EFFECT OF INTERACTION BETWEEN DIFFERENT CONCENTRATONS OF ORYZALIN AND SHAKING AT DIFFERENT TIME DURATION ON CHROMOSOME POLYPLOIDY OF CREPIS CAPILLARIS WITHOUT B CHROMOSME VIA TISSUE CULTURE TECHNIQUE.

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Abstract- These experiments were conducted at Tissue Culture Laboratory/ Faculty of Agriculture and Forestry/ University of Duhok during the period from January 2011 to May 2013. The goal of this study was to study the influence of interaction between shaking different concentrations of oryzalin 0.0, 35, 45 and 55 µm (five and ten days) on chromosome number of Crepis capillaris (without Bchromosomes) through long term callus culture until regeneration of plants from the callus. The result illustrated from tables (1and 2) the interplay between four oryzalin concentrations and two time durations manifest that the treatment of Crepis capillaris calli without B chromosome with 55 µM oryzalin on ten days shaking registered a high percentage of polyploidy; however, the callus remain in diploidy stage during the passage 7 until passage 10.

Index Terms- Crepis capillaris, chromosome, B chromosome, tissue culture.

I. INTRODUCTION

Highlight The diploid cells of Crepis capillaris have only three pairs of large and well distinguishable chromosomes [6]. Chromosome and DNA stability during in vitro culture is rather exceptionally observed, mainly in some non polysomatic plants, but even in such plants the chromosomal stability is not permanent. For example, in callus of Crepis capillaris the chromosomal stability is limited to about one year of culture and then polyploid cells appear successively [8], 2003 and [7]. Recovering fertility and equalizing chromosome numbers by mitotic polyploidization can be achieved by using mitotic inhibitors [3] and [4]. Polyploidy induction has often been used to develop new cultivars [11]. Tetraploid clones of tulip were obtained after treatment of different cultivars of tulip (2n=24) with 0.5 mgl-1 or 1. 44 µM of oryzalin at different time duration (24 and 48 hrs) on 80rpm shaker [2]. Rhododendron L.'Fragrantissimum Improved' is an attractive cultivar with showy. Protocols for in vitro regeneration and polyploid induction were developed for this cultivar as a means to potentially restore fertility and enhance ornamental traits. To induce polyploidy, regenerative callus was treated with 7.5, 15, 30, 60, or 90 µm of the mitotic inhibitor oryzalin for 1, 3, 5, 7, or 14 day in various combinations. Oryzalin significantly affected survival and shoot regenerative capacity. A percentage of homogenous, tetraploid shoots was recovered from treatments of 30 µm oryzalin for 1 (13%) or 3 (13%) days and 7.5 µm oryzalin for 7 (20%) or 14 (7%) days (Hebert et al., 2010).

II. MATERIALS AND METHODS

Selection of Media

The Medium used was Murashige and Skoog media (1962) which has been specially formulated for plant cell, tissue and organ culture. This medium was obtained from HiMedia Laboratories (www.himedialabs.com; info@himedialabs.com). Medium did not contain sucrose and agar; hence, these components had to be added to the medium before use.

Media Preparation

An amount of 4.41 gram of the powdered medium was dissolved in dionized water and other ingredients were added. The pH of the medium was adjusted to 5.7 using diluted HCl or NaOH. The medium was usually prepared in lots of 1000 ml containers and then poured into small sterile glasses at 30 ml of medium for each glass and then autoclaved at 121 0C under a pressure of 1 kg/cm2 for 15 minutes and left in growth room to be used for the next day.

Plant Material and Explants Preparation

Source of the seeds

Seeds of Crepis capillaris were obtained from Herbiseed (for specialist seeds) (www.herbiseed.com; Technical@herbiseed.com).

Seeds of Crepis capillaris (2n=6) and (2n=6 +2B chromosome) were sterilized in a mixture of 5 ml of absolute alcohol + 5 ml of 3% of H2O2 for 5 minutes and then washed several times with sterilized distilled water. The sterilized seeds were cultured in jars containing 30 ml of Murashige and Skoog (1962) solidified basal medium without growth regulators. The cultures were maintained in growth room at 24 ± 1ºC, humidity 60-70% and 16 hours photoperiod (white, natural fluoresced light). After 4-5 weeks, the roots of developed plantlets were fixed to determine the number of chromosome and presence or absence of B chromosomes and then their leaves with an area of 1cm2 were cultured on MS media supplemented with 5.0 mgl-1 NAA +0.2 mgl-1 BA to obtain callus. The

calli of both plants were subcultured every 4-5 weeks. At each passage, only healthy and well growing callus pieces were transferred on to a new medium.

Oryzalin Preparation:

An amount of 0.3463 mg of oryzalin powder was dissolved in 100 ml acetone to obtain (1mM) and then four different concentrations were prepared from this stock solution (0.0, 35, 45, 55 µM) by taking (0.0, 3.5, 4.5 and 5.5 ml) and completing them to 100 ml by sterilized liquid (MS) media.

Treatment of Callus: Four concentrations of oryzalin were used (0.0, 35µM, 45 µM and 55µM) and 10 replications were used for each treatment. The callus at 3rd passage was treated with colchicine in liquid medium for five and ten days duration using shakers (at 90 rpm). And minisart filter (0.45 µm) was used for the sterilization of the treatments. The treated calli were washed with sterilized distilled water 5 times, then cultured on MS medium which was supplemented with 5.0 mgl-1 NAA + 0.2 mgl-1 BA and subcultured every 4-5 weeks until passage 10. At passage 10, the obtained calli were divided into two groups; the first group was subcultured every 4-5 weeks on MS media with the same composition until the end of the protocol. The second group was cultured on regeneration MS media without growth regulators. At passage 12th, the calli were subcultured on ½ strength media without growth regulators for two passages. At passage 14th, the obtained callus was treated with different concentrations of BA and NAA for obtaining organogenesis and plants using half and full MS media.

The treatments used were:

A	3.0 mgl^{-1} BA +0.5 mgl^{-1} NAA.	B	2.0 mgl^{-1} BA +0.5 mgl^{-1} NAA.
C	1.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA.	D	1.0 mgl^{-1} BA +0.5 mgl^{-1} NAA.
E	4.0 mgl^{-1} BA +0.5 mgl^{-1} NAA.	F	5.0 mgl-1 BA +0.5 mgl-1 NAA.
G	6.0 mgl^{-1} BA +0.5 mgl^{-1} NAA.	H	3.0 mgl^{-1} BA +3.0 mgl^{-1} NAA.
I	3.0 mgl^{-1} BA +2.0 mgl^{-1} NAA	J	3.0 mgl^{-1} BA +1.0 mgl^{-1} NAA.
K	2.0 mgl^{-1} BA +0.5 mgl^{-1} NAA.	L	7.5 mgl^{-1} BA +1.25 mgl^{-1} NAA.
M	5.0 mgl^{-1} BA +1.25 mgl^{-1} NAA	N	2.5 mgl^{-1} BA +1.25 mgl^{-1} NAA.
O	2.5 mgl^{-1} BA +1.25 mgl^{-1} NAA	P	10.0 mgl^{-1} BA +0.5 mgl^{-1} NAA.
Q	10 mgl^{-1} BA +1.25 mgl^{-1} NAA	R	12.5 mgl^{-1} BA +1.25 mgl^{-1} NAA
S	15 mgl^{-1} BA +1.25 mgl^{-1} NAA	T	7.5 mgl^{-1} BA +0.75 mgl^{-1} NAA.
U	7.5 mgl^{-1} BA +0.5 mgl^{-1} NAA.	V	7.5 mgl^{-1} BA +0.25 mgl^{-1} NAA.

Both liquid and agar solidified media were used for rooting stage. In case of liquid medium, a filter paper bridge was prepared and insert into the culture tube in such a way that the two arms were dipping into the liquid medium and on which the explants were placed and remained on the above medium. Both media were supplemented with 0.5 mgl-1 NAA. At passage 21th, the roots of developed plantlets were fixed as mentioned previously in order to determine the chromosome number. Moreover, the calli were fixed after 7 days of each passage.

III. RESULTS

Table (1) shows the polyploidy percentage of Crepis capillaris callus without B chromosome after oryzalin treatments for five and ten days in the first and second passages. Concerning characters 3n=9, 6n=18 and 16n=48, the polyploidy percentages were not affected significantly by the interactions of oryzalin after five or ten days in both first and second passages. As for the character 4n=12, each of 45 µM of oryzalin after five and ten days (A1B3 and A2B3), 55 µM of oryzalin after five days (A1B4) and 55 µM oryzalin after ten days (A2B4) were superior in the first passage and they gave (52.54, 50.66 and 50.64%) respectively, while the latter treatment (A2B4) was the best in second passage (50.34%). Also, the same treatment surpassed all the other treatments in character 8n=24 for both passages which gave (16.40 and 10.52%) successively.

Table (1) Polyploidy Percentage of Crepis capillaris Callus Cells without B Chromosome after Oryzalin Treatment for 5 and 10 Days in the First and Second Passages.

	treatments	Polyploidy%					
		2n=6	3n=9	4n=12	6n=18	8n=24	16n=48
Passage 1	A1,B1	100.00 a	0.00 a	0.00 c	0.00 a	0.00 d	0.00 a
	A1,B2	100.00 a	0.01 a	0.01 c	0.00 a	0.01 d	0.00 a
	A1,B3	49.90 b	0.40 a	49.70 a	0.00 a	0.00 d	0.00 a
	A1,B4	39.80 d	0.01 a	50.66 a	0.00 a	9.54 b	0.00 a
	A2,B1	71.10 b	0.40 a	28.50 b	0.00 a	0.01 d	0.00 a
	A2,B2	68.74 b	0.40 a	30.94 b	0.00 a	0.01 d	0.00 a
	A2,B3	41.16 d	0.60 a	52.54 a	0.00 a	5.70 c	0.00 a
	A2,B4	32.16 e	0.01 a	50.64 a	0.00 a	16.40 a	0.00 a

Passage 2	**A1,B1**	100.00 a	0.01 a	0.01 d	0.00 a	0.01 d	0.00 a
	A1,B2	100.00 a	0.01 a	0.01 d	0.00 a	0.01 d	0.00 a
	A1,B3	51.88 d	0.80 a	47.32 ab	0.00 a	0.01 d	0.00 a
	A1,B4	42.10 e	0.40 a	48.30 ab	0.00 a	9.20 b	0.00 a
	A2,B1	72.04 b	0.40 a	27.65 c	0.00 a	0.01 d	0.00 a
	A2,B2	69.76 c	0.40 a	29.64 c	0.00 a	0.01 d	0.00 a
	A2,B3	49.80 d	0.80 a	46.50 b	0.00 a	2.50 c	0.00 a
	A2,B4	38.94 f	0.60 a	50.34 a	0.00 a	10.52 a	0.00 a

Different Letters within a Colum in each for each Passage Represented Significant Differences According to Duncan's Multiple Range Tests at 5% level.

A1: time duration (5 Day) A2: time duration (10 Day)	B1: Concentration of 0.00 µM B2: Concentration of 35 µM B3: Concentration of 45 µM B4: Concentration of 55 µM

Table (2) describes the polyploidy percentage of Crepis capillaris callus cells without B chromosome after oryzalin treatments for five and ten days form passages three to fifteen. The interaction was not significant in each of the seventh, eighth, ninth, tenth and fifteenth passages. While the treatment of 55 µM after 10 days (A2B4) was superior among all treatments in all the passages which gave 39.50, 15.94, 7.22, 3.40, 10.14, 24.60, 28.86 and 38.96% for the third, fourth, fifth, sixth, eleventh, twelfth, thirteenth, and fourteenth respectively followed by the interaction of 45 µM oryzalin after five days (A1B4) in most passages.

Table (2) Polyploidy Percentage of Crepis capillaris Callus Cells without B Chromosome after Oryzalin Treatments for Five and Ten Days from Passage 3 to Passage 15.

Treatment	passages						
	3	4	5	6	7	8	9
A1,B1	0.00 g	0.00 f	0.00 d	0.00 c	0.60 a	0.60 a	0.60 a
A1,B2	0.00 g	0.00 f	0.00 d	0.00 c	0.60 a	0.60 a	0.60 a
A1,B3	22.04 d	7.66 d	4.82 b	0.00 c	0.60 a	0.60 a	0.60 a
A1,B4	32.12 a	12.04 b	5.14 b	2.72 b	0.60 a	0.60 a	0.60 a
A2,B1	9.90 f	6.28 e	4.04 c	0.00 c	0.60 a	0.60 a	0.60 a
A2,B2	15.86 e	8.90 c	5.04 b	0.00 c	0.60 a	0.60 a	0.60 a
A2,B3	28.56 c	9.06 c	5.16 b	0.00 c	0.60 a	0.60 a	0.60 a
A2,B4	39.50 a	15.94 a	7.22 a	3.40 a	0.60 a	0.60 a	0.60 a

Treatment	passages					
	10	11	12	13	14	15
A1,B1	0.60 a	10.00 a	20.30 bc	24.82 c	32.48 b	40.68 a
A1,B2	0.60 a	9.42 ab	22.46 ab	24.18 c	30.60 b	41.64 a
A1,B3	0.60 a	7.78 c	22.70 ab	27.54 b	29.98 b	41.12 a
A1,B4	0.60 a	9.19 b	22.70 ab	28.76 ab	32.50 b	40.46 a
A2,B1	0.60 a	7.70 c	18.28 c	28.78 ab	30.90 b	41.64 a
A2,B2	0.60 a	7.04 c	20.82 b	30.16 a	31.12 b	41.80 a
A2,B3	0.60 a	7.74 c	22.12 ab	28.34 ab	30.18 b	40.96 a
A2,B4	0.60 a	10.14 a	24.60 a	28.86 ab	38.96 a	43.74 a

Different Letters within each Passage Represented Significant Differences According to Duncan's Multiple Range Tests at 5 % Level.

A1: time duration (5 Day) A2: time duration (10 Day)	B1: Concentration of 0.00 µM B2: Concentration of 35 µM B3: Concentration of 45 µM B4: Concentration of 55 µM

IV. DISCUSSION

in the manner of tables (1and 2) the interplay between four oryzalin concentrations and two time durations manifest that the treatment of Crepis capillaris calli without B chromosome with 55 µM oryzalin on ten days shaking registered a high percentage of polyploidy; however, the callus remain in diploidy stage during the passage 7 until passage 10. Our results agree with those reported by Ascough and Staden [1] revealed that the optimum treatment for producing tetraploids on in vitro-grown Watsonia

lepida was 120 µM oryzalin for 24 hrs. Viehmannova et al. [13] detected that the greatest proportions of hexadecaploid plants of Yacon (2n=58) (16%) were obtained after 48 hrs of 25µM oryzalin. Sakhanokho et al. [12] performed that the highest induction frequency (15%) of tetraploidy was achieved when embryogenic callus of Hedychium muluense was exposed to 60 µM oryzalin for 72 hrs. Hebert et al. (2010) also actualized a tetraploid shoots regenerative from callus of Rhododendron L. from treatments of 30 µm oryzalin for 1 or 3 days and 7.5 µm oryzalin for 7 or 14 days. Miguel and Leonhardt [9] attained that higher concentrations and longer treatment durations lowered the survival rates of the explants, but increased the number of polyploids produced for for Dendrobium, Epidendrum, Odontioda, and Phalaenopsis and investigated that the optimal treatments were: 14.4 µM for 6 days in Dendrobium and Odontioda; 57.7 µM for 6 days in Epidendrum; and, 14.4 µM for 3 days in Phalaenopsis.

Conclusions: High concentrations of oryzalin was more effective in increasing polyploidy (%) in Crepis capillaris without B chromosome. Crepis capillaris without B chromosome was easily regenerated to plantlet, after treatment with oryzalin. In spite of the treated calli were polyploidy, all plantlets of Crepis capillaris without B chromosome regenerated from these calli were diploid.

Recommendations: Applying moleculer methods to follow up the changes in molecular level of Crepis capillaris without B chromosome after mutagen treatments. Using other plant species to investigate whether the regenerated plants from polyploidy calli of these species after treatments are diploid or polyploid. Using other mutagens for chromosome doubling.

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