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.

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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. *Robinia* 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. Foresty 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. (2n = 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 (2-4°C) 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 0.1 percent HgCl₂ (Mercuric chloride) for 15 minutes and washed thrice with double distilled water for 5 minutes with gentle swirling. These seeds 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 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 ± 2°C 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 marstmatic 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/mm²) 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 epidermis. 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 mm² 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)**

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2. Chlorophyll b Content \((CH_b) = (25.80) (A_{649\text{ nm}}) - (7.60) (A_{665\text{ nm}})\)

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 Carnoy’s fixative \((ethanol/acetic acid, 3:1)\) 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 13.0–349.1cm²) 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 \textit{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) (cm²)    | 2.415 - 22.418 (20.003)    | 38.593                          | 7.751 ± 2.991                        |
| Largest Leaf Area (LA) (cm²)        | 13.080 - 349.155 (336.075)| 55.487                          | 79.444 ± 44.081                      |
| **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)/mm²        | 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 (DCW) (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 this characteristic, but a significant difference appeared between the impacts of interactions f.
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 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 mg l\(^{-1}\) for 48h) and T5 (2000 mg l\(^{-1}\) for 12h) of colchicine solutions on ungerminated 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 mg l\(^{-1}\) for 12h (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 number obtained in the progenies. 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 mg l\(^{-1}\) | Duration (h) | No. of seeds treated | No. of seed germinated (%) | Ploidy level (No. and %) | Diploid NO % | Mixoploid NO % | Tatraploid NO % |
|-------------------|-------------------------|--------------|----------------------|---------------------------|--------------------------|--------------|---------------|----------------|
| T1                | 0.0 control             |              | 60                   | 56                        | 93.3                     | 56           | 100           | 0              |
| T2                | 500                     |              | 60                   | 54                        | 90.0                     | 42           | 78            | 8              |
| T3                | 1000                    | 12           | 60                   | 56                        | 93.3                     | 48           | 85            | 8              |
| T4                | 1500                    |              | 60                   | 54                        | 90.0                     | 32           | 59            | 8              |
| T5                | 2000                    |              | 60                   | 42                        | 70.0                     | 22           | 52            | 14             |
| T6                | 0.0 control             |              | 60                   | 54                        | 90.0                     | 54           | 100           | 0              |
| T7                | 500                     |              | 60                   | 56                        | 93.3                     | 36           | 64            | 6              |
| T8                | 1000                    | 24           | 60                   | 54                        | 90.0                     | 40           | 74            | 8              |
| T9                | 1500                    |              | 60                   | 42                        | 70.0                     | 22           | 52            | 8              |
| T10               | 2000                    |              | 60                   | 48                        | 80.0                     | 32           | 67            | 8              |
| T11               | 0.0 control             |              | 60                   | 54                        | 90.0                     | 54           | 100           | 0              |
| T12               | 500                     |              | 60                   | 56                        | 93.3                     | 32           | 57            | 20             |
| T13               | 1000                    | 48           | 60                   | 48                        | 80.0                     | 38           | 79            | 0              |
| T14               | 1500                    |              | 60                   | 36                        | 60.0                     | 20           | 56            | 8              |
| T15               | 2000                    |              | 60                   | 42                        | 70.0                     | 30           | 71            | 4              |

| 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%).
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%).

**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
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 mg l⁻¹ | Duration (h) | No. of seedlings treated | No. of seedlings survived (%) | Diploid NO % | Mixoploid NO % | Tetruploid NO % |
|-------------------|------------------|-------------|--------------------------|-------------------------------|-------------|--------------|--------------|
| T1                | 0.0 control      |             | 30                       | 25                           | 83.3        | 25           | 100          |
| T2                | 500              | 12          | 30                       | 23                           | 76.7        | 13           | 57           |
| T3                | 1000             |             | 30                       | 15                           | 50.0        | 5            | 33           |
| T4                | 1500             |             | 30                       | 15                           | 50.0        | 4            | 27           |
| T5                | 2000             |             | 30                       | 11                           | 36.7        | 6            | 55           |
| T6                | 0.0 control      |             | 30                       | 26                           | 86.7        | 26           | 100          |
| T7                | 500              | 24          | 30                       | 17                           | 56.7        | 8            | 47           |
| T8                | 1000             |             | 30                       | 11                           | 36.7        | 7            | 64           |
| T9                | 1500             |             | 30                       | 10                           | 33.3        | 3            | 30           |
| T10               | 2000             |             | 30                       | 8                            | 26.7        | 3            | 38           |
| T11               | 0.0 control      |             | 30                       | 27                           | 90.0        | 27           | 100          |
| T12               | 500              | 48          | 30                       | 15                           | 50.0        | 5            | 33           |
| T13               | 1000             |             | 30                       | 14                           | 46.7        | 4            | 29           |
| T14               | 1500             |             | 30                       | 13                           | 43.3        | 3            | 23           |
| T15               | 2000             |             | 30                       | 8                            | 26.7        | 3            | 38           |

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 colchiphloidy 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 mixoploid 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±18.0mm²) respectively, with increasing in width Approximately (42.2%) and decreasing in density by (-51.8%) in mm² 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 other cells in plant (40). The 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 mg/l | Duration (h) | SG (%) | SL (cm) | SD (mm) | NBPS | NLPS | LIA (cm²) | LA (cm²) | RL (cm) | RD (mm) | NRPS |
|----------------|--------------|--------|---------|---------|-------|-------|-----------|----------|---------|---------|-------|
| 0.0 control    | ± 11.54      |        | ± 2.16  | ± 0.55  | ± 0.00| ± 1.64| ± 0.96   | ± 6.11   | ± 3.32  | ± 0.54  | ± 0.46|
| ± 0.0          | ± 1.26       | ± 1.04 | ± 1.37  | ± 5.4   | ± 4.43| ± 3.65| ± 3.70   | ± 3.7    | ± 0.2238| ± 1.11  | ± 0.37|

500

| ± 17.32       | ± 20.81      | ± 1.24 | ± 0.46 | ± 5.35  | ± 1.49| ± 16.66| ± 19.41  | ± 1.32   | ± 4.16  | ± 0.00  | ± 0.00|
| ± 1.26       | ± 1.04      | ± 1.37 | ± 5.4   | ± 4.43 | ± 3.65| ± 3.70  | ± 3.7    | ± 0.2238| ± 1.11  | ± 0.37|

1000

| ± 11.54       | ± 10.43      | ± 0.97 | ± 0.00 | ± 2.88  | ± 1.27| ± 6.98  | ± 7.33   | ± 1.03   | ± 6.55  | ± 0.00  | ± 0.00|
| ± 1.26       | ± 1.04      | ± 1.37 | ± 5.4   | ± 4.43 | ± 3.65| ± 3.70  | ± 3.7    | ± 0.2238| ± 1.11  | ± 0.37|

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

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Effects of ploidy level on physiological character

The amount of total chlorophyll a and b were significantly increased in the leaves of tetraploid when compare to the diploid and the tetraploid leaves darker than 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)
Chlorophyll is the green pigments in leaves, is very important in plant life through the process of photosynthesis. The result pointed out that polyploidy 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.

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

| Colchicine mg/l | Durati (h) | CH a | CH b | CH ab | STL (mm) | ST W (mm) | NOST (mm²) | FL (mm) | FD (µm) | FDCW (µm) | VL (µm) | VP (µm) |
|----------------|-----------|------|------|------|----------|----------|------------|---------|----------|------------|--------|--------|
| 0.0 control    |           |      |      |      |          |          |            |         |          |            |        |        |
| 12.8           | 0.70      | 3.3  | 16.1 | 12.5 | 7.3      | 165.5    | 0.61       | 9.7      | 5.2      | 130.1      | 49.9   |        |
| 500            |           |      |      |      |          |          |            |         |          |            |        |        |
| 13.1           | 0.26      | 3.9  | 17.0 | 15.7 | 8.7      | 105.5    | 0.78       | 12.0     | 5.9      | 148.0      | 70.4   |        |
| 13.4           | 0.37      | 4.5  | 17.9 | 17.3 | 8.9      | 103.3    | 0.86       | 11.5     | 5.8      | 160.2      | 69.3   |        |
| 1000           | 0.12      | 0.72 | 0.02 | 0.24 | 0.08     | 0.003    | 0.103      | 0.32     | 0.35     | 0.68       | 1.54   |        |
| 1500           | 0.01      | 0.72 | 0.02 | 0.24 | 0.08     | 0.003    | 0.103      | 0.32     | 0.35     | 0.68       | 1.54   |        |
| 2000           | 0.03      | 0.72 | 0.02 | 0.24 | 0.08     | 0.003    | 0.103      | 0.32     | 0.35     | 0.68       | 1.54   |        |
| 24              | 0.01      | 0.72 | 0.02 | 0.24 | 0.08     | 0.003    | 0.103      | 0.32     | 0.35     | 0.68       | 1.54   |        |

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.

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.
The aims of this study were to induce variation through colchicine treatment in seed shoot apical and select some useful variants which can be stabilised and used for developing new varieties. The results appear 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 concentration solution on most vegetative factors, the length of (seed soaking and significant impact of interaction between two that tetraploid Robinia seedling had a clear appearance and 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 mg/l | Duration (h) | SS (%) | SL (cm) | SD (mm) | NBPS | NLPS | LLA (cm²) | LA (cm²) | RL (cm) | R D (mm) | NRPS
|----------------|-------------|--------|---------|---------|------|------|-----------|----------|---------|---------|------|
| **0.0 control** |             |        |         |         |      |      |           |          |         |         |      |
| 83.3 ±5.77     | 38.6 ±9.21  | 3.0    | 0.0     | 0.0     | 2.0  | 4.6  | 57.9      | 38.7     | 3.9     | 2.6     |      |
| 76.6 ±9.7     | 99.7 ±0.0   | 7.6    | +0.67   | +0.01   | +2.0 | +6.2 | +9.0      | +9.2     | +0.31   | +1.52   |      |
| 500 12         |             |        |         |         |      |      |           |          |         |         |      |
| 17.3 ±20.8    | 26.5 ±1.52  | 18.0   | 1.15    | 1.25    | 2.0  | 2.0  | 10.5      | 8.6      | 1.25    | 1.25    |      |
| 1000 12        |             |        |         |         |      |      |           |          |         |         |      |
| 1500 12        |             |        |         |         |      |      |           |          |         |         |      |
| 2000 12        |             |        |         |         |      |      |           |          |         |         |      |

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 and 17 cm²) 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±12.1 cm²) by (341.8 and 159.3%) respectively were induced in T4 (0.15% for 12h), were wider root (10.5mm) recorded in T9 followed 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**
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 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 colchicine 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 mg/l | Durat ion (h) | CH a | CH b | CH ab | STL (mm) | ST W (mm) | NOST (mm) | FL (μm) | FD (μm) | FDCW T (μm) | VL (μm) | VD (μm) |
|---------------|-------------|-----|-----|------|----------|----------|----------|---------|---------|-------------|--------|--------|
| 0.0 control   | 11.4 ±0.31  | 2.1 | 13.5 | 10.6  | 5.9      | 170.6    | 0.51     | 13.5    | 5.5     | 135.9       | 63.1   |
|               | ±0.02       | ±0.41 | ±0.80 | ±0.32 | ±24.2    | ±0.02    | ±1.24    | ±0.51   | ±7.65   | ±16.7       |
| 500           | 15.3 ±0.66  | 3.5 | 18.8 | 17.3  | 8.69     | 84.5     | 0.77     | 15.9    | 7.6     | 158.2       | 89.3   |
|               | ±0.32       | ±0.82 | ±0.82 | ±0.88 | ±14.9    | ±0.04    | ±1.19    | ±0.47   | ±8.93   | ±7.01       |
| 1000          | 14.0 ±0.53  | 12  | 16.9 | 14.8  | 7.1      | 113.7    | 0.71     | 15.9    | 7.4     | 177.7       | 84.7   |
|               | ±0.26       | ±0.68 | ±0.34 | ±0.58 | ±17.14   | ±0.08    | ±1.07    | ±0.34   | ±16.39  | ±14.6       |
| 1500          | 15.8 ±0.73  | 8.8 | 18.7 | 14.3  | 7.9      | 117.0    | 0.69     | 13.8    | 6.9     | 156.5       | 85.9   |
|               | ±0.36       | ±1.03 | ±0.95 | ±0.64 | ±12.9    | ±0.04    | ±1.36    | ±0.52   | ±8.22   | ±9.34       |
| 1500          | 15.3 ±0.76  | 8.8 | 18.7 | 14.3  | 7.9      | 117.0    | 0.69     | 13.8    | 6.9     | 156.5       | 85.9   |
|               | ±0.36       | ±1.03 | ±0.95 | ±0.64 | ±12.9    | ±0.04    | ±1.36    | ±0.52   | ±8.22   | ±9.34       |

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 the 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 following it T10. Were the high number of branches and leaves (20.3) branch and 220.6 leaves per seedling recorded in T8 (1000mg/l for 24h) compare the unearment seedlings. In other hand, the Broader leaflet and leaf (20.3 and 220.6 cm²) 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 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 pseudoacacia were affected by colchicine concentration, 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.

REFERENCES

1. Abdoli, M., A. Moieni, and, H.N, Badi. 2013. Morphological, physiological, cytological and phytochemical studies in diploid and colchicine-induced tetraploid plants of Echinacea purpurea L. Acta Physiol Plant 1–9, doi:10.1007/s11738-013-1242-9

2. Amiri, S., S. K. Kazemtabaar, G. Ranjbar, and M. Azadbakht. 2010. The effect of trifluralin and colchicine treatments on morphological characteristics of jimsonweed (Datura stramonium L.). Trakia journal of sciences. 8 (4): 47–61

3. Balat, M. 2010. Bio-oil production from pyrolysis of black locust (Robinia pseudoacacia) wood. Energy Exploration and Exploitation, 28, 173–186

4. Beest, M., J. J, Le Roux, D. M. Richardson, A. K. Brysting, J. Suda, M. Kubesˇova and P. Pysˇek 2012. The more the better? The role of polyploidy in facilitating plant invasions. Annals of Botany. 109: 19–45

5. Catrina, I. 2005. The Optimal Compositions and Planting Schemes for Shelterbelts. In: Giurgiu V (ed) Optimal Compositions for Romanian Forests. Editura Ceres, București, pp: 193–197

6. Cohen, H., A. Fait and N. Tel-Zur. 2013. Morphological, cytological and metabolic consequences of autopolyploidization in Hylocereus (Cactaceae) species. BMC Plant Biol. 13,173

7. Davis, M.A., J.P. Grime and K. Thompson. 2000. Fluctuating resources in plant communities: a general theory of invisibility. Journal of Ecology 88:528-534

8. Dzialuk, A.; I. Chybicki, M, Welc, E.´Sliwi´nska, and J. Burczyk. 2007. Presence of triploids among oak species. Ann. Bot. 99, 959–964

9. Evans, D.M., C.E. Zipper, J.A. Burger, B.D. Strahm and A.M. Villamagna. 2013. Reforestation practice for enhancement of ecosystem services on a compacted surface mine: path toward ecosystem recovery. Ecol Eng 51:16–23

10. González-García, S., C. Martinez Gasol, M.T. Moreira, X. Garbarrell, J. Rieradevall Pons and G. Feijoo. 2011. Environmental assessment of black locust (Robinia pseudoacacia L.)-based ethanol as potential transport fuel. International journal of life cycle assessment, 16, 465–477

11. Griffin, A. R., H. Twayi, R. Braunstein, G. M. Downes, D. H. Sona and C. E. Harwood. 2014. A comparison of fiber and pulp properties of diploid and tetraploid Acacia mangium grown in vietnam. peer reviewed. 67 (1).

12. Grouh, M. S. H., H. Meftahizade, N. Lotfi, V. Rahimi and B. Baniasadi. 2011. Doubling the chromosome number of Salvia hains using colchicine: evaluation of morphological traits of recovered plants. Journal of Medicinal Plants Research. 5 (19); 4892–4898

13. Han, J. M. Xu, Z. H. Du, G. Y. Li, B. S. Zeng, S. J. Wu and W. Wang. 2011. Polyploidy induction of clone of Eucalyptus grandis with colchicine. African Journal of Biotechnology 10(66), pp. 14711-14717

14. Handayani, Rd. S., M. Yusuf and A. Akma. 2018. Potential changes in watermelon (Citrullus lanatus) ploidy treated by colchicine. J. of Tropical Horticulture 1(1):10-14.

15. Lavania, U. C. and S. Srivastava. 1991. Enhanced productivity of tropane alkaloids and fertility in artificial autotetraploids of Hyoscyamus niger L. Euphytica 52, 73-77

16. Liu F.M., H.Z. Mu, Z.J. Liu, Z.X. Li, J. Jiang and G.F. Liu. 2016. Effects of triploid status on Populus acerifolia and its effect on plant morphology. Euphytica, 157: 145–154

17. Liu G., Z. Li and M. Bao. 2007. Colchicine-induced chromosome doubling in Platanus acerifolia and its effect on plant morphology. Euphytica, 157: 145–154

18. Liao, T., S. Cheng, X. Zhu, Y. Min, and X. Kang. 2016. Effects of triploid status on growth, photosynthesis, and leaf area in Populus. Trees-Struct. Funct. 30, 1137–1147

19. Li, P., Z. Cheng, B. Ma, J. A. Palta, H. Kong, F. Mo, J.Wang, Y. Zhu, G. Lv, A. Batool, X. Bai, F. Li and Y. Xiong. 2014. Dry
land wheat domestication changed the development of aboveground architecture for a well-structured canopy. Plos One. Vol. 9 (9).
20. Li, Y., Y.T. Tian, Y.Q. Qian and S.Z. Wang. 2004. Effect of NAA and IBA on rooting of tetraploid Robinia pseudoacacia in vitro and anatomical observation on adventitious root development. Sci. Silvae Sin. 40: 75–80
21. Lukicˇ S, D. Pantic, S.B. Simic, D. Borota, B. Tubic, M. Djukicˇ and D. Djunisijevic-Bojovic. 2015. Effects of black locust and black pine on extremely degraded sites 60 years after afforestation—a case study of the Grdelica Gorge (Southeastern serbia). iForest 9:235–243
22. Mantovani D., M. Veste, C. Bo`hm, M. Vignudelli and D. Freese. 2015. Spatial and temporal variation of drought impact on black locust (Robinia pseudoacacia L.) Water Status And Growth. iForest 8:743–747
23. Nuţaˇ S.I. and M. Niculescu. 2011. The influence of forest belts on tobacco crops in hydro-ameliorative Sadova-Corabia system. Analele Universită˘ṭii din Craiova, seria Agriculturaˇ-Montanologie- Cadastru, Craiova XLI(2):210–214
24. Omar, O. M. 2008. Induction of chromosomal polyploidy and early evaluation of Ceratonia siliqua L. and Robinia pseudoacacia L. transplants. M.Sc. Thesis, Forest Science, Collages Of Agriculture And Forestry, Mosul University
25. Omidaigbi R., S. Yavari, M.F. Hassani and S. Yavari. 2010. Induction of autotetraploidy in dragonhead (Dracocephalum moldavica L.) by colchicine treatment. J Fruit Ornam. Plant Res. 18(1), 23-35
26. Quinkenstein, A., C. Boehm, E. da Silva Matos, D. Freese and R.F. Huettl. 2011. Assessing the Carbon Sequestration in Short Rotation Coppices of Robinia pseudoacacia L. on Marginal Sites in Northeast Germany. In: Kumar BM, Nair PKK (eds) Carbon Sequestration Potential of Agroforestry Systems: Opportunities and Challenges. Springer, New York, pp: 201–216
27. Pansuksan K., R. Sangthong , I. Nakamura, M. Mii and K. Supaibulwatana. 2014. Tetraploid induction of Mitracarpus hirtus L. by colchicine and its characterization including antibacterial activity. Plant Cell Tissue and Organ Culture, 117: 381–391
28. Papaioannou A., T. Chatzistathis, E. Papaioannou and G. Papadopoulos. 2016. Robinia pseudoacacia as a valuable invasive species for the restoration of degraded croplands. CATENA 137:310–317
29. Roy A.T., G. Leggett and A. Koutoulis. 2001. In vitro tetraploid induction and generation of tetraploids from mixoploids in hop (Humulus lupulus L.). - Plant Cell. Rep., 20: 489-495
30. Sasiree, B, T. Taychasinpitak, S. Wongchoachant and S.Kikuchi, 2013. Effect of colchicines tablets on morphology of Torenia fournieri, Int. Transaction J. of Engg. Management and Applied Sci. & Tech., 4:299-309
31. Schneider, C. A., W. S. Rasband and K. W. Eliceiri, 2012. NIH Image to ImageJ 25 years of image analysis. Nature Methods, 9 (7):671-675, PMID 2293034
32. Soltis, P.S., D.B. Marchant, Y. van de Peer and D. E. Soltis. 2015. Polyploidy and genome evolution in plants. Curr. Opin. Genet. Dev.35, 119–125
33. Straker K.C., L. D. Quinn, T. B. Voigt, D. K. Lee and G. J. Kling. 2015. Blacklocust as a bioenergy feedstock: review. BioEnergy Res 8 (3):1117–1135
34. Talebi, S. F., M. J. Saharkhiz, M. J. Kermani, Y. Sharifi and F. F. Raouf. 2017. Effect of different antimitotic agents on polyploid induction of Anise Hyssop (Agastache foeniculum L.). - Caryologia, 70(2): 184-193
35. Toma, R. H. 2015. Induction of Chromosomal Polyploidy and Early Evaluation of Valonia Oak (Quercus aegilops L.) Transplants. M.Sc. Thesis, Forest Science, Collages Of Agriculture, Duhok University
36. Tulay, E. and M. Unal. 2010. Production of Valonia Oak (Quercus aegilops L.) Transplants. M.Sc. Thesis, Forest Science, Collages Of Agriculture, Duhok University
37. Van Buijtenen, J. P. 2004. Genetics and Improvement of Wood Properties. Texas University. Available at: http://enviro.doe.gov.my/lib/digital/1384867616-3-s2.0-B012145160700082X-main.pdf
38. Van de Peer, Y., E. Mizrachi and K. Marchal. 2017. The evolutionary significance
of polyploidy. Nat. Rev. Genet. 18, 411–424. doi: 10.1038/nrg.2017.26
39. Xing, Sh., X. Guo, Q. Wang, Q. Pan, Y. Tian, P. Liu, J. Zhao, G. Wang, X. Sun and K. Tang. 2011. Induction and flow cytometry identification of tetraploids from seed-derived explants through colchicine treatments in Catharanthus roseus (L.) G. Don. Journal of Biomedicine and Biotechnology. Pp: 10
40. Yang, J., J. Wang, Z. Liu, T. Xiong, J. Lan, Q. Han, Y. Li and X. Kang. 2018. Megasporocyte chromosome doubling in Eucalyptus urophylla S.T. Blake induced by colchicine treatment to produce triploids. Forests 9, 728
41. Yasuda K., H. Kunitake, S. Nakagawa, H. Kurogi, M. Yahata, R. Hirata, Y. Yoshikura, I. Kawakami and Y. Sugimoto. 2008. The confirmation of ploidy periclinal chimera and its morphological characteristics in Meiwa kumquat ‘Yubeni’. - Hort. Res. J., 7: 165-171
42. Yildiz, M. 2013. Plant responses at different ploidy levels. Current Progress in Biological Research. Available at: http://www.intechopen.com/books/current-progress-in-biological-research/plant-responses-at-different-ploidy-levels#article-front
43. Zinan Luo, J. Brian, Iaffaldano and C. Katrina. 2018. Colchicine-induced polyploidy has the potential to improve rubber yield in Taraxacum kok-saghyz Industrial Crops and Products 112, 75–81
44. Zhang, Y., B. Wang, S. Qi, M. Dong, Z. Wang, Y. Li, S. Chen, B. Li and J. Zhang. 2018. Ploidy and hybridity effects on leaf size, cell size and related genes expression in triploids, diploids and their parents in Populus. Planta, 1–12
45. Zhang, Y., B. J. Iaffaldano, X. Zhuang, J. Cardina and K. Cornish. 2017. Chloroplast genome resources and molecular markers differentiate rubber dandelion species from weedy relatives. BMC Plant Biol 17, 34.