Study on callogenesis and plant regeneration from leaf explants of *Leuzea carthamoides*

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Abstract: Leuzea (*Rhaponticum carthamoides*) is a valuable medicinal plant from Asteraceae. The root and rhizomes are used for medicinal purposes, with the biological activity determined by phytoecdystone quantity, including ecdysterone. 20- hydroxy-Ecdison or Leuzine is the most important compound present in ecdysterone. Micropropagation could be a good alternative for the mass propagation of *Leuzea carthamoides*. To investigate the callogenesis of leaf explants, 12 different hormonal combinations including different concentrations of BA and 2, 4-D were studied. In this experiment, the explants were transferred to the Ms medium supplemented with 0.5 mg l^{-1} IAA and 0.5 mg l^{-1} BA 7 days after culture for regeneration. Then, after one month the percentages of callogenesis and the amount of produced callus were measured. For study shoot propagation, callus from H_1, H_2, H_4, H_5, H_6, H_8, H_{10}, and H_{12} treatments were selected and were transferred to shoot propagation treatments. The explant weight, leaf number, length of the greatest leaf, and plantlet height per explants were measured one month after transfer. The maximum callus production was obtained using 1 mg l^{-1} 2, 4-D and 1.5 mg l^{-1} BA. The maximum weight and height of plantlets obtained at 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} BA with 3.16 gram and 2.69 cm per explants, respectively. The highest leaf number per explant was observed at 0.5 mg l^{-1} IBA and 1 mg l^{-1} BA with 73.54 leaves. The maximum leaf length of explant observed at 0.5 mg l^{-1} IBA and 0.2 mg l^{-1} BA with 2.62 cm.
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Introduction

Leuzea (*Rhaponticum carthamoides*) is a valuable medicinal plant from the family Asteraceae (Orlova *et al*., 2000). *Rhaponticum carthamoides* is a perennial herb, commonly known as a maral root or Russian leuzea, which has been used for centuries in eastern parts of Russia due to its marked medicinal properties (Kokoska and Janovska, 2009). The west and east of Siberia, northern Mongolia and central Asia are its natural habitats. It is a medicinal herb with a tonic effect (Selepcova *et al*., 1993). Several different classes of compounds were previously isolated from various parts of *R. carthamoides* of which the main groups are steroids, particularly ecdysteroids, and phenolics (flavonoides and phenolic acids) accompanied with polyacetylenes, sesquiterpene lactones, triterpenoid glycosides and terpenes (essential oil) (Kokoska and Janovska, 2009). 20- hydroxy-Ecdison or Leuzine is the most important compound present in ecdysterone (Omidbaigi, 2007). This plant is a hidden jewel. R. carthamoides extract (RCE) has demonstrated a normalizing effect on central nervous and cardiovascular systems. RCE improves sleep, appetite, moods, mental and physical state, and functional ability of humans under working conditions (Yance, 2004).

In vitro cell and tissue culture methodology is envisaged as a mean for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large-scale revegetation, and for genetic manipulation studies (Nalawade *et al*., 2003).

The conducted researches on micropropagation of *Leuzea carthamoides* are inadequate. Orlova *et al*., (2000) used MS medium supplemented with 1 mgl^{-1} 2,4-D and 1 mgl^{-1} BA for Callus induction. They also used MS medium supplemented with 0.5 mgl^{-1} IAA and 0.5 mgl^{-1} BA for regeneration and MS medium with the addition of 0.5 mgl^{-1} IBA and 0.2 mgl^{-1} BA for shoot propagation. Duskova and Dusek (1995) reported that Calluses derived from the aerial parts grew best on MS media supplemented with 1.0 mg 2,4-D + 0.5 mg IBA/liter (544% increase in FW after 4 weeks). Callus cultures derived from the roots grew less well; the best results were obtained with 1.0 mg 2,4-D + 1.0 mg BA/liter (230%). They also said that Bud formation occurred on partly callused cotyledonary leaflets on media supplemented with 1.0 mg IAA/liter. Akhmetova and Baiburina (2002) reported that in case of micropropagation of *Leuzea carthamoides*, The best results were obtained using the receptacles of young heads of *R. carthamoides* as an explant, and 0.2 mg IBA + 0.2 mg NAA/liter, or 0.5 mg IBA/liter.

The objectives of this research were to determine the effect of different concentrations of (BA) and (2, 4-D) on callus induction and effect of different

concentration of BA + IBA , callus origin on shoot propagation of *Leuzea carthamoides* through the culture of leaf explant.

Materials and methods

Leaf explants of *Leuzea carthamoides* were obtained from Zardband research garden (elevation 1548 m above sea level, latitude 3547 North of Tehran) in June 2008. The leaf explants were transferred to the laboratory after collection. Initially, the explants were washed under running tap water for 30 minutes to 1 hour and then leafs were divided into small pieces and surface-sterilized by immersion in ethanol (70% v/v) for ten seconds continued by 1% (w/v) sodium hypochlorite solution for 18 minutes. Afterward, the plant materials were rinsed in sterile distilled water three times and, finally, the leaf explants were prepared. The explants were cultured in Petri dishes (with 6 cm diameter) containing 12 ml of medium. The medium consisted MS salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, 0.8% agar that was supplemented with 1 mg l^{-1} 2,4-D and 1 mg l^{-1} BAP. The pH of the medium was regulated to 5.7 and autoclaved at 121 ºC for 20 minutes. After one week, the explants were moved to the Ms medium supplemented with 0.5 mg l^{-1} IAA and 0.5 mg l^{-1} BAP. After one month callus produced from leaf explants were transferred to the Ms medium complemented with 0.5 mg l^{-1} IBA and 0.2 mg l^{-1} BAP for regeneration and shoot propagation. Then on Ms medium without growth regulators, plants produced roots. All cultures were incubated at 25±2 ºC under a 16-h photoperiod provided by cool white fluorescent tubes. Finally from this plants were used to provide explants for subsequent experiments.

In this study, to investigate callus induction from leaf explant 12 different hormonal combinations including different concentrations of BA and 2,4-D were studied (The first experiment). These experiments were conducted in factorial based on a completely randomized design (CRD) with two factors and three replications. BA at three levels and 2, 4-D at four levels were used; each replication consisted of one Petri-dish (with 10 cm diameter) with three leaf explants. In this experiment after one week, the explants were transferred to the Ms medium supplemented with 0.5 mg l^{-1} IAA and 0.5 mg l^{-1} BA for regeneration. After one month the percentages of callogenesis (number of callogenic explants/ total number of explants × 100), the rates of explants callogenesis were measured. The rates of explants callogenesis were identified with codes (Code 0: explants producing no callus or gone black; Code 1: explants producing little callus (< 50 mm^2); Code 2: explants producing a little callus (50-100 mm^2); Code 3: explants producing average amount of callus (100-200 $mm^{2)}$; Code 4: explants producing much callus (200-300 $mm^{2)}$; and Code 5: explants producing too much callus (300 <).

In second experiment to investigate shoot propagation, callus produced of H_1, H_2, H_4, H_5, H_6, H_8, H_{10}, and H_{12} treatment from first experiment were selected. This experiment was carried out in factorial based on a completely randomized design (CRD) with two factors and three replications. Callus origin (H) at eight levels and shoot propagation treatments (T) at four levels were applied, each replication consisted of one jam glass (with 10 cm diameter). The plantlets weight per explant, leaf number per explant, length of greatest leaf per explant, plantlet height per explant were measured one month after transfer of callus. Finally, for root production was used MS medium without hormone.

Data were analyzed by one-way ANOVA and the means were evaluated using Duncan's new multiple range test (DMRT) at the 5% level. In related traits such as explants weight, leaf number, length of greatest leaf, and plantlet height data analysis was carried out using SAS Version 9.1 (SAS Institute, 2002). Ranking data were analyzed by Kruskal Wallis nonparametric test.

Results and discussion

In the first experiment about 98.6 percent of explants produced callus. However, the results (Table 1) showed that treatments differed significantly in callus surface. The concentration of 1 mg l^{-1} 2, 4-D and 1.5 mg l^{-1} BA resulted in the highest callus surface (Table 2). Moreover code 1 (explants producing little callus) had the maximum percentages among other codes (Fig 1). In this experiment rapid transmission into medium containing caused the explants regenerated rapidly.

Regeneration was obtained from leaf explants within 18-21 days after transfer into medium containing 0.5 mg l^{-1} IAA and 0.5 mg l^{-1} BA. Results obtained from the first experiment were agreement with the findings of Orlova *et al*., (2000). It was established that using of medium supplemented with 2, 4-D and BA and rapidly transfer from this medium, the efficiency of plant regeneration from leaf explants increased and regeneration occurred rapidly. Besides, Orlova *et al*., (2000) used from MS medium with the addition of 0.5 mg l^{-1} IAA and 0.5 mg l^{-1} BA for regeneration the plant. Fig 2 shows Callogenesis and regeneration of leaf explants.

In the second experiment, the interaction effect of shoot propagation treatments with callus origin was not statistically significant in all four traits studied. Main effect of shoot propagation treatments was statistically significant with respect to the plantlets

weight, leaf number, and Length of greatest leaf. Main effect of shoot propagation treatments was not statistically significant in case of the plantlet height. Main effect of callus origin was statistically significant in all four traits studied (Table 3). The main effect of shoot propagation treatments on studied traits are shown in Table 4. The weight and height of plantlets was the maximum at 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} BA with 3.16 gram and 2.69 cm per explants, respectively. The highest leaf number per explant was observed at 0.5 mg l^{-1} IBA and 1 mg l^{-1} BA with 73.54 leaves. The length of the greatest leaf per explant was the maximum at 0.5 mg l^{-1} IBA and 0.2 mg l^{-1} BA with 2.62 cm. Orlova *et al* (2000) used from the basal medium A contained MS salts, vitamins according to Staba, 8 g l^{-1} agar or agarose, and 30 g l^{-1} sucrose supplemented with 0.5 mg l^{-1} IBA and 0.2 mg l^{-1} BA for the shoot propagation. Table 5 shows main effect of callus origin on traits studied. The concentration of 1 mg l^{-1} 2, 4-D and 1.5 mg l^{-1} BA resulted in the highest plantlet weight (3.84 gr). The medium containing 0.75 mg l^{-1} 2, 4-D and 1.5 mg l^{-1} BA resulted in the highest leaf number (79.75 per explants). The length of greatest leaf, and plantlet height was maximum at 0.25 mg l^{-1} 2, 4-D and 1.5 mg l^{-1} BA with 3.49 cm and 3.05 cm per explant, respectively.

Conclusion

In summary, present experiment showed that use of leaf explants for micropropagation is beneficial. It also can be useful in conservation and genetic transformation studies aimed at improving this plant. In *Leuzea carthamoides*, a combination of BA and 2, 4-D was found to be suitable for induction of callus. However, the yield of shoot regeneration was satisfactory. Regeneration was obtained from leaf explants in MS medium containing 0.5 mg l^{-1} IAA and 0.5 mg l^{-1} BA. Orlova *et al*., (2000) used from MS medium with the addition of 0.5 mg l^{-1} IAA and 0.5 mg l^{-1} BA for regeneration the plant. The present callus regeneration system may also be important for advanced studies on genetic improvement and, in the future, also has considerable potential as an alternative means for production of known and new secondary metabolites.

Table 1. Data analysis of the produced callus surface in leaf explants.

Source of variation	Degree of freedom	Chi-Square of callus surface in first experiment
Treatment	11	19.53*

*, ** significant at 0.05 and 0.01 level, respectively**.**

Table 2. Average rank of the produced callus surface in leaf explants.

Treatment	Average rank in first experiment
H_1 (1 mg l^{-1} 2,4-D + 1.5 mg l^{-1} BA)	3.85
H_2 (1 mg l^{-1} 2,4-D + 1 mg l^{-1} BA)	2.85
H_3 (1 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA)	1.57
H_4 (0.75 mg l^{-1} 2,4-D + 1.5 mg l^{-1} BA)	2.85
H5 (0.75 mg l^{-1} 2,4-D + 1 mg l^{-1} BA)	2.28
H6 (0.75 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA)	2
H7 (0.5 mg l^{-1} 2,4-D + 1.5 mg l^{-1} BA)	1
H8 (0.5 mg l^{-1} 2,4-D + 1 mg l^{-1} BA)	1.57
H9 (0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA)	1.42
H10 (0.25 mg l^{-1} 2,4-D + 1.5 mg l^{-1} BA)	3.42
H11 (0.25 mg l^{-1} 2,4-D + 1 mg l^{-1} BA)	1.28
H12 (0.25 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA)	2.85

Table 3. The results of ANOVA for the effects of shoot propagation treatments and callus origin on plantlet weight, leaf number, length of greatest leaf, and plantlet height of *Leuzea carthamoides*.

Source of variation	df	Mean Square			
		Plantlet weight	Leaf number	Length of greatest leaf	Plantlet height
T (regeneration treatments)	3	0.28 **	9.28 **	0.3 *	0.14 Ns
H (callus origin)	7	0.57 **	7.75 **	0.71 **	0.31 **
T ×H	21	0.091 Ns	2.19 Ns	0.11 Ns	0.05 Ns
Experimental error	64	0.07	2.3	0.11	0.07

*, ** significant at 0.05 and 0.01 level, respectively; Ns: not significant.

Table 4. Main effect of shoot propagation treatments on explant weight, leaf number, length of greatest leaf, and plantlet height of *Leuzea carthamoides*.

Treatments	Mean ± SE			
T	Plantlet weight (gr)	Leaf number	Length of greatest leaf (cm)	Plantlet height (cm)
T_1 (0.5 IBA+0.2 BA)	2.45 ± 0.15^{ab}	49.04 ± 3.8^{b}	2.62 ± 0.27^{a}	2.19 ± 0.19^{a}
T_2 (0.5 IBA+0.5 BA)	3.16 ± 0.28^{a}	69.37 ± 5.63^{a}	2.25 ± 0.27^{ab}	2.69 ± 0.23^{a}
T_3 (0.5 IBA+1 BA)	2.87 ± 0.25^{ab}	73.54 ± 5.08^{a}	1.82 ± 0.27^{b}	2.13 ± 0.18^{a}
T_4 (0.2 IBA+0.5 BA)	2.24 ± 0.22^{b}	60.79 ± 5.52^{ab}	2.11 ± 0.23^{ab}	2.29 ± 0.18^{a}

Within column, means followed by different letters are significantly different at the 5% level of probability (Duncan's multiple range test).

Table 5. Main effect of callus origin on explant weight, leaf number, length of greatest leaf, and plantlet height of *Leuzea carthamoides*.

Treatments	Mean ± SE			
H	Plantlet weight (gr)	Leaf number	Length of greatest leaf (cm)	Plantlet height (cm)
H_1 (1 2,4-D+1.5 BA)	3.84 ± 0.31^{a}	71.08 ± 8.4^{ab}	2.15 ± 0.32^{bc}	2.41 ± 0.4^{ab}
H_2 (1 2,4-D+1 BA)	2.45 ± 0.29^{bc}	61.33 ± 7.79^{abc}	1.81 ± 0.21^{cd}	1.91 ± 0.16^{bc}
H_4 (0.75 2,4-D+1.5 BA)	3.07 ± 0.34^{ab}	79.75 ± 7.32^{a}	1.84 ± 0.2^{cd}	2.25 ± 0.18^{ab}
H_5 (0.75 2,4-D+1 BA)	2.03 ± 0.15^{dc}	62.66 ± 6.19^{abc}	2.3 ± 0.36^{bc}	2.13 ± 0.13^{b}
H_6 (0.75 2,4-D+0.5 BA)	2.6 ± 0.28^{bc}	43 ± 5.07^{c}	3.05 ± 0.49^{ab}	2.75 ± 0.33^{ab}
H_8 (0.5 2,4-D+1 BA)	1.48 ± 0.14^{d}	61.91 ± 6.86^{abc}	0.95 ± 0.12^{d}	1.48 ± 0.13^{c}
H_{10} (0.25 2,4-D+1.5 BA)	3.08 ± 0.37^{ab}	51.41 ± 9.01^{bc}	3.49 ± 0.45^{a}	3.05 ± 0.35^{a}
H_{12} (0.25 2,4-D+0.5 BA)	2.9 ± 0.3^{b}	74.33 ± 4.82^{a}	2.03 ± 0.23^{c}	2.62 ± 0.22^{ab}

Means followed by different letters in each column are significantly different at the 5% level of probability (Duncan's multiple range test).

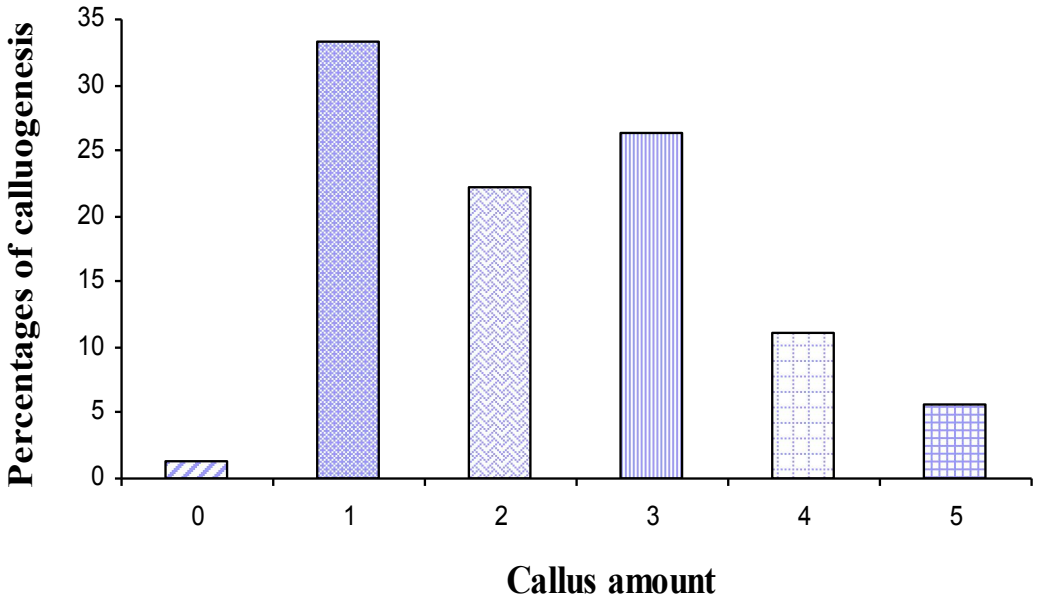

Fig 1. Study of rates of leaf explants callogenesis. (Code 0: explants producing no calli or gone black; Code 1: explants producing little callus (< 50 mm^2); Code 2: explants producing a little callus (50-100 mm^2); Code 3: explants producing average amount of callus (100-200 mm^2); Code 4: explants producing much callus (200-300 mm^2); and Code 5: explants producing too much callus (300 <).

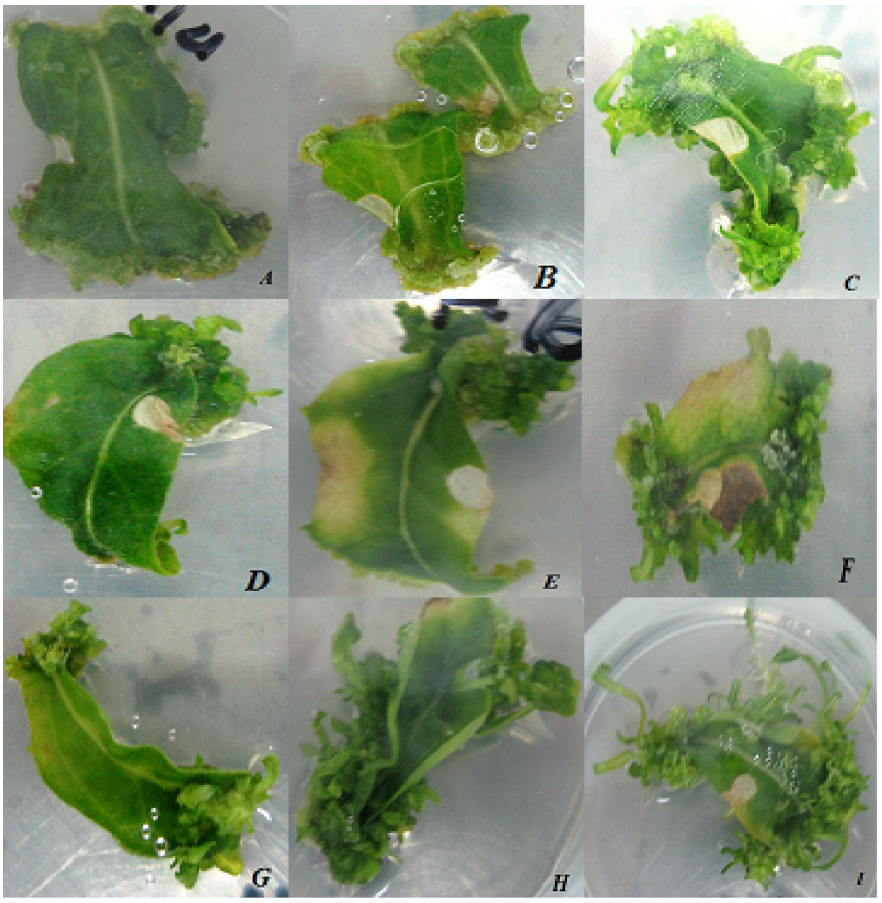

Fig 2. Callogenesis and regeneration of leaf explants.
A) 2,4-D: 1 mg/l, BA: 5/1 mg/l ; **B)** 2,4-D: 1 mg/l, BA: 1 mg/l ; **C)** 2,4-D: 75/0 mg/l, BA: 5/1 mg/l ; **D)** 2,4-D: 75/0 mg/l, BA: 1 mg/l ; **E)** 2,4-D: 75/0 mg/l, BA: 5/0 mg/l ; **F)** 2,4-D: 5/0 mg/l, BA: 1 mg/l ; **G)** 2,4-D: 5/0 mg/l, BA: 5/0 mg/l ; **H)** 2,4-D: 25/0 mg/l, BA: 5/1 mg/l ; **I)** 2,4-D: 25/0 mg/l, BA: 5/0 mg/l.

Fig 3. *In vitro* shoot propagation from leaf explants of *Leuzea carthamoides* on shoot propagation treatments.
A. 0.5 mg l^{-1} IBA + 0.2 mg l^{-1} BA; **B.** 0.5 mg l^{-1} IBA + 0.5 mg l^{-1} BA; **C.** 0.5 mg l^{-1} IBA + 1 mg l^{-1} BA; **D.** 0.2 mg l^{-1} IBA + 0.5 mg l^{-1} BA.

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