POLYPLOIDY INDUCED BY COLCHICINE IN *Robinia pseudoacacia* L. AND IT'S EFFECTS ON MORPHOLOGICAL, PHYSIOLOGICAL AND ANATOMICAL SEEDLING TRAITS

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ABSTRACT
This study was aimed to induce and produce a polyploidy *Robinia pseudoacacia* seedling by different concentrations of colchicine (0.0%, 0.5%, 1.0%, 1.5%, and 2.0%) in various exposure times (12, 24 and 48 h), using in two experiments. In the first, were applied to the seeds by immersing method. In the second, were applied to shoot apical meristems by dropping method. The results showed significant effects of different interactions between colchicine concentration and exposure period on the seed germination, seedling survival rate and seedling characters. By increasing of the colchicine concentration the seed germination and seedling survival rate decreased significantly in colchicine concentrations (0.15 and 0.2%). Frequency of stomata decreased and on the opposite the stomata length and width increased with increases the colchicine concentrations. Polyploidy seedlings show vegetative growth superiority yield compared to diploids seedlings, leaf area in treated plants was larger and with deep green pigmentation as compared to the control seedlings. Seed treatment method was found to be more efficient than shoot apical meristems treatment method in producing tetraploid and mixoploid plants, colchicine solutions at 0.1% and 1.5% in 24 h. and 48 h. was found most effectively produced polyploidy and inducing variation in both investigations.

Keywords: *Robinia pseudoacacia* L. breeding, polyploidy, colchicine, concentration induction.

استحداث التضاعف الكروموسومي بواسطة الكولشيسين وتأثيرها على صفات الشتلات *Robinia pseudoacacia* L. المظهرية والفسلجية والتشريحية

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المستخلص
تهدف هذه الدراسة لأستحداث و أنتاج شتلات روبينيا (*Robinia pseudoacacia*) متضاعف العدد الكروموسومي , من خلال استخدام تركيز مختلفة من الكولشيسين (0.0 ٪ ، 0.5 ٪ ، 1.0 ٪ ، 1.5 ٪ ، و 2.0 ٪) في أوقات التعرض المختلفة (12 و 24 و 48 ساعة) ، وذلك باستخدامها في تجربتين. في البداية، تم تطبيقها على البذور بواسطة طريقة النقع. طريقة الثانية ، تم تطبيقها على على القمة النامية للبادرات عن طريق التنقيط. أظهرت النتائج تأثير معنوي للتداخل بين تراكيز الكولشيسين وفترات التعرض عل معدل النجاة للبادرات وإنبات البذور وخصائص الشتلات. وبزيادة تراكيز الكولشيسين، انخفض معدل النجاة وإنبات البذور بشكل ملحوظ في تراكيز الكولشيسين العالية عند (1.5٪ ، 2.0٪). ومن جانب اخر، انخفض عدد الثغور وعلى العكس زادة طول وعرض الثغور مع زيادة التراكيز. كما اظهرت شتلات المتضاعفة تفوقا معنويا في النمو الخضري مقارنةً بشتلات الغير المتضاعف، وكانت مساحة السطحية للاوراق في النباتات المعالجة أكبر و ذات تصبغ أخضر داكن مقارنةً بالشتلات غير معالجة. ووجد أن معاملة البذور كان أكثر كفاءة مقارنة بمعاملة القمم المرستيمية بمادة كولشيسن في أنتاج نباتات رباعية و ثلاثية التضاعف الكروموسومي, محلول كولشيسين عند تركيز (1.0٪ و 1.5٪) وباوقات (12 ساعة و 48 ساعة) كانت أكثر فعالية في أستحداث الأختلافات وأنتاج نباتات متضاعف الكروموسومي (polyploidy) في كل التجارب.

الكلمات المفتاحية: *Robinia pseudoacacia* L., تربية, تضاعف الكروموسومي, كولشيسين تراكيز الأستحداث

***Received:12/6/2019, Accepted:25/9/2019**

INTRODUCTION

Robinia pseudoacacia L. are leguminous family, one of a fast growing, nitrogen fixing, broad leaves and deciduous tree species. It is native to southeastern North America but has been naturalized at temperate regions around the world. *Robina* is the second most widely planted hardwood genus in the global. It is only species that produce white flowers from the other *Robinia* species. From to twenty species under the genus *Robinia* have been recorded in world over, but the only multipurpose and economic tree recognized globally is *Robinia pseudoacacia* Linn. Because easily propagation and produces large amounts of dense and good texture wood with high combustion potential on a short rotation, it is preferred for many wood products. According to Davis (7), species invasions (Black locust) are one of the main ecological consequences of global changes in climate and land use, the importance of this species is adaptability to poor soils with high temperature variations is expected to increase. Moreover *R. pseudoacacia* are adaptable to environmental extremes such as drought, air pollutants and high light intensities, efficiency for improving soil chemical properties, fertility, reclaiming and rehabilitating degraded soils, the role of improvement the soil due to its nitrogen-fixing abilities, with roots nodulated by diverse Rhizobium communities (17, 22, 28). More than, ability to sequestering carbon (26), improving biodiversity (9) more than had being exploited as a potential source for bio-oil production (3, 10). Moreover, black locust also is important to establish crop protection shelterbelts and providing as habitats for small mammals (5, 23). Else, the tree is widely planted for honey production. The induction of polyploids is an effective and useful tool for doubling the chromosome number of a species. Forestry ploidy breeding is a very important method to acquire new variety (13). Whereas, Zhang *et al.*, (44) indicated the inducing artificial polyploidy is an effective method to encourage genetic enhancements in forestry breeding. Van de Peer *et al.*, (38) believed that the polyploidization to be a major mechanism of adaptation, speciation and recognized as a major force in evolution. Autopolyploidy is usually related with increased plant, organ, and cell sizes, so polyploids generated through plant breeding have been used as tools to increase crop yields. Induction of polyploidies has been achieved by using different chemical reagents, such as colchicine ($C_{22}H_{25}NO_6$), which considered one of the efficient chemical agents of chromosome doubling on any actively growing tissue such as, root, apical meristem, flower buds, germination seed and bud consist of two self-perpetuating tissues, the outer tunica and the inner corpus. In addition, it is widely applied and successfully used to modify the chromosome numbers in diverse plant species (16, 17, 27). This study is aimed at developing an effective polyploidization system in the *R. pseudoacacia* using colchicine treatment in seeds by soaking and shoots growing tips by dropping, to explore the possibility of generating new varieties with improved morphological, physiological and anatomical characteristics.

MATERIALS AND METHODS

This study was conducted in the laboratory and nursery of the Forestry Department, Collage of Agriculture, during 2016-2017. In this used two independent experiments with same polyploidization substanc. Therefore the experimental designed were be two factorial with one mitotic inhibitors (colchicine), five concentrations and three time durations (5x3) with three replications using Randomize Complete Block Design (RCBD).

Plant materials and seed preparation

Healthy and brown mature pods of *Robinia pseudoacacia L.* (2*n* = 2x=24) (24), were harvested from five open-pollinated, vigor trees, taller straighter bole and fine attractive branching habit were selected in November 2016. This grows at the field of Collage of Agriculture, University of Duhok (42°, 52′, 02″ E, 36°, 51′, 38″ N, and altitude 456m. over sea level) of Kurdistan region, North of Iraq. The pods were cleaned; seeds were extracted by crushing the dried pods and removing from the dust, putted in moisture-proof containers, such as polyethylene bags and stored in a cooling chamber in a low temperature (2-4C°) until using within experiments

Tetraploid induction: Dry and clean seeds were immersed in tap water for 24 h, at room temperature. Those seed which became visibly swollen were selected and surface sterilised by immersion in in 0.1 percent $HgCI_2$ (Mercuric chloride) for 15 minutes and washed thrice with double distilled water for 5 minutes with gentle swirling. These seed were subsequently used as the starting material for two alternative methods for colchicine application.

Colchicine treatment of seeds and axillary buds

Seeds immersed directly in aqueous solution of colchicine, five concentrations are (zero, 500, 1000, 1500 and 2000 mgL^{-1}) and different periods of seeds immersion in aqueous concentrations of colchicine in three periods (12, 18, and 24 hours). The disinfected seeds were placed on filter paper in 90 mm Petri dishes (20 seed in each Petri dish) and provided with an aqueous solution of colchicine, so that the filter paper was fully saturated. 900 seeds were treated. After that, the seeds were washed with sterile water and transferred to fresh filter paper. Seeds treated throughout only with distilled water provided the control sample. The seeds were then allowed to germinate in darkness incubator at (25 ± 2°C); for study germination percentage and chromosome number. The five treated seeds with colchicine solution were planted in polyethylene sacks (30 cm in length and 10 cm in diameter) filled with a sandy soils, between February and March 2017 inside a lath house, where the percentage of light was about 50%. In the second method, colchicine was applied to the apical meristem of growing seedlings. Sterilized seeds were sown directly into the mixed pea, perlite medium and placed in seedling bags (5 X 5 X 10 cm), at 25 ± 2C° with three seeds in each bag, and allowed to germinate in a forestry laboratory. When the seedlings will at the two cotyledons true leaf stage, colchicine solution was applied using plastic dropper placed on apical buds. Various concentrations of colchicine solution (0.0%, 0.5%, 1.0%, 1.5% and 2.0% w/v), formulated by 1% dimethyl sulfoxide (DMSO) will applied to the apical marstimatic cells in three times each day at 12, 24 and 48 during the early morning for three consecutive days. Control seedlings received distilled water, after that the seedlings were washed with tap water, transferred to greenhouse conditions, planted and labelled in the polyethylene sacks.

Growth and maintenance of seedlings

The field trial was has established in the green house of Forestry Department. Seedlings of each experiment were lebled, maintained, irrigated and weed whenever it is necessary. In the end of November (2017), we selected best (5) seedlings in each experiment units for all treatments to study and compared between tetraploid plants and diploid plants for each study. A rule (with accuracy 1mm) and Verner digital caliper (with accuracy 0.1 mm) were used in this study.

Measurement of stomata

Stomatal characteristics (numbers/mm^2) and size of stomata (length and diameter) were measured by the method of Omidbaigi *et al.*, (25). Well expanded, mature and enlarged leaves were taken from both control and treated plants. Nail varnish technique was used to isolate samplings from surface epidermises. The epidermis were mounted on glass slides and a light microscopy "Olympus U-DA" with a DinoXcope digital camera support on MAC, was used to photograph and measure stomata dimensions with magnification of 100x and 400x respectively. The 15 readings for each treatment will scored in addition to the average, the stomata number will count per mm^2 by use a gaged lens**.**

Chlorophyll determinations

The pigments in the leaves were extracted by dissolving 0.5g of fresh mature leaf sample in 100 ml of absolute ethanol alcohol. The leaves after removing the middle vein cut into small pieces, and putted in flasks of 50ml capacity which include to 30ml of the absolute ethanol alcohol and then they kept in darkness for 24 hrs. The operation of extraction was repeats more than three times to guarantee the chlorophyll extraction completely, after that; the final volume was reached 90ml and the volume complete to 100ml. The absorption of solution was measured in two wavelengths (665 nm and 649 nm) by using Spectrophotometer, which was utilized to study the following characteristics:

1. Chlorophyll a Content (CH a) = (13.70) (A 665 nm) – (5.76) (A 649 nm)

2. Chlorophyll b Content (CH b) = (25.80) (A 649 nm) – (7.60) (A 665 nm)

3. Total Chlorophyll Content = Chlorophyll a + Chlorophyll b

Morphological parameters of seedling characteristics

After identification of tetraploid plants, morphological, physiological and anatomically characteristics where mention blew, as well as growth behavior of both tetraploid and diploid plants were recorded in order to characterize the differences and compared with control plants. The largest leaf of transplant were selected and calculated via (ImageJ 1.52a) program according to (31), each sample was counted accurately.

Anatomical Parameters of Seedlings Wood Characteristics

The wood cells of seedlings in each experiment were separated from each other by using the maceration method. The equal amount of glacial acetic acid (CH_3COOH) and hydrogen peroxide (H_2O_2) by volume 1:1 were used to macerate the small piece of the wood stem of seedling. The macerate samples later they washed by distilled water to remove the remaining of solution, and then stained by Safranine stain (1%). After that, the anatomical characteristics of wood will studies by use Olympus microscope with magnification of 100x and 400x respectively. Fifteen readings for each treatment they taken in addition to the average.

Detection of ploidy level in progeny

Study of cytogenetic event was implemented based on counting the set of chromosomes in individual plant cells of diploid and tetraploid plants

The ploidy level of all putative triploid plants was finally confirmed through somatic chromosome counting of root and stems tips cells, confirmed by meiosis observation.

A- Germinated seeds with minimum root length of 3 cm, were pretreated in a saturated solution of 8-hydroxyquinoline 0.02 M at 4°C for 4 h and then at room temperature in for 1 h. the samples were then fixed in dark and cold freshly prepared Carney's fixative (ethanol and glacial acetic acid (3:1) at room temperature for 24 h and stored in 70% ethanol at 4°C for until used. After three rinses by distilled water for 5 min, later the root tips were soaked in hydrolyzing by 1 N HCl at 60°C for 10 min and then moved to distilled water. After that, the root tips were squashed for chromosome observation for meiotic investigation; ends of root tips (3mm) were fixed and stained in 2% (w/v) aceto-carmine solution.

B- Stem tips were removed from the treated and untreated seedlings and pretreated in a saturated solution of 8-hydroxyquinoline 0.02 M at 4°C for 4 h, then washed once and fixed in fresh Carnoy's fixative (ethanol/acetic acid, 3:1) for at least 24 h at 4°C. After that, samples were hydrolyzed in 38% HCl/ethanol (1:1) for 10 min at room temperature. After washing in distilled water three times for 15min, samples were squashed and stained with aceto-carmine solution. Images of mitotic chromosomes in the root and stem tip were observed with the light Olympus microscope at 100X under oil immersion objective lens, and the best metaphase views were photographed with DinoXcope digital camera support on MAC.

Statistical analysis

This study was conducted using factorial base experiment within randomized complete block design (RCBD). The collected data were analysed with the SAS 9.1 for windows software package (Statistical). Means were compared using Duncan Multi ranged test at the 5% and 1% probability levels.

RESULTS AND DISCUSSION

Descriptive statistics of standard deviation and coefficient of variation presented in Table 1, indicates there was wide range of variation within the studied traits, a high variability within seed and seedlings characters it is clear index of impact of different interactions between colchicine concentrations and soaking, drooping periods on seed germination (%), seedling survival (%), and cytological, morphological, physiological and anatomical seedling characteristics of the two experiments. The larger value of the variance coefficient is a good indicator of the wide variance on which the best seedlings can be selected. In contrast, the standard deviation is assumed to be less valuable. This means that most experimental unit values are consistent when this standard deviation is significant. The value is within each experimental unit and in such case the individual selection is the

most useful. This positive variations resulted is a great opportunity to allow the use of such a trait as a tool for estimation and screening and then for the specific selective improvement of plant. The range in germination percentage was 50%, seedling survival 90%, shoot length 22.1–212.8cm, leaflet and leaf area found to be (2.4–22.4 and 13.0–349.1cm^2) respectively, while the ranges in fiber length distributed between 0.46-1.02mm, in chlorophyll content, stomata and seedling characteristic studies the ranges distributed show in Table 1. This changes in the this characteristics study such as plant height, leaflet area, leaves area, chlorophyll content stomata size and stomata numbers were important indicators for the detection of ploidy levels in this species.

Table 1. Total data of *Robinia pseudoacacia* L. seedlings characteristics (seeds and apical treatments).

Characters	Minimum, Maximum and Range	Coefficient of Variation (C.V.)	Average ± Standard Deviation (SD.V.)
Seed Germination (SS) (%)	50.00 - 100.000 (50.00)	18.442	82.826 ± 18.442
Seedling Survival (SS) (%)	10.00 - 100.000 (90.00)	48.351	52.289 ± 25.282
Shoot System Characteristics			
Stem Length (SL) (cm)	22.100 - 212.800 (190.700)	45.780	65.991 ± 30.211
Stem Diameter (SD) (mm)	1.860 - 15.910 (14.050)	45.918	4.830 ± 2.218
Number of Branches / Transplant (NBPS)	1.000 - 25.000 (24.000)	109.473	2.190 ± 2.398
Number of Leaf / transport (NLPS)	10.000 - 268.000 (258.000)	99.087	33.869 ± 33.560
Largest Leaflet Area (LLA) (cm^2)	2.415 - 22.418 (20.003)	38.593	7.751 ± 2.991
Largest Leaf Area (LA) (cm^2)	13.080 - 349.155 (336.075)	55.487	79.444 ± 44.081
Root Systems Characteristics			
Root length (RL) (cm)	6.300 - 156.200 (149.900)	44.830	55.036 ± 24.673
Root diameter (RD) (mm)	2.000 - 45.600 (43.600)	49.329	6.357 ± 3.136
Number of secondary roots (NRPS)	1.000 - 24.000 (23.000)	66.415	6.069 ± 4.031
physiological Characteristics of Leaves			
Chlorophyll (a) Content (CH a)(mg/g)	11.6325 - 19.8957 (8.2632)	14.0292	14.724 ± 2.0656
Chlorophyll (b) Content (CH b) (mg/g)	1.8288 - 5.9344 (4.1056)	28.1498	3.662 ± 1.0307
Total Chlorophyll Content (CH ab) (mg/g)	13.8738 - 25.4053 (11.5315)	15.6957	18.385 ± 2.8857
Anatomical Characteristics of Leaves			
Stomata Length (STL) (µm)	8.6723 - 23.4509 (13.8431)	10.1140	13.843 ± 1.4001
Stomata Width (STW) (µm)	4.808 - 11.920 (7.535)	8.328	7.535 ± 0.628
Number of stomata (NOST)/mm^2	58.440 – 224.02 (165.58)	12.778	140.7290± 35.9658
Anatomical Characteristics of Shoot Wood			
Fiber Length (FL) (mm)	0.4646 - 1.0241 (0.5595)	15.6635	0.677 ± 0.1061
Fiber Diameter (FD) ((µm)	8.4652 - 21.9859 (13.5207)	16.3390	11.798 ± 1.9277
Double Cell Wall Thickness of Fiber (DDCW) (µm)	3.8084 - 10.8912 (7.0828)	15.4731	5.598 ± 0.8663
Vessel Length (VL) (µm)	16.3133 - 311.2400 (294.9267)	16.2097	145.132 ± 23.5254
Vessel Diameter (VD) (µm)	26.7118 - 134.6647 (107.9529)	21.7345	68.6868 ± 14.9287

The results of ANOVA between two factors were described in blue of Table 4, 5, 6 and 7. Explains the values of (F) calculated for each characters study, the table showed there were highly significant variances and affected of the interaction between a colchicine concentrations solution and soaking/dropping time on most seedling traits at ($P < 0.05$ and 0.001). Moreover, non-significant effect of this interaction on seed germination and seedling survival, despite of absence the significant effect in in this characteristic, but a significant difference appeared between the impacts of interactions f

The effects of ploidy level on cytological character

Chromosome counting and ploidy level determination

Chromosome counting is the most direct method of ploidy analysis from mitotic cells in

roots and apical meristems, which illustrated in Figure 1, the phases of the mitotic cell division of *Robinia pseudoacacia* roots. Data present in Table 2, appears that were among the 900 colchicine-treated ungerminated seeds only 752 seed were germinated in laboratory examination. Approx. 88 plants or (11.7% of the germinated seeds) were found to be tetraploid (4n= 4x = 48) chromosome and 100 plants (13.3%) of germinated seed also were also identified to be mixoploid (3n =3x = 36) chromosomes, were other remaining treatments chromosome number have their original diploid number of chromosome (2n = 2x = 24) according to chromosome counting from the root tips. Results showed the application of T12 (500 mgl^{-1} for 48h) and T5 (2000 mgl^{-1} for 12h) of colchicine solutions on unterminated seed of block locust tended to induce most mixoploid plants in the number and rate of 20 (36%) and 14 (33%) respectively (Figure, 1). whereas the highest percentages of tetraploid plants were induced at the 1500 mgl^{-1} for 12 h (T4) Period of immersion in colchicine solution about 14 (26%) followed it the treatment T15 in 10 (24%) from total survival seedlings in this treatment (2000mg l^{-1} for 48h). No tetraploid was obtained from the T3 treatments (0.1% dose at 12h). In the other test, when solution of colchicine (0.05–0.2%) in different dropping time was applied to the seedling apical meristem of *Robinia* seedlings observed from 450 seedlings treated only 238 seedlings survived, a total of 45 seedlings (18.9%) mixoploids were the 51 (21.4%) of seedling tetraploid were obtained in the progenies obtain in Table 3. This result show the potential of seed and shoot apical block locust in responding to colchicine's treatments reacted differently, so the certain of plants can't preserve their chromosome sets in balance within the cells, in particular at the high concentration rate of colchicines in exposure period.

Table 2. Effect of interaction between different colchicine concentrations and Soaking Periods on seed germination rate and ploidy level induction of *Robinia pseudoacacia L*

No. of Treatments	Colchicine mgl^{-1}	Duration (h)	No. of seeds treated	No. of seed germinated	seed germinated (%)	Ploidy level (No. and %)					
						Diploid		Mixoploid		Tatraploid	
						NO	%	NO	%	NO	%
T1	0.0 control		60	56	93.3	56	100	0	0	0	0
T2	500		60	54	90.0	42	78	8	15	4	7
T3	1000	12	60	56	93.3	48	86	8	14	0	0
T4	1500		60	54	90.0	32	59	8	15	14	26
T5	2000		60	42	70.0	22	52	14	33	8	19
T6	0.0 control		60	54	90.0	54	100	0	0	0	0
T7	500		60	56	93.3	36	64	6	11	4	7
T8	1000	24	60	54	90.0	40	74	8	15	6	11
T9	1500		60	42	70.0	22	52	8	19	12	29
T10	2000		60	48	80.0	32	67	8	17	8	17
T11	0.0 control		60	54	90.0	54	100	0	0	0	0
T12	500		60	56	93.3	32	57	20	36	4	7
T13	1000	48	60	48	80.0	38	79	0	0	10	21
T14	1500		60	36	60.0	20	56	8	22	8	22
T15	2000		60	42	70.0	30	71	4	10	10	24
Seed Number and rate			900	752	83.5	558	73.0	100	13.7	88	12.6

The highest rate of tetraploid seedling (54%) produced in T14 (0.15% for 48h) highest induction efficiency had survival (43.3%) followed it T13 (0.1% colchicine solution for 48h) had survival (46.7%) in the rate of (43%). While, the greatest percentages of mixoploid plants were produced at the T4 treatment (0.15% for 12h) of drooping with seedlings rate (47%).

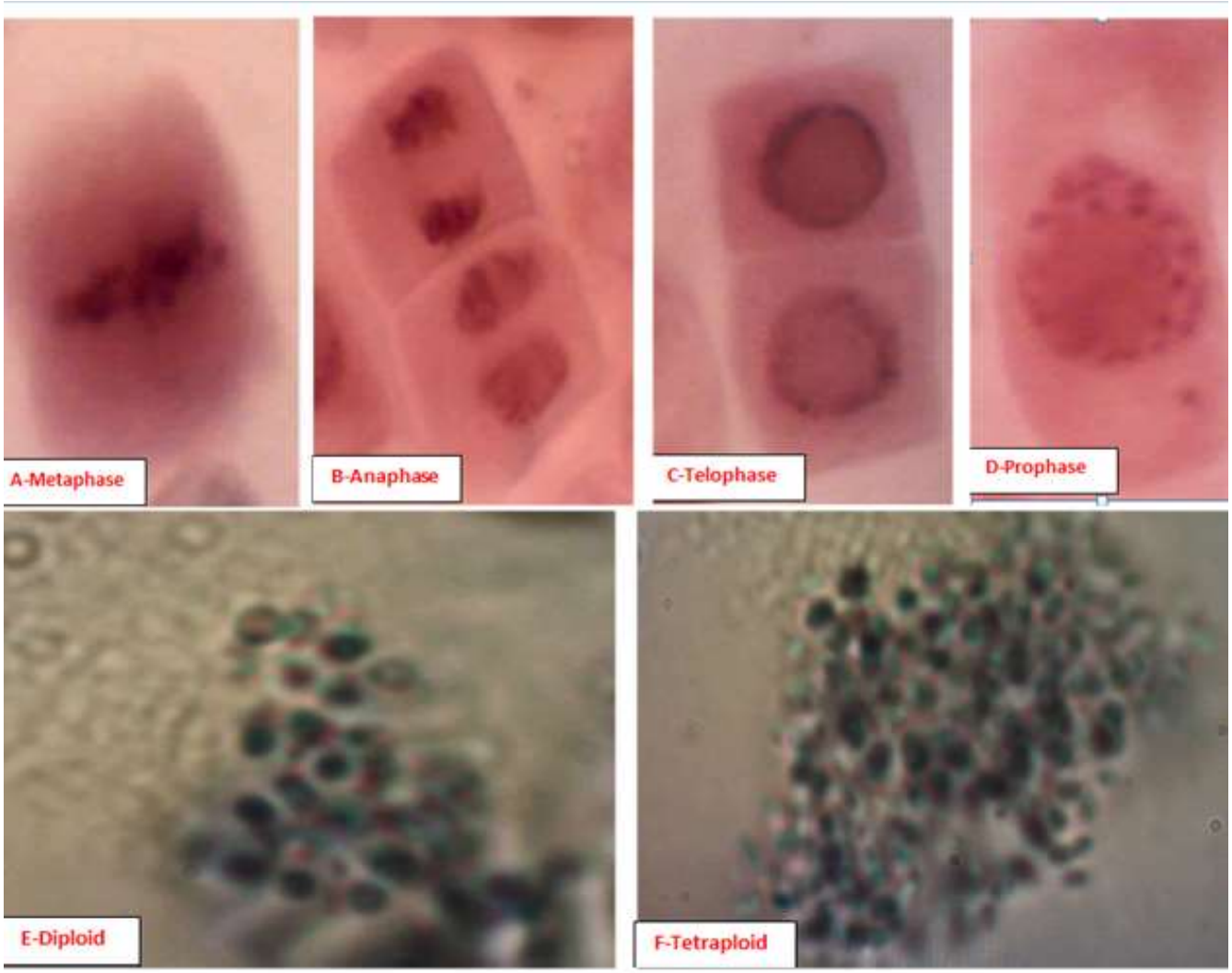

Figure 1. cell divisions stage in mitotic root cell A- Metaphase B- Anaphase C- Telophase D- Prophase And chromosome counting of *Robinia pseudoacacia L*. E- Diploid plant and F- Tetraploid plant

Abdoli *et al.,* (1) mentioned that the colchicine is an antimitotic agent that inhibits the formation of spindle fibers and effectively arrests mitosis at the metaphase stage leading to polyploidy and, as such, has been widely used to induce polyploidy in plant breeding. Liu *et al*., (17), when applied colchicine concentrations on ungerminated seed of London Plane, founded the frequency of tetraploid seedlings increasing from 16.7% (0.1% colchicine) to a maximum of 40% (0.4% colchicine). Were Omar, (24) in his study the induction of chromosomal polypoid in *Robinia pseudoacacia* transplant by colchicine treatment in seed found the 34.6% of tetraploid plants produced in 1000mgl^{-1} and 2000mgl^{-1} of colchicine induced triploid seedlings (3n) in rate of (32.7%). Flow cytometry was used by Z-Luo *et al.,* (43) to identify induced tetraploids in rubber dandelion, when used colchicine to treat seeds. The optimal induction rate of 4.92% was obtained after 0.2% colchicine for 24h, followed by 0.1% for 48h with an induction rate of 3.77%. However, 0.1% colchicine for 48h resulted in higher induction efficiency (56.6%) than in 0.2% for 24h (46.45%).

Table 3. Effect of interaction between different colchicine concentrations and dropping Periods on seedling survival rate and ploidy level induction of *Robinia pseudoacacia L*

No. of Treatments	Colchicine mgl^{-1}	Duration (h)	No. of seedlings treated	No. of seedlings survived	Seedling survived (%)	Ploidy level (%)					
						Diploid		Mixoploid		Tatraploid	
						NO	%	NO	%	NO	%
T1	0.0 control	12	30	25	83.3	25	100	0	0	0	0
T2	500		30	23	76.7	13	57	5	22	5	22
T3	1000		30	15	50.0	5	33	7	47	3	20
T4	1500		30	15	50.0	4	27	7	47	4	27
T5	2000		30	11	36.7	6	55	2	18	3	27
T6	0.0 control	24	30	26	86.7	26	100	0	0	0	0
T7	500		30	17	56.7	8	47	3	18	6	35
T8	1000		30	11	36.7	7	64	1	9	3	27
T9	1500		30	10	33.3	3	30	3	30	4	40
T10	2000		30	8	26.7	3	38	2	25	3	38
T11	0.0 control	48	30	27	90.0	27	100	0	0	0	0
T12	500		30	15	50.0	5	33	5	33	5	33
T13	1000		30	14	46.7	4	29	4	29	6	43
T14	1500		30	13	43.3	3	23	3	23	7	54
T15	2000		30	8	26.7	3	38	3	38	2	25
Seedling Number and Rate			450	238	52.9	142	51.4	45	22.5	51	26.0

The effects of ploidy level on seed germination and seedling survival percentage

In Table 4, 5, 6 and 7, indicate the germination % of the colchicine-treated seeds, especially at higher concentration levels of colchicine (0.15% and 0.2%) soaked in 48 h, recorded (60 and 70%) germination respectively, were lower than controls (0.0%, and 12h soaked in water) which were germination (93.3%), and germination was delayed. In the other hand, the highest number of mortality 66.7 and 73.3 % were recorded (0.15 and 0.2 %) respectively when applying colchicine to shoot apical meristems of seedlings. Moreover, the survival rate of seedlings, ranging from 10 to 100%, was greatly affected and inhibited by colchicine treatment, also especially at concentrations 0.15% and 0.2%, were the survival rate recorded (33.3 and 26.6%) in dropping period (24 and 48h) respectively, versus to the control treatment the survival was 90.0%. The germination rate of seeds and survivorship of seedlings decreasing when the colchiploidy concentration and duration time increased. The observation indicated there is a positive correlation between various concentrations of colchicine and soaked or dropping time applications with mortality in seedlings. This study implemented to induce polyploidy in *Platanus Acerifolia* by (17), *Robinia pseudoacacia* L. and *Ceratonia silique* L. by (24), in *Quercus aegilops* L. by (35) and *Agastache foeniculum* L. by (34) was indicated that a high concentration of colchicine and longer duration of immersion provided the reduction the rate of seed germination and plant survival. The high dose of colchicine used as a mutation agent for plant, toxic contamination and abnormality became main cause of death in the plants. Sasiree *et al*., (30) suggested the decrease in survival rate with increase in concentration of colchicine may be due to the cause of tissue necrosis when exposure in different solution of colchicine concentration solution.

Effects of ploidy level on Stomata character

In the initial screen for putative tetraploids in the field, the stomata characters could be used a best predicted and as rapid technique for identification between tetraploid and diploid plants population. A result appears in Table 4

and 5, the stomatal length (STL) and stomata width (STW) was significantly greater and have a lower stomata density in tetraploid and mixoploid plants compared with diploids. However, fewer stomata per unit leaf area were observed in tetraploid than mixoplid plants than in diploids plants in both seed and shoot apical treatments (Figure 2). The results in Table 4 demonstrate that the length stomata (21.2±2.08) contributed (55.7%) recorded in T14 (0.15% of colchicine for 48h) which were significant (P<0.0001) from other treatments, while the width and lower density of stomata showed in T7 (0.05% for 24h) treatment with means (10.5±1.43 and 79.1±18.0mm^2) respectively, with increasing in width Approximately (42.2%) and decreasing in density by (-51.8%) in mm^2 from diploid plants, and also the T7 treatment significantly differed from other treatments at (P<0.05 and 0.008) sequentially. In second method of induction polyploidy plants by tip growing we found there were highly significant effect of interaction between soaking and colchicine concentration on STL, STW and NOST at (P<0.0001, 0.017 and 0.001) respectively between different treatments, the longest stoma tetraploid were appear in treatment T9 (0.15% for 24h) as tall as (18.7±1.61) increased by (68.7%) compere to diploid T6 (11.1±0.44µM), were wide stomata showed in T2 (0.05% for 12h) in width (8.69±0.88 µM) by (45.6%) from the diploid plant (5.9±0.32). moreover, the T2, T5, T13 and T5 treatments showed less stomatal number per unit of leaf and significant difference from other treatment and control (Table 6). Roy *et al.*, (29) was suggested that the length and diameter of the stomata were set to be standard parameters for identification of tetraploid plants. Other reports on stomata size in the guard cells indicated that these cells are more dependent on genetic than environmental factors as compared to other cells in plant (40). The results suggested that stomata in tetraploid plants were larger but fewer (in area unit of leaf) compared to the control plants. These results are consistent with other studies (6, 8, 17, 24, 34, 35, 40). The increased cell size as exemplified by enlarged stomata size in triploid may play an important role in increasing the size of the leaves, which was also observed in this study. Liao *et al.*, (18) indicated the higher photosynthetic efficiency of these triploid plants may be able to explain their significant faster growth in plant height and ground diameter.

Table 4. Impact of interaction between colchicine concentrations and soaking periods of seeds treatments on means of germination percentage and morphological traits of seedling

Colchicine mgl^{-1}	Duration (h)	SG (%)	SL (cm)	SD (mm)	NBPS	NLPS	LLA (cm^2)	LA (cm^2)	RL (cm)	R D (mm)	NRPS
0.0 control	12	93.3 ± 11.54 a	32.5 ± 2.16 H	2.70 ± 0.55 g	1.0 ± 0.00 b	13.7 ± 1.64 c	5.4 ±0.96 g	44.3 ±6.11 g	36.5 ±3.32 e	3.70 ±0.54 f	3.7 ±0.46 d
500		90.0 ±17.32 ab	74.6 ± 20.81 cdefg	5.6 ± 1.24 cdef	1.2 ± 0.46 b	31.8 ± 5.35 bc	9.30 ±1.49 fcde	74.6 ±16.66 defg	67.9 ±19.41 abcd	6.6 ±1.32 bcde	17.2 ±4.16 a
1000		93.3 ±11.54 a	63.3 ± 10.43 cdefgh	4.6 ± 0.97 defg	1.0 ± 0.00 b	18.3 ± 2.88 c	7.6 ±1.27 efg	66.7 ±6.98 efg	39.1 ±7.33 e	6.4 ±1.03 cde	18.0 ±6.55 a
1500		90.0 ±10.00 ab	101.6 ± 23.47 Bc	7.1 ± 1.47 bc	2.6 ± 0.57 b	30.3 ± 5.50 bc	10.8 ±2.63 cde	195.9 ±17.52 a	94.7 ±12.18 a	8.8 ±0.96 ab	12.3 ±2.88 ab
2000		70.0 ± 10.00 bc	97.2 ± 12.19 bc	5.3 ± 1.35 cdef	2.0 ± 1.00 b	27.3 ±10.78 bc	10.39 ±2.41 gde	129.5 ±34.64 bc	55.6 ±26.38 cde	7.8 ±2.12 bcd	4.3 ±3.21 cd
0.0 control	24	90.0 ± 10.00 ab	48.2 ± 7.97 fgh	3.6 ± 0.16 efg	1.0 ± 0.00 b	15.0 ± 2.53 c	6.6 ±0.62 fg	49.1 ±12.81 fg	39.0 ±5.77 e	3.9 ±0.78 f	6.0 ±2.00 bcd
500		93.3 ± 11.54 a	55.4 ± 20.07 defgh	4.14 ± 1.18 efg	1.0 ± 0.00 b	17.7 ± 4.72 c	12.5 ±1.93 bc	74.5 ±31.67 defg	51.0 ±15.40 de	6.7 ±1.48 Bcde	16.3 ±3.05 a
1000		90.0 ± 10.00 ab	50.33 ± 14.94 efgh	4.1 ± 1.44 efg	1.0 ± 0.00 b	18.3 ± 4.04 c	10.1 ±2.15 cde	83.6 ±21.50 def	50.8 ±13.12 de	5.5 ±1.44 Def	7.66 ±2.88 bcd
1500		70.0 ± 17.32 bc	144.3 ± 55.13 a	8.7 ± 2.51 ab	2.6 ± 2.88 b	45.0 ± 18.02 b	11.5 ±0.98 bcd	109.8 ±20.90 cd	85.9 ±19.40 ab	10.5 ±2.36 a	11.3 ±5.68 abc
2000		80.0 ± 10.00 abc	94.1 ± 13.36 bcd	5.9 ± 1.29 cde	1.0 ± 0.00 b	27.0 ± 12.29 bc	11.2 ±2.35 bcd	107.1 ±18.83 cd	67.9 ±12.60 abcd	8.6c ±0.70 abc	7.1 ±2.34 bcd
0.0 control	48	90.0 ± 10.00 ab	41.8 ± 2.27 gh	3.3 ± 0.46 fg	1.0 ± 0.00 b	17.3 ±2.08 c	5.8 ±1.39 g	38.7 ±15.27 g	41.1 2.05de	4.5 ±0.65 ef	4.0 ±2.64 d
500		93.3 ± 5.77 a	81.9 ± 22.49 cdefg	4.9 ± 1.29 cdefg	1.0 ± 0.00 b	27.0 ±6.08 bc	14.3 ±2.88 ab	107.2 ±26.71 cd	62.9 ±10.92 bcde	6.7 ±1.71 bcde	9.0 ±1.00 bcd
1000		80.0 ± 20.00 abc	129.6 ± 13.74 ab	9.3 ± 2.31 a	7.3 ± 1.15 a	99.0 ± 23.06 a	17.0 ±2.13 a	151.9 ±22.80 b	78.8 ±17.03 abc	10.5 ±1.89 a	9.3 ±5.13 bcd
1500		60.0 ± 10.00 c	91.0 ± 30.25 bcde	6.8 ± 2.15 bcd	6.3 ±2.51 a	44.3 ± 24.13 b	8.7 ±1.06 defg	91.8 ±18.27 de	63.5 ±21.05 bcde	8.0 ±1.72 bc	5.6 ±3.05 bcd
2000		70.0 ± 10.00 bc	83.7 ± 11.90 cdef	5.1 ± 1.69 cdefg	2.6 ± 0.58 b	33.0 ±6.08 bc	10.4 ±2.06 cde	108.4 ±28.70 cd	91.2 ±19.12 a	7.8 ±1.40 bc	12.3 ±4.16 ab
P-value		0.2238	<.0016	<0.001 3	<0.000 1	<.0001	0.0003	<.0001	0.0062	0.0027	0.0042

Note: Means ± standard deviations of 3 independent replications in the same column followed by the same letters do not differ significantly (p<0.05) as determined by Duncan's multiple range test.

Red indicates the lowest mean of seed germination and seedling morphological characters

Blue indicates the highest mean of seed germination and seedling morphological characters

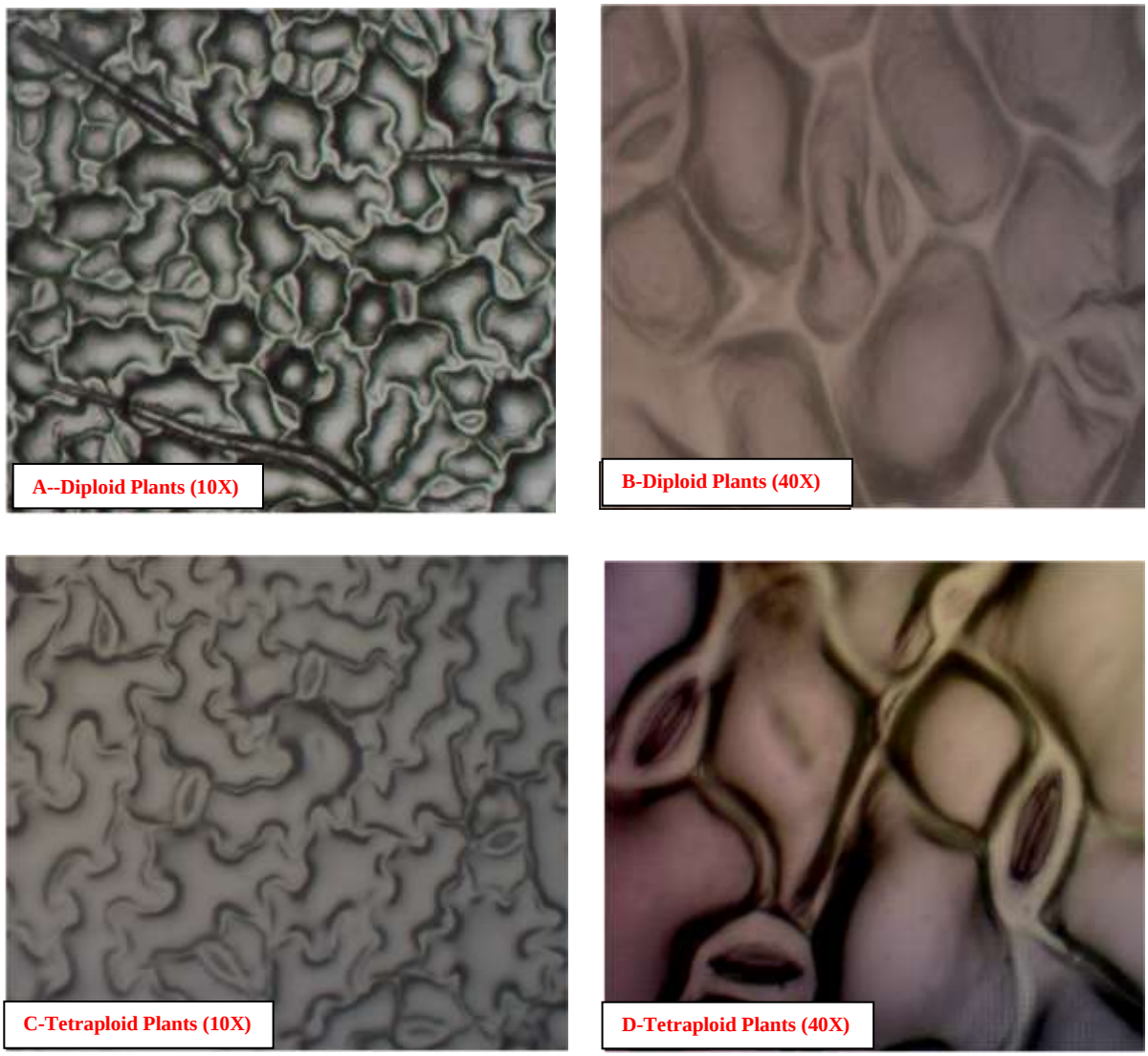

Figure 2. Stomata size and frequency of Diploid plant (control) shows in A and B figure and in Tetraploid plant shows in C and D

Effects of ploidy level on physiological character

The amount of total chlorophyll a and b were significantly increased in the leaves of tetraploid when compere to the diploid and the tetraploid leaves darker then the diploid leaves. The accumulation of high chlorophyll contents in the leaves of tetraploid plants may be relevant to increase number of chloroplasts in the stomata guard cells. ANOVA test proved in seed treatment test investigated that the chlorophyll (a, b and total) content of tetraploid plants were also significantly changed with ploidy levels at ($P<0.003$, 0.006 and 0.001) respectively resulted of affect the interaction in both colchicine concentration and exposure time were appear in table 6. The highest content of chlorophyll a (18.3mg/g), b (5.4 mg/g) and total (23.7mg/g)

Table 5. Impact of interaction between colchicine concentrations and soaking periods of seeds treatments on means of physiological and anatomical traits of seedling

Colchicine mgl^{-1}	Duration (h)	CH a	CH b	CH ab	STL (mm)	ST W (mm)	NOST (mm^2)	FL (mm)	FD (µm)	FDCWT (µm)	VL (µm)	VD (µm)
0.0 control	12	12.8 ±0.70 b	3.3 ±0.14 e	16.1 ±0.56 c	12.5 ±0.87 e	7.3 ±0.24 f	165.5 ±9.00 a	0.61 ±0.03 d	9.7 ±1.03 d	5.2 ±0.32 ef	130.1 ±6.58 e	49.9 ±1.54 f
500		13.1 ±0.26 b	3.9 ±0.20 de	17.0 ±0.45 c	15.7 ±1.64 bc	8.7 ±0.72 cdef	105.5 ±11.9 cbd	0.78 ±0.05 bc	12.0 ±0.26 bc	5.9 ±0.38 cde	148.0 ±13.3 cd	70.4 ±6.23 cd
1000		13.4 ±1.02 b	4.5 ±0.37 cd	17.9 ±1.15 c	17.3 ±2.24 b	8.9 ±1.40 abcd	103.3 ±14.21 cbd	0.86 ±0.05 ab	11.5 ±1.29 bc	5.8 ±0.72 cde	160.2 ±8.02 cd	69.3 ±3.18 cde
1500		18.2 ±1.17 a	5.4 ±0.50 a	23.6 ±1.59 a	16.4 ±1.29 b	9.2 ±1.04 abcd	107.1 ±25.38 bc	0.70 ±0.08 cd	13.7 ±0.59 b	6.4 ±0.69 bcd	151.1 ±12.9 cd	68.5 ±6.63 cde
2000		17.6 ±1.58 a	5.3 ±0.52 ab	23.0 ±1.62 ab	16.1 ±1.57 b	8.5 ±0.90 cdef	104.8 ±16.13 cbd	0.83 ±0.11 b	11.4 ±1.55 cd	5.7 ±0.70 def	128.5 ±6.06 e	64.3 ±8.57 de
0.0 control		13.1 ±0.35 b	3.7 ±0.42 E	16.8 ±0.76 c	13.1 ±1.03 De	7.4 ±0.31 ef	164.1 ±11.5 a	0.63 ±0.02 d	9.6 ±0.62 d	4.9 ±0.68 f	124.1 ±5.71 e	51.1 ±1.99 f
500		12.8 ±0.51 b	3.8 ±0.29 de	16.6 ±0.76 c	20.0 ±0.70 a	10.5 ±1.43 a	79.1 ±18.0 d	0.84 0±.08 b	17.0 ±1.71 a	7.4 ±0.63 a	207.5 ±10.8 b	83.4 ±10.0 a
1000	24	17.3 ±1.01 a	4.9 ±0.30 abc	22.3 ±1.31 ab	16.2 ±1.78 b	8.5 ±0.29 cdef	110.6 ±26.85 b	0.84 ±0.07 b	11.5 ±0.89 cd	5.8 ±0.23 cde	145.7 ±7.02 d	60.5 ±2.09 e
1500		18.0 ±1.91 a	5.3 ±0.22 abc	23.4 ±1.69 a	16.4 ±1.17 b	8.9 ±0.68 cde	108.5 ±10.0 bc	0.82 ±0.10 b	11.2 ±1.98 cd	5.9 ±0.85 cde	161.6 ±13.5 c	68.5 ±15.6 de
2000		16.8 ±1.73 a	4.9 ±0.52 abc	21.7 ±2.19 ab	15.2 ±1.08 cbd	8.7 ±0.82 cdef	123.1 ±19.8 b	0.76 ±0.05 bc	11.4 ±0.86 cd	5.6 ±0.44 def	206.3 ±13.1 b	79.4 ±4.10 b
0.0 control		12.6 ±0.43 b	3.5 ±0.32 e	16.1 ±0.71 c	13.6 ±0.64 cde	7.40 ±0.36 ef	162.6 ±10.9 a	0.62 ±0.05 d	10.5 ±0.46 cd	5.2 ±0.82 ef	122.6 ±6.72 e	52.2 ±3.57 f
500		15.9 ±1.25 a	4.7 ±0.45 abc	20.6 ±1.67 b	16.6 ±1.32 b	8.9 ±1.60 cde	123.3 ±5.62 b	0.79 ±0.03 bc	12.2 ±1.33 bc	6.6 ±0.55 bc	154.7 ±8.70 cd	75.2 ±6.79 be
1000	48	18.3 ±1.21 a	5.4 ±0.48 a	23.7 ±1.37 a	16.0 ±1.71 b	9.5 ±1.12 abc	110.3 ±14.8 b	0.82 ±0.08 b	11.2 ±0.95 cd	5.8 ±0.21 cde	147.3 ±9.45 cd	68.8 ±7.80 cd
1500		17.5 ±1.50 a	4.6 ±0.43 bc	22.1 ±1.56 ab	21.2 ±2.08 a	10.4 ±1.31 ab	82.5 ±24.2 cd	0.97 ±0.09 a	13.5 ±0.55 b	6.9 ±0.93 ab	235.4 ±17.3 a	70.4 ±10.6 cde
2000		16.6 ±2.86 a	4.9 ±0.49 abc	21.6 ±3.35 ab	15.0 ±2.47 cbd	7.9 ±1.69 def	119.9 ±24.2 b	0.78 ±0.08 bc	10.8 ±0.94 cd	5.4 ±0.45 ef	147.7 ±6.11 cd	64.7 ±7.77 cde
P-value		0.0037	0.0069	0.0012	<.0001	0.0502	0.0088	0.0063	<.0001	0.0084	<.0001	<.0001

Note: Means ± standard deviations of 3 independent replications in the same column followed by the same letters do not differ significantly (p<0.05) as determined by Duncan's multiple range test

Red indicates the lowest mean of seedling morphological, physiological and anatomical characters

Blue indicates the highest mean of seedling morphological, physiological and anatomical characters

Chlorophyll is the green pigments in leaves, is very important in plant life through the process of photosynthesis. The result pointed out that polyploidy plants leaves were darker than diploid plants and they contain higher content of chlorophyll a, b and total chlorophyll than control treatment. The results of chlorophyll were agreement with other researchers (12, 24, 36, 35). On the other, the results were disagreeing with Yildiz (42) who mentioned that there is a negative correlation between chloroplast number and ploidy level and the chlorophyll a, chlorophyll b and total chlorophyll contents of tetraploid sugar beet genotypes were found to be lower than diploid. Xing *et al.*, (39) demonstrated the existence of genetic variation for the physiological response to different concentrations of colchicine. Were Amiri *et al.*, (2) documented that the amount of chlorophyll produced affected by environmental conditions and genetic composition of the plant

Effects of ploidy level on morphological observation characteristics of seedling

The results revealed that morphological changes in treated plants were reliable and accurate indicators for identification of tetraploid plants from diploid plants. Table 1, indicates high variability of morphological characters produced result of colchicine

treatment effect. Were the aims of this study also to induce variation through colchicine treatment in seed shoot apical and select some useful variant which can be stabilised and used for developing new variety. The results appears in Table 4, 5, 6 and 7, demonstrated that tetraploid Robinia seedling had a clear advantage in vegetative growth. The ANOVA results showed that there was highly significant impact of interaction between two factors, the length of (seed soaking and dropping period) and the colchicine concentration solution on most vegetative characters at ($P < 0.01$), plant height is the demonstration of high yield, also genetically controlled. Duncan Multiple test reveal in Table 4 and 5 in seed treatment for maximum stem length 144.3±55.1cm by 212.4% from the control was achieved in a 12-hour colchicine treatment with 0.15% of colchicine concentration were the minimum 32.5cm shoot length was recorded in control at 12h. While the greatest of thicker stem, branch number, leaf number and wider leaflet area (9.3mm, 7.3 branch, 99.0 leaves and17 cm^2) by (177.8, 633.3, 471.1 and 189.7%) exists in T13 (0.1% for 48h) while the lowest in T1 and T11 diploid control treatment (Table 3). The larger leaves and length root (195.9±17.5 cm2 and 94.7±12.1cm) by (341.8 and 159.3) % respectively were induced in T4 (0.15% for 12h), were wider root (10.5mm) recorded in T9 followed it T13, while the huge number of secondly root (18.0±6.5) by 382% in T3 (0.1% for 12) followed it T2 by 360.7% from diploid plants.

Table 6. Impact of Interaction between colchicine concentrations and dropping Periods on apical buds treatments on means of survival percentage and morphological traits of seedling

Colchicine mgl^{-1}	Duration (h)	SS (%)	SL (cm)	SD (mm)	NBPS	NLPS	LLA (cm^2)	LA (cm^2)	RL (cm)	R D (mm)	NRPS
0.0 control	12	83.3 ±5.77 ab	38.6 ±9.21 h	3.0 ±0.77 h	2.0 ±0.0 e	37.3 ±5.5 g	4.6 ±0.67 g	57.9 ±8.62 g	38.7 ±9.20 g	3.9 ±0.31 H	2.6 ±1.52 E
500		76.6 ±20.8 abc	99.7 ±16.6 efg	7.6 ±0.69 ef	8.6 ±2.08 bc	172.0 28.2 b	12.2 ±1.04 cd	134.6 ±13.6 cde	97.4 ±10.3 cde	10.4 ±1.19 Cdef	10.0 ±2.0 Abc
1000		50.0 ±17.3 cd	159.8 ±26.5 abc	9.8 ±0.72 cde	4.3 ±1.52 de	108.3 ±14.0 ef	8.6 ±1.50 cde	110.6 ±10.5 defg	84.6 ±9.87 ef	9.7 ±2.12 Cdef	6.0 ±1.0 D
1500		50.0 ±10.0 cd	203.1 ±9.14 a	14.6 ±1.39 a	6.0 ±1.00 cd	133.6 ±19.5 cde	10.2 ±1.80 cde	290.6 ±51.8 a	112.5 ±13.5 abcd	15.9 ±1.66 a	13.0 ±2.64 a
2000		36.6 ±11.5 d	82.8 ±12.2 fgh	5.9 ±0.65 f	7.6 ±1.52 Bcd	112.0 ±16.5 ef	10.0 ±1.77 cdef	173.9 ±26.8 bcd	67.3 ±12.6 f	7.4 ±1.68 fg	6.3 ±1.52 d
0.0 control	24	86.6 ±15.3 ab	55.1 ±3.0 gh	3.4 ±0.37 h	1.3 ±0.577 e	24.6 ±4.51 g	6.0 ±0.51 efg	71.6 ±3.50 fg	41.7 ±6.92 g	4.9 ±0.39 gh	2.6 ±1.52 e
500		56.6 ±28. bcd	154.3 ±49.2 bcd	7.8 ±1.06 ef	8.0 ±2.0 Bcd	159.0 ±16.5 bc	17.3 ±5.56 a	302.6 ±31.5 a	125.9 ±29.5 ab	12.2 ±2.46 bcd	8.0 ±1.0 bcd
1000		36.6 ±15.3 d	107.4 ±38.6 def	10.3 ±2.20 bcde	20.3 ±4.16 a	220.6 ±28.0 a	13.1 ±1.94 bc	198.1 ±57.3 bc	117.9 ±17.3 abc	9.1 ±1.52 def	13.0 ±2.0 a
1500		33.3 ±20.8 D	111.1 ±22.7 cdef	9.0 ±1.64 de	7.0 ±1.73 bcd	147.0 ±18.3 bcd	10.8 ±1.28 cd	138.4 ±29.0 cde	89.0 ±9.87 def	9.1 ±1.93 def	10.6 ±1.52 ab
2000		26.6 ±5.7 d	164.8 ±18.8 ab	12.7 ±1.54 ab	10.6 ±1.52 b	152.6 ±19.6 bcd	11.4 ±2.31 cd	140.3 ±60.2 cde	96.0 ±12.3 cde	11.4 ±2.79 bcde	8.6 ±1.52 Bcd
0.0 control	48	90.0 ±17.3 a	46.0 h ±5.84	3.8 ±0.80 h	1.3 ±0.57 e	28.3 ±1.52 g	5.6 ±0.91 fg	63.7 ±5.73 fg	36.3 ±2.15 g	4.7 ±0.51 gh	2.6 ±1.0 E
500		50.0 ±17.3 Cd	140.9 ±26.1 bcde	11.9 ±2.49 bc	9.3 ±2.51 bc	141.6 ±17.9 bcde	11.2 ±1.79 cd	160.5 ±36.0 bcde	117.0 ±13.0 abc	14.5 ±4.13 ab	7.6 ±1.52 bcd
1000		46.6 ±20.8 cd	106.8 ±8.7de f	7.7 ±1.40 ef	7.6 ±1.52 bcd	92.3 ±22.5 f	9.0 ±2.60 cdefg	121.6 ±32.7 def	83.5 ±9.46 ef	8.5 ±1.14 ef	7.0 ±2.64 cd
1500		43.3 ±20.8 cd	153.4 ±41.9 bcd	12.2 ±1.65 abc	9.3 ±5.5 bc	123.0 ±18.0 def	16.5 ±4.17 ab	202.1 ±26.7 b	135.1 ±20.3 a	12.9 ±1.59 abc	12.6 ±1.52 a
2000		26.6 ±11.5 d	123.7 ±28.90 bcdef	10.8 bcd ±1.36	6.6 ±1.15 bcd	99.3 ±16.9 f	7.9 ±1.11 defg	108.8 ±25.0ef g	99.7 ±23.4 bcde	8.7 ±1.69 def	8.6 ±2.52 bcd
P-value		0.8801	0.0001	<.0001	<.0001	<.0001	0.0098	<.0001	0.0030	0.0009	0.0007

Note: Means ± standard deviations of 3 independent replications in the same column followed by the same letters do not differ significantly (p<0.05) as determined by Duncan's multiple range test
Red indicates the lowest mean of seedling survival and seedling morphological characters
Blue indicates the highest mean of of seedling survival and seedling morphological characters

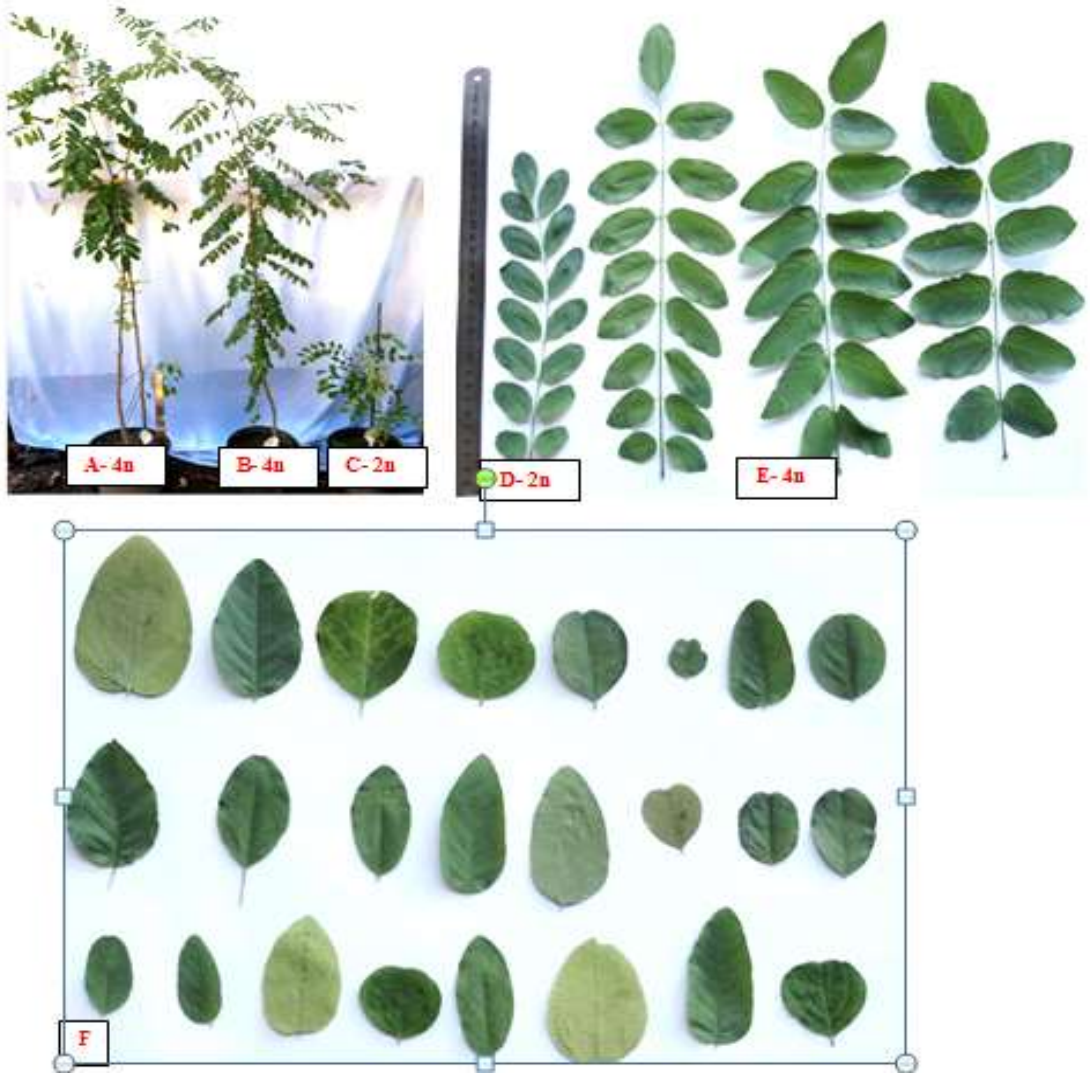

Figure 3, *Robinia pseudoacacia* L. Seedling and leaves grown in greenhouse at 8 months old. (A, B) Triploid plant.(C) Diploid plant. (D) control leaf were (E) treatment with colchicine leaves. (F) untreatment leaflet were others are the deformed leaflet of treated seedlings with different colchicine concentration with different exposure time

This study was investigated that the tetraploid seedlings are more productive had a longest and thicker stem and root with large number of leaflet per leaves, different leaflet shapes, have more branch and secondary root per transplant, also the leaves in colchiploid plants were thicker, broader and darker green in appearance than those of the diploids which appears in Figure 3. Similar results were also reported by (15, 18, 24, 35, 43) revealed that doubling level of chromosome from diploid to tetraploid caused to increase the stem height, stem diameter, root length and root width, wider leaves, fresh and dry weight of root and shoot In polyploid plants, the larger number of chromosomes causes the cell size and cell nucleus to grow larger. Larger cells produce larger parts of the plant such as leaves, flowers, fruits, and plants (14). Whereas the increase in leaf area leads to increase the biological processes such as photosynthesis which in turn leads to increase the growth of plant (4, 19).

Table 7. Impact of interaction between colchicine concentrations and dropping periods on apical buds treatments on means of physiological and anatomical traits of seedling

Colchicine mgl^{-1}	Duration (h)	CH a	CH b	CH ab	STL (mm)	ST W (mm)	NOST (mm^2)	FL (mm)	FD (µm)	FDCWT (µm)	VL (µm)	VD (µm)
0.0 control		11.4 ±0.31 g	2.1 ±0.22 g	13.5 ±0.41 e	10.6 ±0.80 e	5.9 ±0.32 d	170.6 ±24.2 b	0.51 ±0.02 c	13.5 ±1.24 c	5.5 ±0.51 cd	135.9 ±7.65 ef	63.1 ±6.17 e
500		15.3 ±0.66 cd	3.5 ±0.32 Ab	18.8 ±0.82 c	17.3 ab ±0.82	8.69 ±0.88 a	84.5 ±14.9 f	0.77 ±0.04 ab	15.9 ±1.91 ab	7.6 ±0.47 a	158.2 ±8.93 cbd	89.3 ±7.01 bc
1000	12	14.0 ±0.53 ef	2.9 ±0.26 cde	16.9 ±0.68 d	14.8 ±0.34 cd	7.1 ±0.58 cd	113.7 ±17.14 def	0.71 ±0.08 b	15.9 ±1.07 ab	7.4 ±0.84 ab	177.7 ±16.39 cbd	84.7 ±14.6 bcd
1500		15.8 ±0.73 Bcd	2.8 ±0.36 de	18.7 ±1.03 c	14.3 ±0.95 d	7.9 ±0.64 abc	117.0 ±12.9 def	0.69 ±0.04 b	13.8 ±1.36 bc	6.9 ±0.52 ab	156.5 ±8.22 de	85.9 ±9.34 bcd
2000		15.3 ±0.55 cd	3.6 ±0.32 a	18.9 ±0.31 bc	16.2 ±0.82 bc	7.2 ±0.53 bcd	82.9 ±12.8 f	0.71 ±0.05 b	12.9 ±0.13 c	7.7 ±0.54 a	155.5 ±11.0 def	114.5 ±14.9 a
0.0 control		12.0 ±0.40 g	2.2 ±0.26 fg	14.2 ±0.26 e	11.1 ±0.44 e	6.05 ±0.36 d	164.3 ±11.0 b	0.50 ±0.02 c	12.9 ±0.60 c	5.0 ±0.60 d	138.1 ±8.24 ef	65.4 ±5.91 de
500		16.2 ±0.71 Bc	2.8 ±0.17 e	19.1 ±0.61 bc	16.4 ±1.37 bc	7.2 ±0.79 cd	97.5 ±9.75 ef	0.70 ±0.04 b	16.5 ±0.96 a	6.9 ±0.72 ab	186.7 ±16.4 bc	92.4 ±12.4 bc
1000	24	13.6 ±0.66 F	2.6 ±0.21 ef	16.3 0.83 d	10.5 ±1.55 e	8.4 ±0.74 ab	203.1 ±31.9 a	0.77 ±0.07 ab	13.9 ±2.77 bc	7.5 ±0.58 a	163.4 bcde ±7.92	96.7 ±12.3 bc
1500		16.5 ±0.71 b	3.4 ±0.25 abc	20.0 0.47 b	18.7 ±1.61 a	8.0 ±0.34 abc	98.7 ±26.3 ef	0.84 ±0.05 a	12.8 ±1.35 c	7.0 ±0.63 ab	160.4 ced ±11.82	83.6 ±11.3 cbd
2000		15.4 ±0.55 cd	3.1 ±0.33 abcde	18.5 0.82 c	14.9 ±0.61 cd	7.5 ±0.68 abc	121.7 ±16.8 de	0.88 ±0.07 a	13.5 ±0.96 c	7.5 ±0.65 ab	217.7 ±25.8 a	93.4 ±13.9 bc
0.0 control		11.3 ±0.55 g	2.3 ±0.20 fg	13.6 0.73 e	11.5 0.69 e	5.9 0.44 d	168.7 ±7.56 bc	0.56 ±0.02 c	11.6 ±0.75 c	5.0 ±0.55 d	128.5 ±9.30 f	75.9 ±6.28 bcd
500		15.3 ±0.63 Cd	3.3 ±0.25 abcd	18.6 ±0.88 c	13.9 ±0.42 d	6.9 ±0.78 cd	134.8 ±12.2 cd	0.84 ±0.07 a	13.8 ±1.07 bc	7.4 ±0.92 ab	189.5 ±18.1 b	101. ±12.2 b
1000	48	13.5 ±0.58 F	3.0 ±0.20 bcde	16.5 ±0.78 d	16.5 ±1.47 bc	7.9 ±0.79 abc	81.2 ±15.6 f	0.80 ±0.09 ab	13.1 c ±1.50 c	6.4 ±0.49 bc	159.7 ±12.3 cde	95.4 ±10.4 bc
1500		17.7 ±0.44 a	3.5 ±0.42 ab	21.3 ±0.93 a	17.4 ±1.01 ab	8.63 ±0.64 a	93.9 ±24.1 ef	0.87 ±0.08 a	17.7 ±2.01 a	7.9 ±0.64 a	167.5 ±18.3 bcd	92.6 ±13.1 bc
2000		14.7 ±0.29 de	3.4 ±0.27 abcd	18.2 ±0.13 c	17.2 ±1.11 ab	8.2 ±0.78 abc	81.2 ±22.9 f	0.80 ±0.07 ab	13.4 ±0.73 c	5.9 ±0.41 cd	179.1 ±28.3 bcd	82.46 ±11.3 bcd
P-value		0.0107	0.042	0.0083	<.0001	0.017	<.0001	0.0259	0.0003	0.0077	0.0054	0.0034

Note: Means ± standard deviations of 3 independent replications in the same column followed by the same letters do not differ significantly (p<0.05) as determined by Duncan's multiple range test

Red indicates the lowest mean of seedling morphological, physiological and anatomical characters

Blue indicates the highest mean of seedling morphological, physiological and anatomical characters

Through the research work it is shown that treatment the shoot apical growing with mutagenic agent had been more effect on morphological traits then when seed treatments. Duncan analyses test (Table, 6) proved that the treatment apical growing at two true-leaf with 0.15% of colchicine for 12h dropping period (T3) they will produce length and width shoot stem (203.1cm and 14.6mm) by (307.7 and 425.4%) sequentially followed it T10. Were the high number of branches and leaves (20.3) branch and 220.6 leaves per seedling recorded in T8 (1000mgl^{-1} for 24h) compere the untearment seedlings. In other hand, the Broader leaflet and leaf (20.3 and 220.6 cm^2) were existed in T8 (0.1% of colchicine and soaked for 24h). Were the length root 135.1±20.3cm by 271.9% in 0.15% for 48h (T14) followed it T6 (0.0 plants 5% for 48h). While wider root and have more large number of secondary roots per plants in treatment (T4) 0.15% of colchicine solution for 12h. by (15.9mm and 13.0 branches per transplant) nearly increased by (301.7 and 387.5%) consideration to the diploid plants.The morphological changes also included the deformity in some leaves as a result of the effect of different colchicine

concentrations. The deformed leaves were various in their shapes when comparing with control, as shown in f Figure 3. In seedlings which treated with different colchicine concentrations has been observed that some of normal leaf shape changed to the abnormal shape. Li *et al*., (20), in his study to compare between tetraploid and normal diploid *Robinia pseudoacacia* they found a significantly higher yield with larger leaves and higher leaf protein content. It is polyanthus, long blossoming, and suitable for feeding and beekeeping. Additionally, and suggested the tetraploid black locust is fast growing and able to tolerate harsh environments including salt, drought, and nutrient deficiencies. Thus, it has high ecological value and is widely planted to improve the soil.

The effects of ploidy level on anatomical characteristics of seedling wood stem

The results of observations on fiber dimensions and vessel element of *Robinia pesdioacacea* were affected by colchicine concertation, exposure time and interaction between them. Table 5 and 7 shows the fiber length, fiber width, cell wall thickness, vessel length and vessel diameter were increased in different the ploidy level and found there were statistically significant analysis between tetraploid plants and diploid plants at ($P < .05$ and .01). This reinforces the indication that Black locust seed and shoot apical of seedlings treated with colchicine have changed the chromosome set from diploid to tetraploid because in polyploid plants, the larger number of chromosomes causes the cell size and cell nucleus to grow larger. Larger cells produce larger parts of the plant such as leaves, flowers, fruits, and plants. In the seed treatment the maximum values of fiber and vessel length depend in Duncan Multiple test were observed in T14 (0.15% for 48h) with a mean of (0.91mm and 235.4µm) respectively were significant different with diploid plants at ($P < 0.001$) while the minimum value in T1 and T11 control seedling. The superior values of both fiber and vessel diameter and double cell wall thickness are also observed to be 17.0, 83.4 and 7.4 µm receptively in T5 (0.05% for 24h) also highly significant with controlled plants, while the minimum value of fiber and vessel diameter were found to be 9.7 and 49.9µm respectively in T1 (0.0% for 12h), also the minimum value of double cell wall thickness (4.9µm) were recorded in T6 (0.0% for 24 h). In apical growing treatment method we recognized there were high significant differences between colchiploid plants and diploid at ($P< 0.05$) result of effect the interaction between dropping time and colchicine concentration solution in anatomical characters of seedling stem wood were present in table, 7. Indicated the length fiber and vessels with means 0.88 mm and 217.7µm by 74% and 57% respectively were achieved in T10 (0.2% for 24h) followed it T14 and T12. While shorter fibers and vessels found in untreated shoot apical T6 and T10. Moreover, the wider fiber (17.7±2.01µm) by 52% and thicker cell wall (7.9±0.64µm) by 57% were existed in T14 (0.15% for 48h) treatment followed T6 and T10. On the contrary, thinner fiber and cell wall were recorded in control treatment T10 and T6. In the end, the wider vessels (114.5±14.9µm) by 81% produced when treated the growing apical with $2000mgl^{-1}$ of colchicine solution for 12h soaked were significant differed with control and other treatments ($p = 0.003$). This result conformed with (11, 35) were founded that the tetraploid plants produced longer, wider and greater cell wall thickness in libiform fiber and fiber tracheid, in addition to the length and diameter of vessel, compared to the diploid plants which were less than treated seedlings. According to the results the increased in fiber dimensions and vessel element area were probably due to the fact that cells with a larger complement of chromosomes grow larger to maintain a constant ratio of cytoplasmic to nuclear volume, and express more proteins with the presence of more genes (2). Superiority the tetraploidy on these traits contribute to breeding and improvement of trees for economic purposes, for example, the long fibers are more suitable for manufacturing of cellulose pulp and paper.

CONCLUSION

The results showed many superior traits in tetraploids as compared to control seedlings depending on the interaction. Also there were significant high variations in those characteristics within the treatments at different concentrations and periods of

colchicine solution. These changes in seedling characters suggested ploidy manipulation as a rapid, effective method for enhancing genetic diversity and metabolite production and to use in breeding program. It is concluded from this study that seed treatment method is more efficient than tip meristem treatment method to produce highest number of stable triploids and tetraploids plants.

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