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EFFECT OF DIFFERENT COLCHICINE CONCENTRATIONS ON CHROMOSOME POLYPLOIDY BEHAVIOR OF GALL OAK (*QUERCUS INFECTORIA* OLIV.) BY USING MOLECULAR BIOLOGY

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ABSTRACT

Gall oak has a large ecological adaptability landscape and social importance and it is share of the natural wealth and has great economic value. Polyploidy deducted in this study through pre-screened directly by determining stomata guard cell length then confirmed by quantifying the DNA content using SCoT-PCR analysis. Depending in the stomata length as method we found the maximum of tetraploids (4n) and mixoploids (3n) plants were induced in chlciploidy treatments 2000 and 3000 mgl^{-1} of colchicine and soaked for 96 h. and 24 h. SCoT-PCR can detect polyploidy in the Gall Oak and provides an alternative method for analyzing polyploid genotype and breeding. Ungerminated seeds increased with increasing colchicine concentrations and exposure period especially in colchiploidy (2000 and 3000 mgl^{-1} for 72 and 96 h.) as a result of the toxicity of the colchicine substance. Significant differences were observed between polyploids and diploids plants. The tetraploid plants, show superiority in it is phenotypical treaties were created taller and thicker seedlings stems and roots, had more yields of branch, leaves and roots per seedlings, thicker leaves and bigger leaves. Whereas, chemical and physiological characteristics, significant contents of chlorophyll increased in polyploid plants, which leads to greener leaf color, but decreased in the of tannins amount. Also, the stomata guard cells size increased but decreased in number per area. Inducing novel features by ploidy breeding is a powerful tool that can lead to commercial success in forest tree species.

KEYWORD: Quercus infectoria Oliv, Polypoid breeding, Seedling characters, SCoTPCR.

INTRODUCTION

Quercus infectoria (Gall oak) is considered important hosts prone to such abnormal outgrowth, long-lived tree, foliage deciduous, widely distributed, native tree species among the most common mountain oak forest communities which make to 90% of the total forest cover Kurdistan region of Iraq *with Q. aegilops*, Q. *libani*, and *Q. macranthera* (Khwarahm, 2020). Where conform much and grown vastly in Middle Eastern countries like Cyprus, Syria, Turkey, Iraq and Iran deciduous forests (Askari, *et al.,* (2020).

The Oak trees have large ecological adaptability landscape and socioeconomic importance. Throughout history, plays a vital role to provide humans and wildlife with shelter, enjoyment, food, preventing soil erosion, and preserving water resources. It's wood used for construction, furniture, mine timbers, plywood, pulp chips, and the production of coal (Stein 1990; Niemiec *et al.,* 1995). High-content tannin Gallnuts are collected annually for exporting purposes. Also different parts of oak were uses in folk medicine (Sariozlu and Kivanc, 2011).

Shang *et al.,* (2020) investigate that the forest are rich in evolutionary diversity, and could leverage breeding. Hybridization introgression and polyploidy (Mason and Wendel, 2020) have already pumped morphological novelty by testing more genetic compatibilities than humans ever will (Cortés *et al.,* 2020). Moreover, Kang and Wei (2022), proved polyploidy breeding is one of the most demonstrated, documented achievements in this aspect, especially triploid production.

The application of biotechnology can overcome many of the drawbacks associated with conventional breeding strategies. This may be achieved using polyploidy methods. The traits of a plant can be altered by changing the

chromosomal groups and the number of genes in a cell, with an outcome that may be desirable or undesirable. Polyploidy occurs in more than 80% of plant species and is responsible for 2–4% of speciation in flowering plants (Madani *et al.,* 2021). Also, Mason & Wendel, (2020), demonstrated polyploidy is a major force in plant evolution and speciation. Therefore, polyploidy induction is one such approach that may introduce phenotypic characteristics of interest to the market and industry (De Moura and others, 2020). So, research aimed to study the effectiveness of colchicine at inducing polyploidy in *Quercus infectoria* Oliv. and its effect on the characteristics of seedlings will investigate to establish a working protocol for polyploidization in this species.

Material and Methods

1. Location of Study

This study was conducted in Forest Department and Laboratory Research Centers of College of Agricultural Engineering Sciences/ University of Duhok during growing seasons (2021-2022), located in the district of Sumail (42°, 52′, 02″ E, 36°, 51′, 38″ N, and altitude 456m. over sea level and average annual rainfall 400 – 450 mm) and in the greenhouse of Malta Nursery belong to the Directorate General of Forestry and Rangeland located in (36° 51'43.879 N), (42° 5616'.827" E) and in 522 m above sea level in Duhok Province

2. Acorns and seed collecting

Healthy and Mature brown acorns of this species were collected in the begging of November (2021), at the time of natural seed dissemination, from opened pollinate trees in natural stands in Jamanky location (36°, 55′, 37.46″ N º), (43°, 26′ , 16.96″ Eº) in altitude 920m above sea level in N-E Dohuk province. Seeds were extracted from the acorns, cleaned, and kept in a cooling chamber at a low temperature (2-4°C) until experimented.

3. Seeds Treatment with Colchicine

Colchicine ($C_{22}H_{25}NO_6$) from (Bio-world 4150 Tuller Rd., Ste 228 Dublin, OH 43017, USA) a chemical inducer that was used in this study. The experiment of this investigation involved the treatment of *Q. infectoria* seeds with different concentrations of colchicine (Zero, 500, 1000, 2000, 3000 mg/l) and soaked time (24, 48, 72, and 96 hrs.). The treated seeds were planted in polyethylene sacks at the end of February (2022) inside a greenhouse, where the percentage of light is about 50%.

4. Growth of Seedlings

In the greenhouse seedlings after 8 months of planting the following characteristics were studied:

A. Seed Germination percentage (SGP)

The percentage of the germination and living plants after treated with colchicine solutions were accounted and data were transferred to the Angular or Arcsine Transformation before analysis.

B. Morphological characteristics of seedling

Shoot Stem length (SST), Stem diameter (SD), Internode Length (IL), Branch Number (BN), Leaf Number (LN), Root Length (RL), Root Diameter (RD), and Secondary Roots Number (SRN).

C. Leaf morphology characteristics

Five leaves of the middle part from 5 seedlings were collected in each treatment to measure leaf morphology, chemical, and anatomical measurements.

Leaf Length (LL), Leaf Width (LW), Leaf thickness (LT), Leaf Length / Leaf Width Ratio (LR), and Largest leaf area (LLA).

D. Stomatal Length (SL), Stomatal Width (SW), and Stomatal Density (SD):

According to the Li *et al.,* (2018) stomata measurements, the abaxial leaf surface of mature leaves about 0.1 cm^2 was coated with transparent nail polish, and then the lower epidermis was peeled off and put on a glass microscope slide for stomata analyses. The number of intact stomata, and stomatal apparatus length and width was measured by using the Olympus microscope connected to DinoXcope digital camera. Five mature leaves were chosen from each treatment of control diploid and polyploid plants. Stomatal length, width, and density were measured and compared between diploid and polyploid plants with 15 stomatal apparatus measured for each leaf. Number of Stomata per mm^2.

E. Chemically Character of Seedlings Leaf

1- Chlorophyll a (Ch a), Chlorophyll b (Ch b), Total Chlorophyll ab (Ch ab):

The chlorophyll quantity was measured via using a spectrophotometer tool, through taking leaves from each transplant in fresh weight of 0.5 g after removing the middle vein of the leaf. The leaves were later cut into small pieces and put in flasks of 50ml capacity which include 30 ml of the absolute ethanol alcohol and then they were kept in darkness for 24 h. The operation of extraction was repeated more than three times to guarantee the chlorophyll extraction completely,

after that; the final volume reached 90ml and the volume completed to 100ml (Knudsen *et al.*, 1977). The absorption of the solution was measured in two wavelengths (665 nm and 649 nm) by using Spectrophotometer, which was utilized to study the following characteristics:

2- Estimation of Tannin Content (TC) in Oak Leaf

According to the Paaver *et al., (*2010) the total tannins were estimated. Tannin quantitative content in samples was estimated by the method of Paaver *et al.,* (2010) who depended on Price and Butler (1977), method with some modifications. In short, 0.1 g of a dried ground was held in a glass beaker of leaf from treated and untreated plants were removed to 100 ml flask; 50 ml water was added, and boiled for 30 min. After filtration with cotton filter the solution was further transferred to a 500 ml flask and water was added ad500 ml mark. 0.5 ml aliquots were finally transferred to vials, 1 ml 1% $K_3Fe(CN)6$ and 1 ml 1% FeCl3 were added, and water was added ad 10 ml volume. After five min time period, the solutions were measured spectrophotometrically at 720 nm. The actual tannin concentrations were calculated on the basis of the optical absorbance values obtained for the standard solutions in range 5 - 25 µg /10 ml.

F. Detection of Ploidy Level in Progeny (Polyploidy evaluation)

There were several methods to detect polyploidy. In forest trees, cytological techniques are standard, but they are difficult to perform because of the small size of tree chromosomes.

1. Stomata Length (SL)

According to (Beck *et al.*, 2005; Maritz 2008; and Suliman 2020) the lengths of stomata for identification of ploidy level were used as pre-screening or directed method for deduction polypoid plants.

2. Simple Sequence Repeat (SSR) markers and DNA Extraction

1- DNA Extraction

The DNA extraction process was carried out in molecular genetic analysis laboratories, El Shorbagy, Giza, Egypt. Leaves of 11 treatments (1 diploids plants (control treatment) and 10 treatments were treated with different colchicine concentrations and exposure for various periods) of *Quercus infectoria* Oliv. were collected separately from this experiments which had descriptive morphological characters from other plant treatments, then DNA extraction was performed using DNeasy plant Mini Kit (Bio Basic).

2-DNA isolation

Isolation protocol of DNA adapted by Mohamed *et al.,* (2015) was as follows:

Genomic DNA was isolated from freshly leaves by DNeasy plant mini kit (bio basic). DNA quality was checked by means of absorbance ratios A_{260}/A_{280} through a UV-spectrophotometer where DNA is pure with a ratio A_{260}/A_{280} from 1.8- 2.0. Moreover, using electrophoresis in 1% agarose gel with ethidume bromied, a qualitative check for DNA samples was done.

3-Polymerase Chain Reaction

Genomic DNA was used as a template for Polymerase Chain Reaction (PCR) amplification using 8 SCoT primers in molecular assessment for the treatments. SCoT primers were designed from consensus sequence derived from the previous studies by Joshi *et al.* (1997); Collard and Mackill, (2009) and Mohamed *et al*., (2015) and procured from Biobasic Com. All SCoT primers were 18-mer and were from Dataset I which based on highly expressed genes as described by Sawant *et al.,* (1999). For SCoT primers design, the start codon ATG (+1, +2, and +3), 'G' at position +4, 'C' at position +5, and A, C, C and A at positions +7, +8, +9 and +10, respectively, were fixed (5´-----ATGGCTACCA---3´).

4-Polymerase Chain Reaction

Amplification reactions for the SCoT technique were achieved as described by Fathi *et al.,* (2013) and Xiong *et al*., (2011) respectively, and were carried out in Techni TC-512 Thermal Cycler as follows: One cycle at 94 ºC for 4 min followed by 40 cycles of 1 min at 94 ºC, 1 min at annealing temperature 57 ºC for the 2 min. at 72 ºC, followed by 72º C for 10 min, the reaction was finally stored at 4 ºC.

4-Gel Electrophoresis

Amplified products were loaded and separated on a 1.5% agarose gel with Ethidium bromide and from 50bp to 1500bp DNA Ladder markers 50 bp. The run was carried out for about 30 min at 100 V in mini-submarine gel BioRad.

5-Gel reading and analysis

DNA banding pattern photos were photographed using Bio-1D Gel Documentation system and were analyzed by GelAnalyzer3 software which scoring clear amplicons as present (1) or absent (0) for each primer and entered in the form of a binary data matrix. From this matrix, DNA-profiles were performed for

ISSR and SCoT techniques according to Adhikari *et al.* (2015). Depending to binary data matrix, Molecular distances MD (Dissimilarity) were calculated by Dice coefficient (Nei and Li, 1979) and cluster analysis was performed agglomerative hierarchical clustering (AHC) analysis derived from Unweighted pair-group average UPGMA method using XLSTAT.7 software.

RESULT AND DISCUSSION

- **Effect of colchicine efficiency on variability characters of seeds and seedlings:**

Can notice from the Table (1) that the seed germinations percentage (SGP) ranged from 25% to 100% with mean 61.45%, while the broadest range was attributed to the Shoot Stem length (SST) in mean, maximum, minimum, and range (18.36, 63.5, 6.5. and 57.0 cm) respectively, also the number of leaf per seedling (LN) ranged between 52.0 and 6.0 leaf, where largest leaf area (LLA) ranged from 29.58 to 2.84 with mean 11.29 cm^2. The variation data of the other characters studied in this experiment also present in Table (1). Positive variation between seed and seedlings characters was obtained from this study it is a good opportunity and important for selection and breeding among Gall Oak tree seedling populations. The increase in cell size is due to the increase in nuclear content, which causes cell splitting reduction whilst their growth results on account of cell enlargement. Moreover, Sattler *et al.,* (2016) and Manzoor *et al.,* (2018) proved that polyploidies are faced with increasing plant body size in terms of morphological characteristics.

Table (1):- Show the variation in seeds and seedlings of *Quercus infectoria* Oliv. characters among treated and untreated seeds with colchicine.

Seed and Seedling Characters	Mean	Maximum, Minimum, and Range	Standard Deviation (SD.V.)	Coefficient of Variation (C.V.)
SGP (%)	61.458	(100.000 - 25.000) 75.000	32.922	20.233
SST (cm)	18.361	(63.500 - 6.500) 57.000	38.874	7.137
SD (mm)	4.443	(8.920 - 1.710) 7.210	23.696	1.053
IL (cm)	1.19014	(2.985 - 0.450) 2.535	0.443	37.198
BN	1.247	(6.000 - 0.000) 6.000	117.516	1.465
LN	18.983	(52.000 - 6.000) 46.000	45.569	8.651
LL (cm)	5.961	(9.200 - 3.100) 6.100	19.241	1.147
LW (cm)	3.045	(5.300 - 1.500) 3.800	22.190	0.676
LBR	1.992	(2.826 - 1.319) 1.507	14.924	0.297
LT (mm)	0.174	(0.260 - 0.120) 0.140	11.544	0.020
LLA (cm^2)	11.297	(29.586 - 2.842) 26.744	38.098	4.304
SDW (g)	2.231	(8.710 - 0.543) 8.167	49.079	1.095
RL (cm)	51.982	(89.10 - 20.00) 69.100	23.395	12.161
RD (mm)	5.588	(8.650 - 2.100) 6.550	24.461	1.367
SRN	2.437	(8.000 - 0.000) 8.000	82.210	2.003
RDW (g)	4.825	(12.959 - 0.736) 12.223	43.677	2.107
Ch a (mg/g)	11.314	(17.872 - 7.219) 10.653	23.673	2.678
Ch b (mg/g)	3.5250	(6.293 - 0.703) 5.589	32.5121	1.1460
Ch ab (mg/g)	14.839	(24.165 - 9.883) 14.282	24.764	3.675
TC (mg/g)	1.38	(2.08 – 0.83) 1.25	23.127	0.032
SL (µm)	26.775	(56.413 - 15.562) 40.851	21.234	5.685
SW (µm)	17.542	(41.835 - 10.155) 31.68	20.904	3.667
SD (per 1 mm^2)	246.546	(482.74 - 43.10) 879.29	26.941	66.423

- Effect of interactions between colchicine concentrations and exposure times on of seeds and seedlings characters:

1. Seed germination percentage

The results show in Table (2) the maximum seed germination percentage was found in when seed treated with distilled water for 72 h. in the rate of 92.5%, while the lowest percentage of seed germinations (37.50%) in treatment combinations (3000 mg/l of colchicine at 92 h), and in less than (59.5%) from the control. However, a high colchicine concentration and a longer immersion provided a reduction in germination percentage; his indicated that was an inversely related between seed germination percentage with colchicine concentrations and the soaking period and reduce the living population of explants in some treatments to less than half.

Zlesak *et al.,* (2005) inspected that the decrease in germination percentage of treated seedlings with colchicine could be back to the poor seedling vigor, resulting in an ability to overcome the toxic effect of colchicine on

germination percentage. Whereas, Chaicharoen and others (1995) investigated that the colchicine not only prevented the function of the division mechanism but also may kill cells and tissues of plants. Reduction in percentage germination with increasing concentration has also been reported in other forest tree species, for example, in *P. acerifolia* by (Liu *et al.*, 2007), *R. pseudoacacia* L. and *C. silique* L. by (Omar 2007), Eucalyptus app. by Maritz (2008), *Q.aegilops* L. by(Toma 2015) and *A. foeniculum* L. (Talebi *et al.*, 2017), *R. pseudoacacia* L. and *C. siliquastrum* L. by Suliman (2020), *N. cadamba* by Eng, *et al.*, (2021).

2. Seedling Morphological Characters

Results show in Table (2) the taller shoot stem (50.03 cm) founded in combination treatment (2000 mgl^{-1} for 72 h), while thicker shoot stem (8.04 mm) achieve it by colchicine solutions 1000 mgl^{-1} for 48 hour of soaking. Also, the longer distance between leaves was obtained in interaction treatment 2000 mgl^{-1} for 92 h, although the best seedlings produced higher biomass of branches (4.33 branches). Maximum number of leaves (45 leaves) were produced when seeds induced in colchicine treatment 3000 mgl^{-1} for 48 h and 24 h separately, and greater dry weight of shoot system (4.87 gm) obtained in the treatment 1000 mgl^{-1} for 48 h. variation in morphological characters which appears in Figures (1 and 2).

Producing seedlings that had longer and wider stem in this study it is an important morphological feature as the larger stem will result in higher productivity Oak trees, especially for timber tree species in features. The "Gigas" phenomenon mentions to the size of cells (larger organ size) due to the doubling of the chromosomes number (Eng and Ho, 2019; Salma *et al.*, 2017). Although, Iannicell *et al.*, (2020) mentioned the cell size in polyploidies is usually larger and increasing cell size is the most well-known polyploidy effect in plants. However, the final biomass size may not always increase in plants.

3. Root system affected by colchicine

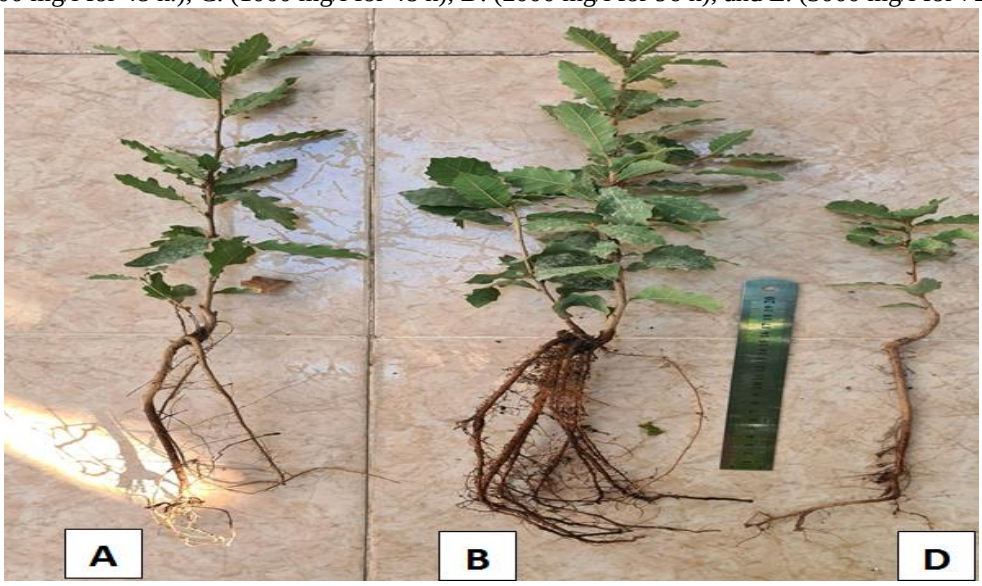
Duncan's multiple range tests of interaction between colchicine concentrations dose and period of seed soaking which illustration in Table (2) indicate the combination treatment (3000 mgl^{-1} for 24h) gave length root (81.63 cm), whereas the seedlings from colchicine treatment combinations (1000 mgl^{-1} through 48 h.) show more efficiency to induction on root diameter character and recorded the higher means values (8.04 mm) by comparison to the control plants (3.88 mm). On the other hand, plants with a greater mean number of secondary roots (6.33) produced from seeds treated with 500 mgl^{-1} of mutagenic substance soaked for 24 hour. While, the lowest mean number of secondary roots per seedling (0.33) was from 0.00% colchicine for 72 h. of immersion. While advanced root dry weight (8.39 gm) was detected in seedlings treated with 500 mg l-1% of colchicine and soaked for 48 hours and opposite of this, the minimal dry weight of root (2.08 gm) was recorded in control plant treated itis seeds with 0.0 mgl-1 of colchicine solution for 24 hours (controls). Polyploids plants may have larger leaves and flowers, thicker stems and roots, darker green leaves, an increased width-to-length ratio of the leaves, a more compact growth habit, and a higher tolerance to environmental stress (Liu *et al.*, 2007; and Lavania *et al.*, 2012). Moreover, Dudits *et al.*, (2016) described on some traits of autotetraploid willow genotypes as their larger leaf and root systems, greater net photosynthetic CO2 uptake, improved photosynthetic functions, slower primary growth, and increased shoot diameter might jointly determine biomass yield. This data agree with the data of Omer, (2008); Hamid Reza *et al.*, (2013); Toma, (2015); Dudits *et al.*, (2016); Suliman, (2020) and disagreed with Kerdsuwan and Te-chato (2012).

Table(2):- Combination between colchicine concentrations with seed soaking duration effects on seed and morphological characters of *Quercus infectoria Oliv*. seedlings

Colchicine mgl-1	Duration (h)	Treatment No.	SG	SL	SD	IL	BN	LN	RL	RD	RN
0.00	**24**	**T1**	87.50 a	11.23 i	3.50 e-h	0.84 hi	0.67 e	9.00 h	49.27 cde	4.19 i-j	0.67 gh
0.00	**48**	**T2**	81.67 ab	12.30 i	2.56 h	0.73 i	1.00 de	11.33 gh	44.80 cde	3.88 i-j	0.33 h
0.00	**72**	**T3**	**92.50 a**	12.24 i	3.77 e-h	0.80 hi	0.67 e	12.00 gh	37.92 e	4.35 g-j	2.00 d-h
0.00	**96**	**T4**	89.17 a	11.47 i	2.91 gh	0.68 i	1.33 cde	10.00 h	38.50 e	4.27 h-j	1.33 e-h
500	**24**	**T5**	79.17 abc	27.53 cd	4.79 b-f	1.86 b	3.33 abc	32.00 bc	67.83 ab	6.71 a-d	5.00 abc
500	**48**	**T6**	57.50 c-f	23.07 edf	3.39 gh	1.37 d-g	3.00 a-d	28.67 bcd	59.93 bc	6.89 abc	4.00 a-e
500	**72**	**T7**	75.83 a-d	24.90 de	4.82 b-f	1.77 bc	2.00 e-d	24.00 cde	67.50 ab	**7.95 a**	**6.33 a**
500	**96**	**T8**	76.67 abc	24.63 de	3.64 e-h	1.01 ghi	1.67 cde	22.00 e-f	38.73 e	6.25 b-f	0.33 h
1000	**24**	**T9**	54.17 e-f	24.00 de	4.82 b-f	1.29 e-g	2.00 b-e	17.33 e-h	43.90 de	6.32 b-f	2.33 c-h
1000	**48**	**T10**	75.00 a-d	33.40 bc	**8.04 a**	1.85 bcd	3.33 abc	27.00 cde	68.67 ab	**8.04 a**	3.33 b-g
1000	**72**	**T11**	54.17 e-f	26.77 d	5.80 b	1.30 e-g	2.67 a-c	28.67 bcd	68.70 ab	5.43 d-i	2.33 c-h
1000	**96**	**T12**	50.00 efg	21.57 d-g	5.65 bc	1.30 e-g	4.00 ab	28.00 bcd	45.33 cde	5.71 c-g	2.00 d-h
2000	**24**	**T13**	54.17 e-f	21.77 d-g	4.24 b-g	1.04 f-i	1.67 cde	25.00 cde	59.27 bcd	6.48 b-e	3.33 b-g
2000	**48**	**T14**	65.50 b-e	19.08 e-h	4.07 c-h	1.25 fg	1.00 de	21.00 d-g	48.58 cde	5.66 c-h	4.33 a-d
2000	**72**	**T15**	62.50 e-f	38.90 b	4.73 b-f	1.65 b-d	3.00 a-d	**42.67 a**	67.50 ab	7.06 abc	3.67 a-f
2000	**96**	**T16**	50.00 e-f	**50.03 a**	4.61 b-f	**2.30 a**	2.67 a-e	37.67 ab	56.73 bcd	6.93 abc	6.00 ab
3000	**24**	**T17**	63.33 b-e	28.10 cd	5.11 b-e	1.42 c-d	2.67 a-e	45.00 a	81.63 a	7.46 ab	1.00 fgh
3000	**48**	**T17**	52.50 efg	16.10 ghi	5.78 b	1.14 fgh	**4.33 a**	28.00 bcd	56.70 bcd	5.13 e-i	3.33 b-g
3000	**72**	**T19**	40.83 fg	17.03 f-i	5.35 bcd	0.98 ghi	2.00 e-d	20.00 d-g	69.10 ab	6.45 b-e	4.00 a-e
3000	**96**	**T20**	**37.50 g**	14.68 hi	4.17 b-g	0.72 i	1.67 cde	13.67 fgh	40.92 e	4.98 f-i	1.33 e-h

- **Note: The columns with the same letters do not differ**

Fig(1):- Variations in shoot length, diameter, number of branches and leaves per seedlings of Gall Oak seedlings treated with different colchicine treatments. A. (0.0 mg/l for 24h), B. (2000 mg/l for 24 h), and C. (1000 mg/l for 48 h.), C. (1000 mg/l for 48 h), D. (2000 mg/l for 96 h), and E. (3000 mg/l for 72 h).

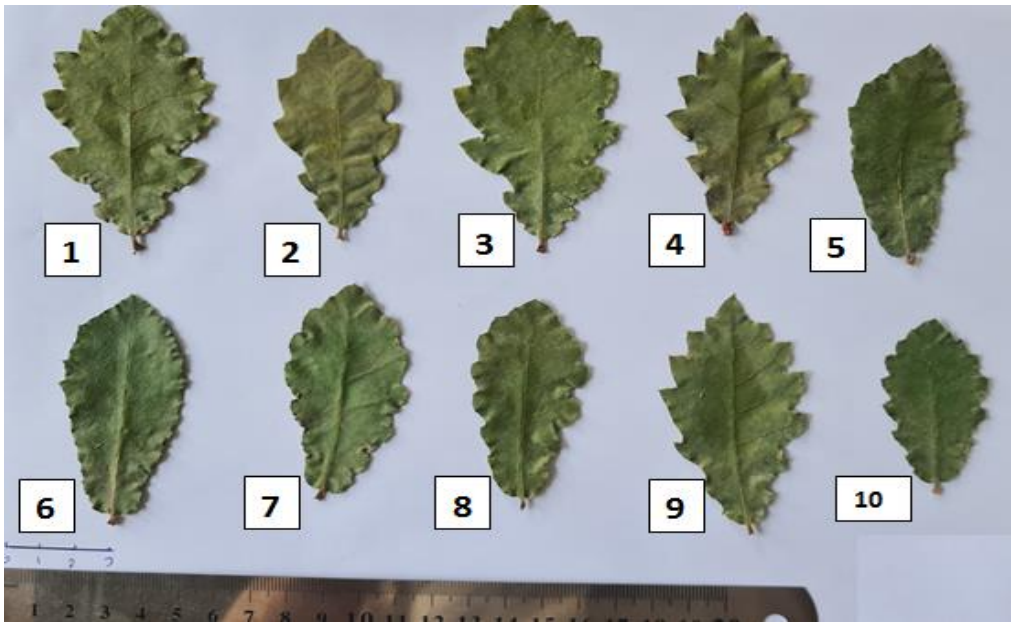


Fig(2):- Variations in root system of Gall Oak seedlings treated with different colchicine treatments such as A. (500mg/l for 72 h), B. (2000mg/l for 92 h.), and C. (0.00mg/l for 24 h).

4- Leaf morphological effected by colchicine

According to Table (1) high variation in leaf characteristics was observed in the greenhouse results of colchicine treatments. The phenotyping experiment demonstrated that the autotetraploid plants of Gall Oak developed a larger foliage area and play an important role in photosynthetic activity, and was found higher in polyploid genotypes in the field (Fig. 3). Credence on Table (3) of Duncan multiple range test, longer leaf was achieved (7.90 cm) when seeds treated with ($500mgl^{-1}$) of colchicine concentration for 24 h. of soaking period, while width leaf (4.30 cm) and the largest plant leaf area ($23.23\ cm^2$) found in colchicine treatment also in colchicine concentrations 500 mg/l of but in immersion time for 96 h. colchicine concentration for 12 h. whereas, the thicker leaf recorded in ploidy level treatment ($3000\ mgl^{-1}$). While, smallest leaves in length, width, area, and thickness were established in untreated seeds as show in Table (3).

Xu *et al.,* (2016); Zhou *et al.,* (2017); and Suliman (2020) published that polyploid plants have thicker leaves. The leaf thickness can be analysed using transverse sections of paraffin with embedded leaves (Zhou *et al.,* 2017). Similarly, Major goal in many breeding programs is the development of compact growing plants, the polyploidy in plants causes an increase in the size of vegetative structures.



Fig(3):- Variations in leaf shape, size, and color of Gall Oak seedling at 8 months old, leaves treated with different colchicine treatments.

5-

Leaf chemically contain effected by colchicine

The chlorophyll in the leaf is the key parameter for the characterization of the physiological performance of plants, including the determination of vegetation indices with woody species (Lu *et al.,* 2015). Under greenhouse conditions, the leaves of the treated plants contained significantly greater concentrations of chlorophylls and were darker than the diploid plants.

For the blends between colchicine concentrations with soaking periods impacted by using Duncan's multiple range tests which appear in Table (4), designates significant differences between treated and untreated seedlings for the amount of chlorophyll in leaves. The maximum amount of chlorophyll a and totally chlorophyll (ab) were observed in combination (3000 mgl^{-1} colchicine for 24 h. of soaking) with value (16.2o and 21.17 mg/g) respectively. Where, the greater amount of chlorophyll b was achieved from leaves of seedling induced by interaction of 2000 mgl^{-1} of colchicine concentrations with 48 h period of soaking. And as usual the minimum value of chlorophyll contain were recorded in the control treatment. Eng *et al.,* (2021) investigated the green leaves from explants treated with colchicine can be related to the chloroplast number in cells and leaf thickness. Darker green leaves have been observed in several colchicine induced polyploids plants, such as *A. andraeanum* (Chen *et al.,* 2011); *M. esculentum* (Zhou *et al.,* 2017); *P. ternate* (He *et al.,* 2012); *Q. aegilops* L. (Toma 2015); *A. foeniculum* L. (Talebi *et al.,* 2017); *R. pseudoacacia* L. and *C. siliquastrum* L. (Suliman 2020); and *N. cadamba* by Eng, et al., (2021).

6- Tannins contain affected by colchicine

Table (3) revealed the ploidy levels combination (500 mgl-1 and 48 h) provided that was more efficient to effect in this character and produced leaves contain higher amount of tannins by 2.0 mg/g. Contrary to that found the poorer quantity of tannins (0.90 mg/g) was produced in leaves of seedling treated its seeds soaked for 72 h in 2000 mgl^{-1} of colchicine. It is very important from this result to produce a genotype that leaves contains less amount of

these compounds to produce trees in the future producing leaves, branches, and seeds of this species more palatable by domestic animals and other organisms.

Tannins are natural polyphenolic compounds broadly distributed in the plant kingdom in the leaves, bark, fruits, and other parts. Additionally, those tannins have many biological functions in humans and animals and are used mainly in the pharmaceutical and cosmetic industries. Oaks tree species have been an important source of tannins and dyes, Oak bark and leaves were often used for tanning leather (Amr and others, 2021). Other products include fuel, fodder, and building materials throughout their ranges (Nixon, 1997).

The genetic variability of trees affects the chemical composition of tissues. This determines herbivore impact and, consequently, herbivore performance (Solla *et al.,* 2016). On the other hand, Ghasemi *et al.,* (2021) mentioned these polyploidy plants can also be used as a new species or genotype, which can be used in future reproduction programs to improve products. Due to the great importance of polyploidy, it has been induced in many economically important products (Lertsutthichawan *et al.,* 2017; Manzoor *et al.,* 2019).

7- Physiological character affected by colchicine

As shown in Table (1) and Figure (4), there was high variation in stomata length, width, and numbers on the abaxial surface of the leaves, also a great significant effect of different colchicine treatments by Gall Oak seeds on this characters, the stomata of tetraploid *Q. infectoria* were bigger than those of the diploid plants and stomatal number was significantly decreased in the tetraploid plants compare to diploid plants. Table (3) showed significant effect of mitotic inhibiter colchicine with soaking periods in stomata characters, from this test appear that immersed seeds in high colchicine concentration and duration time (3000 mgl^{-1} foe 72 h.) showed the superior size of stomata (47.25 µm length and 34.41 µm width) and lower density (66.21 guard cell per $1mm^2$) than plants treated with low and no colchicine conditions which produce plants had smallest and high number of stomata.

Islam *et al.,* (2022) investigated the successful technique for developing novel traits is a polyploidization method. Polyploidy causes significant transcriptomic and regulatory changes that bring physiological and morphological changes. Similar results were founded in other studies such as Thao *et al.,* (2003); Omar (2008); Dudits *et al.,* (2016); Anitha *et al.,* (2017); Parsons *et al.,* (2019); and Suliman (2020).

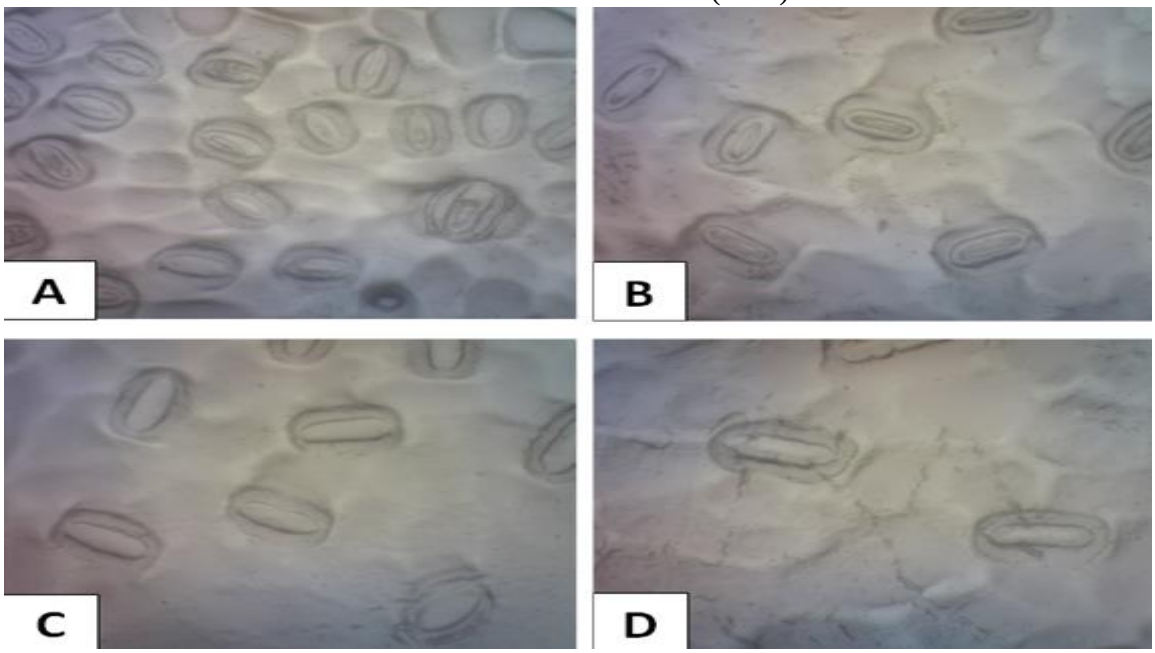


Fig (4):- Show the variation on stomata size and frequency at 40X for different polyploidy level: A. For control plants (0.00 mgl-1 for 48h), B. for (1000 mgl-1 for 24h), D. for (2000 for 48h), and D. for (3000 for 72h).

Table (3): -Combination between colchicine concentrations with seed soaking duration effects on morphological, chemical, and physological characters of *Quercus infectoria Oliv*. seedlings leaves

Colchicine mgl-1	Durat (h)	Treat No.	LL	LW	LT	LLA	Ch a	Ch b	Ch ab	TC	SL	SW	SD
0.00	**24**	**T1**	5.17 e-f	2.50 gf	0.17 c-f	10.33 efg	8.80 hij	2.68 d-h	11.49 ghi	1.5 d	18.48 e	12.69 f	428.38 a
0.00	**48**	**T2**	4.87 e-f	2.83 d-g	0.15 f	8.85 g	8.45 ij	2.38 fgh	10.83 hi	1.5 ed	17.77 e	12.26 f	413.78 a
0.00	**72**	**T3**	5.00 e-f	2.47 g	0.15 ef	10.73 efg	9.66 f-j	2.54 fgh	12.01 f-i	1.5 ed	18.71 e	12.25 f	370.68 a
0.00	**96**	**T4**	5.43 c-f	2.67 e-g	0.16 d-f	11.34 efg	9.32 h-j	2.11 h	11.44 ghi	1.51 d	19.89 e	12.74 f	376.43 a
500	**24**	**T5**	**7.63 a**	3.77 abc	0.19 cd	14.47 d-g	12.14 def	4.00 a-f	16.13 cde	1.9 b	27.00 cd	18.67 cde	234.13 bc
500	**48**	**T6**	**7.90 a**	4.10 ab	0.18 c-f	14.21 d-g	13.12 bcd	4.08 a-f	17.21 b-e	**2.0 a**	29.89 cd	16.17 ef	224.13 bcd
500	**72**	**T7**	**7.70 a**	4.10 ab	0.18 c-f	20.15 abc	12.43 b-e	3.16 d-h	15.59 de	1.8 b	28.48 cd	19.09 cde	233.33 bc
500	**96**	**T8**	6.63 a-d	**4.30 a**	0.17 e-f	**23.23 a**	7.73 j	2.91 d-h	10.64 i	1.7 c	33.33 c	20.33 cde	216.34 b-e
1000	**24**	**T9**	6.67 a-d	3.57 a-d	0.18 cde	12.97 d-g	11.05 d-h	3.66 a-h	14.71 d-g	1.4 e	26.67 d	18.00 cde	242.64 b
1000	**48**	**T10**	7.43 ab	3.87 ab	0.18 cde	19.36 a-d	12.35 cde	4.23 a-e	16.57 cde	1.3 f	29.64 cd	19.45 cde	176.78 cde
1000	**72**	**T11**	7.13 ab	3.57 a-d	0.19 cd	16.17 b-e	10.23 e-j	3.85 a-g	14.09 e-h	1.3 fg	27.33 cd	18.33 cde	209.77 b-e
1000	**96**	**T12**	4.33 f	2.40 g	0.18 cde	9.05 fg	12.44 b-e	4.31 a-d	16.75 cde	1.1 h	28.67 cd	16.33 def	212.75 b-e
2000	**24**	**T13**	6.73 a-d	3.50 a-e	0.17 e-f	12.85 d-g	14.68 abc	4.49 ab	19.17 abc	1.1 hi	31.64 cd	20.10 cde	163.33 de
2000	**48**	**T14**	7.37 ab	3.40 b-e	0.18 cde	12.23 efg	12.58 b-e	**5.00 a**	17.58 bcd	1.1 i	28.53 cd	20.55 cd	175.28 cde
2000	**72**	**T15**	7.33 ab	3.37 b-e	0.18 cde	16.76 a-e	9.38 g-j	2.20 gh	11.58 ghi	0.9 j	32.61 cd	19.38 cde	198.27 b-e
2000	**96**	**T16**	7.67 a	4.20 ab	0.18 c-f	15.74 b-f	11.41 d-g	3.49 a-h	14.90 def	1.3 g	41.51 b	25.11 b	178.16 cde
3000	**24**	**T17**	6.33 a-e	3.50 a-e	0.23 ab	21.34 ab	14.91 ab	4.03 a-f	18.94 abc	1.1 hi	32.01 cd	20.87 c	156.32 e
3000	**48**	**T17**	6.83 abc	3.93 ab	0.23 ab	21.78 ab	10.94 d-i	3.41 a-h	14.35 d-g	1.1 hi	32.43 cd	20.77 c	162.06 de
3000	**72**	**T19**	6.33 a-e	3.33 b-f	**0.24 a**	14.42 d-g	15.64 a	4.74 ab	20.38 ab	1.2 h	**47.25 a**	**34.41 a**	**66.21 e**
3000	**96**	**T20**	5.83 b-f	2.93 c-g	0.20 bc	11.30 efg	**16.20 a**	**4.97 a**	**21.17 a**	1.0 i	28.29 cd	19.23 cde	161.39 e

- **-Note: The columns with the same letters do not differ**

9- Polyploidy detections

A. Stomata length

Measurement of stomatal length, width, and density in the green house showed they were high variations between polyploidy and diploidy plants and could use to prescreen the selected *Q. infectorias* seedlings, to identify putative polyploids (Table, 1). In general, the putative polyploids displayed a significant increase in stomatal guard cell length and width with and decrease in stomatal frequency compared with the diploid control seedlings as shown in Figure (4).

To summarize results, the stomata length for 300 plant leaves was ranged from 15.56 to 56.41 µm with overall mean 26.77 ± 5.68 µm, whereas, diameter of stomata varied from 10.15 to 41.3 µm with mean 17.54 ± 3.66 µm, and the maximum number of guard cells in per area was 482.74 stomata and less number was 43.10 cells with average 246.54 ± 66.42 stomata in imm^2, the data present in Table (1). Similarly, it was noted from table (3) there where great significant inductions and effects at 1% probability level of mutagenic material colchicine solutions on stomata guard cells elongation.

As the results show the length of stomata for (60) leaves of diploid plants was 18.23 ± 2.40 µm and for autopolyploid plants treated with colchicine was 29.33 ± 5.61 µm. therefore according to the data that we got it and the researcher as aBeck et al., 2003; Gu et al. 2005; Stanys et al. 2006; Martiz, 2008; and Suliman 2020, the length of stomata guard cell for the diploids was between 15.00 - 21.00µm, while for triploid plant were from 22.00 to 32.00 µm,

whereas for tetraploid ranged between 33.00 to 43.00 µm, and greater from 44.00 µm stomata length for petaploid Gall Oak plants.

According to the Table (4), the higher triploids plants (3n) were induced when seeds treated with 500 and 2000 mgl^{-1} for 24 h. of soaking in value (7) with rate (46.67 %), whereas the ploidy level combination (2000 and 1000 mgl^{-1} of colchicine concentrations and to expose it for 48 h.), produced maximum tetraploids plants (4n) in number 5 and 4 and at a rate 33.33 and 26.67% respectively, also depending on the stomata length for detection polyploidy plants the only one pentapolid (54.08 µm stomata distance) was achieved in plants were treated with followed by colchicine solutions 2000 mg/l for 96 h. The total number of diploids, triploids, tetraploids and pentaploid among 300 plants were (186, 79, 34 and 1seedlings) separately (Table 4).

Cohen and Yao 1996; Beck *et al.*, 2003; Gu *et al.*, 2005; Stanys *et al.*, 2006; Liu *et al.*, 2007, Maritz, 2008; Suliman, 2020, use stomatal length and frequency as dependable indicators of ploidy in a number of species, including woody plants

Table (4):- Combinations among altered colchicine concentrations and soaking periods effect on rate of poly level induction by seeds of *Q. infectoria* Oliv.

Colchicine Concentration Mg/L	Soaking Duration (h)	No. of Seedling Scanned	Ploidy level							
			Diploid (2n)		Triploid (3n)		Tetraploid (4n)		Pentaploid (5n)	
			No.	%	No.	%	No.	%	No.	%
0.0 control	24	15	15	100.00	0	0.00	0	0.00	0	0.00
500		15	7	46.67	7	46.67	1	6.67	0	0.00
1000		15	11	73.33	2	13.33	2	13.33	0	0.00
2000		15	6	40.00	6	40.00	3	20.00	0	0.00
2000		15	5	33.33	7	46.67	3	20.00	0	0.00
0.0 control	48	15	15	100.00	0	0.00	0	0.00	0	0.00
500		15	7	46.67	6	40.00	2	13.33	0	0.00
1000		15	7	46.67	4	26.67	**4**	**26.67**	0	0.00
2000		15	7	46.67	3	20.00	**5**	**33.33**	0	0.00
3000		15	6	40.00	6	40.00	**3**	**20.00**	0	0.00
0.0 control	72	15	15	100.00	0	0.00	0	0.00	0	0.00
500		15	11	73.33	4	26.67	0	0.00	0	0.00
1000		15	11	73.33	4	26.67	0	0.00	0	0.00
2000		15	9	60.00	5	33.33	1	6.67	0	0.00
3000		15	7	46.67	6	40.00	2	13.33	0	0.00
0.0 control	96	15	15	100.00	0	0.00	0	0.00	0	0.00
500		15	9	60.00	5	33.33	1	6.67	0	0.00
1000		15	8	53.33	5	33.33	2	13.33	0	0.00
2000		15	5	33.33	6	40.00	3	20.00	1	6.67
3000		15	10	66.67	3	20.00	2	13.33	0	0.00
Seed Number		300	186		79		34		1	

B. SCoT-PCR

Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) has been evidenced to be valuable in assessment the inter-population variations and phylogenetic relationships (Dilipan et al., 2017). While Ganie et al., (2015) mentioned it is the most widely used molecular technique which produces a PCR products fingerprint to distinguish between species and characterized by its simple, rapid, and inexpensive processing. Furthermore, it does not require previous knowledge and the sequences of the target DNA. The Gall Oak leaves in this study were used from 11 ploidy level plants treatments (1 untreated seed and 10 treated seeds with different colchicine solutions in various periods) and were young leaves for DNA isolation. In total, out of 12 SCoT primers 8 have shown amplifications and out of 8, eight primers revealed some degree of polymorphism among selected species. 8 SCoT primers were used to produce 33 RAPD bands (The total number of produced bands) with an average of 4.125 per primer. The produced bands ranged from 100 and 1000 bp (base pair).

Figure (5) show different SCoT primers that the DNA bands produced are clear and bright and no smears significantly affect the visualization of DNA bands. So, the identification and classification of monomorphic and polymorphic DNA bands can be done. DNA bands that have relatively the same thickness show that the primers used are specific to amplifying the target DNA.

Primer SCoT 2 (ACC ATG GCT ACC ACC GGC) and SCoT 14 (ACC ATG GCT ACC AGC GCG) produced the highest number of bands (7 and 6) respectively (Table 9 and Figure 6 and 7). Table (5) shows that primers altogether produced 14monomorphic bands for the studied five strains, while, the number of polymorphic bands was 19 leading to 57.57% polymorphism. The polymorphic bands included (3) polymorphic unique bands and (16) polymorphic without unique bands. In the other hand, the higher percentage of polymorphic was recorded in SCoT primers 6 and SCoT primers 10 in value 100 and 75% respectively where less polymorphic percentage in SCoT primers 14 in the rate 50%.

Amplification band patterns show genetic variation among Gall Oak plants in this study. Genetic diversity has a key role in forest tree population adaptation to changing climate, as well as planning future conservation programs (Kesic *et al.*, (2021). Therefore the genetic variations based on the pattern of DNA band results of this amplification can be used as a basis in the process of breeding of plants.

Random amplified polymorphic (RAPD) and inter-simple sequence repeat (ISSR) markers have been used often by several researchers to

investigate genetic diversity between species among the DNA markers; such as microsatellite loci repeats will amplified with the primers ssrQpZAG9, ssrQpZAG110 (Steinkellner *et al.*, 1997), ssrQrZAG7, ssrQrZAG20 (Kampfer *et al.*, 1998), MSQ4 and MSQ13 (Dow *et al.*, 1995) — by the PCR multiplex protocol described by Dzialuk *et al.*, (2005). Furthermore, the primers (QrZAG11, 96, 112, QpZAG110, QrZAG30, 87 and 101, QrZAG101 QrZAG30 and 87, MSQ13 and QpZAG9, QpZAG1/5, QrZAG15 and 20) and PCR amplification according to Sheet *et al.*, (2018); and Kesic *et al.*, (2021).

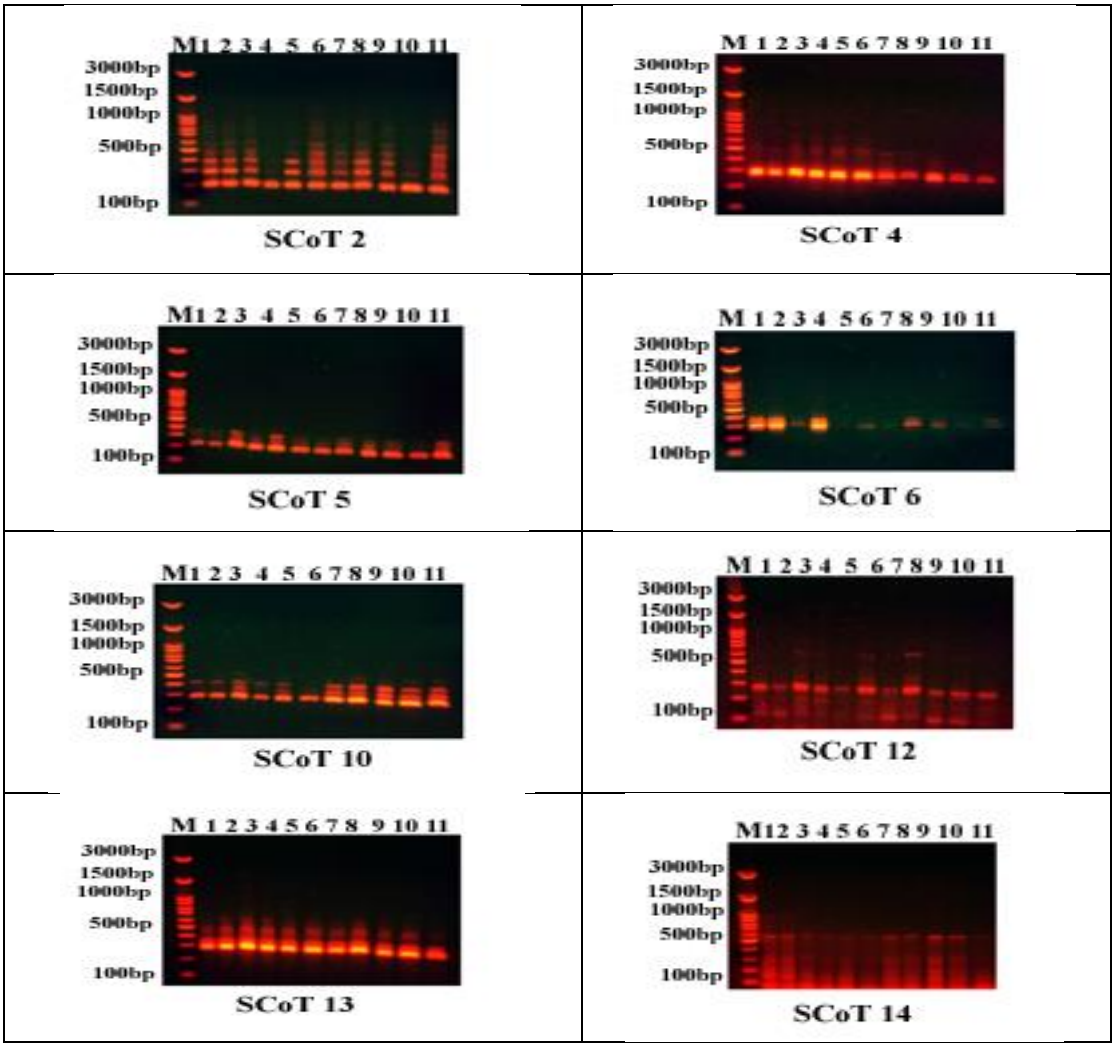


Fig (5):- Agarose gel electrophoresis of RAPD banding of *Quercus infectoria* Oliv. plants using primers SCoT2 to SCoT14. Lanes from 1 to 5 refer to the different genotypes of diploid plants controls and polypoid plants treated plants. 1. Represent dioploid plants treated with (disttlid water for 48 h), 2. Treated seedling with (500 mgl^{-1} of colchicine and soaked for 48h), 3. (500 mgl^{-1} for 72h.), 4. (1000 mgl^{-1} for 48h.), 5. (1000 mgl^{-1} for 48h.), 6. (2000 mgl^{-1} for 24h.), 7. (2000 mgl^{-1} for 48h.), 8. (2000 mgl^{-1} for 72h.), 9. (2000 mgl^{-1} for 92h.), 10. (3000 mgl^{-1} for 24h.), and 11. (3000 mgl^{-1} for 72h.).

Table(5):- of primer name, sequences,totalbands number, monomorphic bands, polymorphic bands, unique bands, and percent of polymorphic bands

Primer Name or Code	Sequences	Total Band	Monomorphic Band	Polymorphic band	Unique Band	Polymorphic %
SCoT 2	ACC ATG GCT ACC ACC GGC	7	3	4	1	57.14%
SCoT 4	ACC ATG GCT ACC ACC GCA	3	1	2	-	66.66%
SCoT 5	CAA TGG CTA CCA CTA GCG	3	1	2	-	66.66%
SCoT 6	CAA TGG CTA CCA CTA CAG	2	-	2	-	100%
SCoT 10	ACA ATG GCT ACC ACC AGC	4	1	3	1	75%
SCoT 12	CAA CAA TGG CTA CCA CCG	5	2	3	1	60%
SCoT 13	ACC ATG GCT ACC ACG GCA	3	3	-	-	-
SCoT 14	ACC ATG GCT ACC AGC GCG	6	3	3	-	50%
	Total	33	14	19	3	57.57%

CONCLUSIONS

The chromosomal duplication of forest species aims to maximize economic interest features, such as those related to development and forest gain. Keeping in mind the above views, this study aims at developing an effective polyploidization system in the *Quercus infectoria* Oliv. using colchicine treatment in seeds by soaking, to explore the possibility of generating new varieties with improved morphological, physiological, and anatomical characteristics.

High variability in phenotypical, chemical, physiological, anatomical, and cytological traits was obtained between treated plants and untreated plants, resulting in significant effects of chromosome doubling colchicine concentrations and period of inductions in character studies. An efficient polyploidization protocol for the studied was set up, and tetraploids were characterized for their morphological traits and plant architecture.

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