cell walls if used in high concentration or for an extended duration. This effect is consistent with the findings of those who reported that prolonged exposure to high concentrations causes damage to plant cells [18].

3.2 The influence of 6-benzyl adenine concentration on bud formation and growth

The results of the effects of BA concentration (mg/L) on the percentage of unfolding, the rate of vegetative branch number, and branch length (cm) are presented in Table 2. After 90 days of callus planting, the garlic plant showed a significant increase in the percentage of callus developing into vegetative branches, as well as in the average number and length of the branches. A concentration of 5.0 mg/L BA was most effective in promoting callus formation and increasing the average number of branches. The highest callus detection rate reached 80%, with an average of 6.6 branches formed. In contrast, a concentration of 2.5 mg/L BA resulted in the longest average branch length, measuring 1.80 cm, while the control treatment and the highest concentration of 10.0 mg/L BA resulted in lower values [19].

Table 2. Effect of 6-benzyladenine concentrations on plant growth

BA (µM)	Effect on Plant Growth	No. of New Shoots	Effect on Hormonal Balance in Plants
0.5	Stimulates leaf and bud growth	30	Positive effect on plant hormones
1.0	Stimulates leaf and bud growth	45	Positive effect on plant hormones
2.0	Noticeable increase in bud growth	60	Increased hormonal activity (positive)
5.0	Inhibits growth	20	Negative effect on hormonal balance
10.0	Significant growth inhibition	10	Severe negative effect on hormonal balance

BA: 6-benzyl adenine concentration

Benzyl adenine is a hormone that induces the production of lateral buds and general plant growth. In the present study, it was observed that varying BA concentrations influenced hormonal activity in plants, inducing leaf or bud growth. Low concentrations of BA significantly increased the number of new shoots, whereas high concentrations tended to inhibit growth by disrupting the plant's hormonal balance. This result agrees with an earlier study [20], which showed that BA can increase plant growth at a certain range of concentrations but inhibit growth at high concentrations. The reason for these effects may be attributed to the addition of BA, which stimulates the growth of adventitious buds. However, high concentrations reduced the stimulation rate and the average number of branches due to their inhibitory effect when exceeding the optimal level. This increase in concentration causes an accumulation that leads to an increase in catabolism. Additionally, these effects may be due to genetic variations in the varieties used [21].

3.3 RAPD analysis and genetic stability

Total DNA was extracted from actively growing young leaves of the *Allium sativum* L. hybrids subjected to the treatments described in the experimental methods. Cetyltrimethylammonium bromide (CTAB) was utilized in the extraction solution according to the method described by Jo et al. [18], resulting in a sufficient quantity of DNA for subsequent electrophoresis on agarose gel. It should be noted that each material used in DNA isolation and extraction functions to remove unwanted cellular components without causing damage. Due to the thickness of plant cell walls, cell disruption was achieved by manual crushing in the presence of liquid nitrogen, which inhibits the activity of nuclear enzymes released during cell wall breakdown [20, 21]. The OPA-05 and OPA-08 gene fragments (Table 3) were used in genetic analysis to determine genetic variation among treated samples. The results showed a clear difference in genotype among the different treatments, which indicates that the sterilization process and the activity of BA can influence the genetic makeup of plants. Such genetic variations can also be indicative of the plants' reaction to a particular environmental variation, e.g., sterilization or exposure to hormones, allowing for the isolation of genetic sets that are possibly more resistant or compatible with certain environmental factors. These observations are consistent with previous studies demonstrating that genetic diversity can serve as a key indicator of adaptation to unfavorable environmental pressures, as shown in study [22] on the impact of environmental factors on plant genetic diversity.

Table 3. Primer products from the total and polymorphic band with their efficiency ratios and their differential ability

Discriminating Ability of the Primer (%)	Primer Efficiency (%)	Polymorphism (%)	Number of Total Polymorphic Bands	Number of Total Bands	Primer Code
8.808031	31.50847	97.77778	14	18	OPA-05
8.705031	26.42373	93.33333	14	15	OPA-08
4.402516	24.11864	43.75	7	16	OPB-06
4.433962	25.42373	20	15	15	OPB-08
			138	159	

Table 4. Molecular weight detection of leaf sample bands using primer OPC-03

MW (bp)	*Allium sativum* L1	*Allium sativum* L2	*Allium sativum* L3	*Allum sativum* L4
2000	0	1	1	0
1750	0	1	0	1
1500	1	1	1	1
1200	1	1	1	1
1100	0	1	1	0
1000	1	1	1	0
800	1	1	1	0
725	1	1	1	0
700	1	1	1	0
675	1	1	1	1
600	1	0	0	0
575	0	1	0	0
525	1	1	1	0
475	0	1	0	1
400	0	0	0	1
375	1	1	1	0
350	0	1	0	0
257	0	0	0	1
250	0	1	1	1

The number of bands recorded in the OPC-03 primer was 19 bands with sizes ranging from 250 to 2000 base pairs.

CTAB present in the extraction solution forms a complex with nucleic acids (CTAB complex), enhancing its resistance to degradation and thereby preserving its fundamental structure, which facilitates its separation from proteins. EDTA, a chelating agent, functions by sequestering positively charged ions such as Mg^{2+}, which are essential for the activity of nuclear enzymes involved in nucleic acid degradation, thereby inhibiting their enzymatic function [22]. Chloroform plays an important role in removing CTAB and denaturing proteins in the subsequent stage of the extraction process. It also aids in the elimination of polysaccharides and other cellular substances, such as chlorophyll, through centrifugation. Meanwhile, isoamyl alcohol prevents foam formation during the extraction process by reducing surface tension. For the materials involved in the extraction process, sodium chloride salt plays an important role in preserving DNA by providing it with appropriate osmotic conditions. Consequently, the DNA remains in the aqueous phase and is precipitated using refrigerated isopropyl alcohol. Subsequently, the precipitated DNA was purified from residual water and other contaminants by washing with 70% ethanol [15]. Mercaptoethanol serves to denature DNase enzymes and prevent the oxidation of phenolic compounds, thereby inhibiting the development of a brown coloration in the extract [23].

The results in Table 3 indicated that the four primers—OPA-05, OPA-08, OPB-06, and OPB-08—did not produce any bands on the agarose gel. In contrast, the primer OPC-03 generated clear amplification in the hybrid plant samples. To confirm the reliability of this finding, the experiment was repeated three times (Table 4). For DNA samples from *Allium sativum* L. hybrid treatments amplified with the OPC-03 primer, the number of resulting bands, their migration distances (in mm), and their estimated molecular sizes were measured. Analysis and comparison of DNA samples from treatments 2, 3, 4, and the control, each of which produced 10 bands, led to three key observations [24].

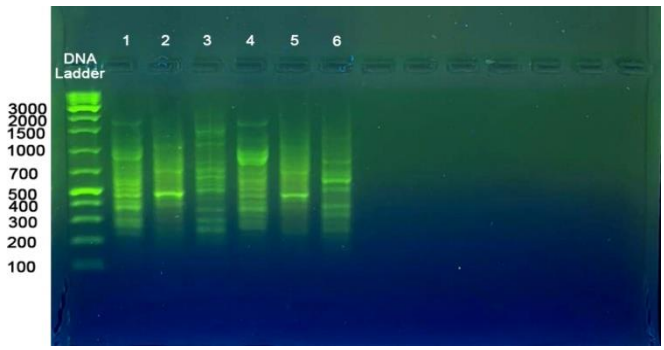

Figure 1. The PCR product of the primer

The primer was electrophoresed on 2% agarose at 5 volts/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder 1000 bp to reach genetic stability with primer OPC-03 on agarose gels, DNA bands appeared clear, well-defined, and consistent across repeats.

According to Figure 1, the molecular weight and estimated molecular sizes of the duplicated bands also varied within each treatment. The largest molecular size was 1835 base pairs (bp) for treatments 1, 2, and 3, whereas it was 1910 base pairs for treatment 4 (Table 4). The other bands in the four *Allium schoenoprasum* treatments were consistent in molecular size according to the study [25]. The analysis of genetic relationships relies on the presence or absence of bands produced by the duplication of specific genome segments in the studied plants, as well as on the molecular weights of these

bands, consistent with findings reported in previous studies [26]. Differences in the fluorescence intensity of the bands were not taken into account [27].

Although the method has several drawbacks, the RAPD used in this investigation proved helpful in identifying genetic diversity in Allium sativum. Since the genetic markers are unable to distinguish between homozygous and heterozygous individuals, they are regarded as dominant and are unable to offer complete genetic information. Additionally, the markers are extremely sensitive to environmental factors, which prevents them from being reproduced. Furthermore, as observed in the current investigation, where just one of the five primers (OPC-03) was responsive, indicating varying primer efficiency, there are issues with false positives and non-specific amplification. In contrast, other molecular markers like SSR (Simple Sequence Repeats) and ISSR (Inter Simple Sequence Repeats) are more trustworthy. Although ISSRs are also prevalent, they are more dependable and specific than RAPD because they employ longer microsatellite-based primers. On the other hand, SSRs are codominant, have a huge polymorphism ability, and are more reproducible, thereby generating more genetic data. Because of their excellent consistency and repeatability, the ISSR and SSR molecular markers are better than RAPD for obtaining complete genetic data [28].

3.4 Analysis of microreproduction success rate and acclimatization

The highest percentage of plant tissue induction was achieved by planting 0.5 cm-long garlic segments, excised from 8–10 cm shoots, on MS medium supplemented with 100 µM 2,4-D. The induction rates for the studied variety reached 70% and 40%, respectively. For propagating the induced plants, the highest fresh weight (286.3 mg) was achieved when the tissue was transferred to MS medium supplemented with 100 µM 2,4-D, which was significantly higher than the fresh weight of 210.5 mg obtained at the same concentration of NAA. Regarding the formation of adventitious buds, the results indicated that the highest percentage of plants with bud formation reached 80%, with an average of 16.4 buds in MS medium supplemented with 10.0 µmol and 5.0 µmol of NAA. The liquid medium further enhanced bud formation, increasing the number to 24.8 buds, which was significantly higher than within the same medium.

This approach yielded optimal outcomes, enhancing the acclimatization success rate to 70% and 80% for the two studied varieties during the agricultural micropropagation process. Auxin promotes enzyme activation and facilitates active intracellular movement, thereby increasing organ development and exposure [29]. This observation can be explained by the influence of growth regulators in the culture medium, which serves as the primary factor promoting morphological changes in tissue culture. Specifically, cytokinins such as BA stimulate the proliferation of axillary buds, while kinetin plays a crucial role in branch formation during micropropagation, thereby increasing the number of branches. Cytokinins are particularly vital for branch growth, with higher concentrations resulting in greater branch yields, consistent with previous studies [29, 30]. More so, the difference in domestication success rate (70% vs. 80%) may also be attributed to the influence of breed specificity or environmental adaptability.

3.5 Future research applications

3.5.1 Improving sterilization methods for plants in agriculture

The findings on the effects of sodium hypochlorite concentration and sterilization time on plant cell health can be applied to improve sterilization methods for plants, including seedling culture and agricultural experiments. By determining the best concentration and sterilization time (0.8% for 20 minutes), sterilization effectiveness can be improved while minimizing negative effects on plant cells. By applying this technology in agricultural production, the costs and time involved in sterilizing plants and enhancing the quality of plant products will decrease [31]. Through the sterilization of greenhouse-grown plants, both greenhouse sterilization procedures and closed-culture system processes can be improved, and pathogen-free seedlings produced. Application of this information to select the optimum sterilization conditions of seedlings and avoid contamination improves agricultural output [32].

3.5.2 Applications for optimizing plant reproduction and growth

According to Table 5, the effect of different BA concentrations on budding percentage, branch number, and branch length of BA on the growth and reproduction of plants reflects the significance of this hormone in optimizing the yield of plants. Discoveries on the effect of different BA concentrations can be utilized in promoting growth in specific plants, such as agricultural produce or medicinal plants. Data on the most appropriate concentration of BA for growth promotion can assist in enhancing agricultural yield or the cultivation of plants with major medicinal applications [33]. Increased crop yields can be achieved through the use of BA to promote growth in major agricultural crops such as fruits and vegetables and by enhancing the yields of medicinal plants through growth and reproduction stimulation via the application of plant hormones like BA [34].

Table 5. Effect of different BA concentrations on budding percentage, branch number, and branch length

BA Concentration (mg/L)	Budding Percentage (%)	Average Branch Number	Branch Length (cm)
0 (Control)	40	3.2	1.2
2.5	60	4.5	1.8
5.0	80	6.6	1.5
10.0	50	3.0	1.1

3.5.3 Application in genetic analysis and environmental resistance increase

Comparing genetic information using individual markers such as OPA-05 and OPA-08 may help explain how and why plants respond to environmental factors, such as sterilization or hormone treatment. It may be instrumental in increasing disease and environmental resistance to drought or pollution in the plant. This analysis helps to identify plants with environmental tolerance or resistance to extreme conditions such as pollution or environmental stress [35]. By identifying increased resistance of plants, genetic variation can help to identify plant species that are able to resist extreme environmental conditions, such as high temperatures or environmental pollution through genetic improvement of plants, genetic diversity can be used to identify traits that enhance the resistance of plants to chemicals or harmful

environmental factors [36].

3.5.4 Enhancing the use of chemicals in agriculture

According to Figure 2, the line chart showing hormone concentrations and responses, the findings of this study can support more effective use of chemicals in agriculture. For example, identifying the best and safest chemicals for plant sterilization can help minimize environmental impact while improving crop yields [37]. Effective and safe sterilization in commercial agriculture can be achieved by identifying the most appropriate chemicals for the process [38]. Additionally, optimizing the use of these chemicals is essential to minimize harmful effects on the surrounding soil and vegetation. Future studies should also investigate the impacts of sterilizing chemicals on plants. These findings could open new avenues for research on the effects of other chemicals, such as pesticides or synthetic fertilizers, on plant health. These effects may be investigated with possible new solutions for promoting the resistance of plants and agricultural productivity in addition to investigating the impact of other chemicals, such as pesticides and fertilizers, on plants and their optimization in agriculture, the long-term effect should also be studied to acquire greater insight into the plant sterilization and hormonal influences [39].

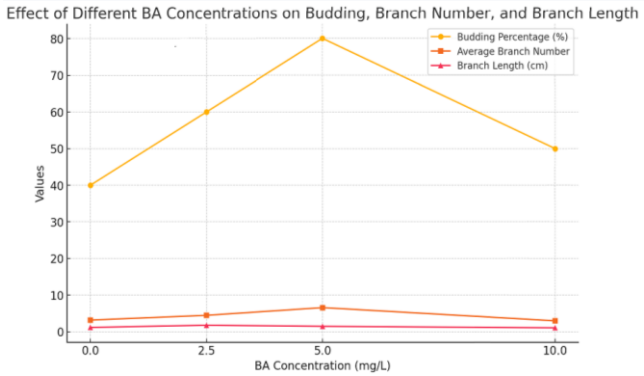

Figure 2. Line chart showing hormone concentrations and responses

4. CONCLUSIONS

This study successfully established an efficient protocol for the micropropagation of Iraqi garlic (*Allium sativum* L.) using optimized concentrations of sodium hypochlorite (0.8% for 20 minutes), 6-benzyl adenine (5.0 mg/L), and 2,4-D (100 µM), achieving high rates of contamination-free explants (64%), callus induction (80%), and bud formation (up to 24.8 buds). The RAPD analysis using OPC-03 primers confirmed the genetic stability of the propagated plants, with consistent banding patterns and minimal variation. These findings underscore the reliability of tissue culture for clonal propagation and genetic fidelity, providing a robust framework for large-scale garlic production and conservation in Iraq.

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