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Study on callus induction and plant regeneration of *Leuzea carthamoides* via tissue culture system

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Leuzea (*Rhaponticum carthamoides*) is a valuable medicinal plant from Asteraceae. 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 16-benzylaminopurine (BA) and 2,4-dichlorophenoxyacetic acid (2, 4-D) were studied in two separable experiments. In both experiments, the explants were transferred to the Ms medium supplemented with 0.5 mg L^{-1} indole acetic acid (IAA) and 0.5 mg L^{-1} BA for 7 and 50 days after culture for regeneration, respectively. Then, after one month the percentages of callogenesis and the amount of produced callus were measured. In other experiment to investigated regeneration of root explants, 9 different hormonal combinations were studied including different concentrations of BA and IAA. The number of leaf per explants, length of greatest leaf per explant and regeneration percentage were measured one month after culture. The maximum callus production was obtained using 1 mg L^{-1} 2, 4-D and 1.5 mg L^{-1} BA and 0.25 mg L^{-1} 2, 4-D and 1.5 mg L^{-1} BA in first experiment and second experiment, respectively. In the third experiment, root explants had direct regeneration and medium with 0.5 mg L^{-1} BA + 1 mg L^{-1} IAA can be suitable medium.

Key words: *Leuzea carthamoides, in vitro*, regeneration, callus induction.

INTRODUCTION

Leuzea (*Rhaponticum carthamoides*) is a valuable medicinal plant from the family Asteraceae (Orlova et al., 2000). *R. 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 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

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as a means 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 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2, 4-D) and 1 mg L^{-1} 16-benzylaminopurine (BA) for Callus induction. They also used Murashige and Skoog (MS) medium supplemented with 0.5 mg L^{-1} Indole acetic acid (IAA) and 0.5 mg L^{-1} BA for regeneration and MS medium with the addition of 0.5 mg L^{-1} IBA and 0.2 mg L^{-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 supple-mented 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 present study aims to determine the effect of different concentrations of (BA) and (2,4-D) on callus induction of *L. carthamoides* through the culture of leaf explants and investigate the effect of different concentrations of BA and IAA on regeneration of root explants.

MATERIALS AND METHODS

Leaf explants of *L. 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 min to 1 h 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 min. Afterward, the plant materials were rinsed in sterile distilled water three times and finally, the leaf explants were prepared. The medium consisted MS salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, 0.8% agar that was supplemented with different hormonal combinations in each experiment. The pH of the medium was regulated to 5.7 and autoclaved at 121°C for 20 min. All cultures were incubated at 25 ± 2°C under a 16 h photoperiod provided by cool white fluorescent tubes.

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 in two distinguishable experiments (Table 2). 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 first 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 and the percentages of regeneration (number of regenerated explants / total number of explants × 100) were measured.

In second experiment after 20 days, the explants were transferred to the same medium for one additional month. Then 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, 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 to 100 mm^2); Code 3: explants producing average amount of callus (100 to 200 mm^2); Code 4: explants producing much callus (200 to 300 mm^2); and Code 5: explants producing too much callus (< 300). To shoot propagation, plantlets obtained from firth experiment were transferred to MS medium supplemented with 0.5 mg L^{-1} IBA and 0.2 mg L^{-1} BA. Finally, MS medium without hormone was used for root production of plantlets.

In third experiment, root explants were taken from these plantlets. In this experiment, to study regeneration of root explants, 9 different hormonal combinations were applied including different concentrations of BA and IAA (Table 6). This experiment was carried out in factorial based on a completely randomized design (CRD) with two factors and four replications. Each replication consisted of one Petri dish (with 6 cm diameter) with three leaf explants. The number of leaf per explants, length of greatest leaf per explant and regeneration percent were measured one month after culture. For acclimatization, the rooted plantlets were taken out and tenderly washed in tap water to remove all traces of the media. Subsequently, they were planted in plastic cups (upper diameter 7.5 cm × length 8 cm, with a volume of 240 cm^3) filled with peat moss purchased from Slovenia. Then they were kept in greenhouse at 29°C in day and 25°C in night and relative moisture more than 90% at the first 14 days and 70% at the next 30 days.

Data were analyzed by one-way analysis of variance (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

About 98.6 and 94.44% of explants produced callus in the first experiment and second experiment, respectively. However, the results (Table 1) showed that treatments differed significantly in callus surface. In first experiment, the concentration of 1 mg L^{-1} 2, 4-D and 1.5 mg L^{-1} BA resulted in the highest callus surface. The concentration of 0.25 mg L^{-1} 2, 4-D and 1.5 mg L^{-1} BA resulted in the most callus surface in second experiment (Table 2). In first experiment, Code 1 (explants producing little callus) had the maximum percentages among other codes.

Whereas in second experiment code 3 (explants

Table 1. Data analysis of the produced callus surface in leaf explants in first experiment and second experiment.

Source of variation	Degree of freedom	Chi-Square of callus surface in first experiment	Chi-Square of callus surface in second experiment
Treatment	11	19.53*	45.06**

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

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

Treatment (2,4-D+BA) (mg L^{-1})	Average rank in first experiment	Average rank in second expriment
H_1 (1 + 1.5)	3.85	2.5
H_2 (1 + 1)	2.85	3.8
H_3 (1 + 0.5)	1.57	2.8
H_4 (0.75 + 1.5)	2.85	3.5
H_5 (0.75 + 1)	2.28	3.6
H_6 (0.75 + 0.5)	2	3.6
H_7 (0.5 + 1.5)	1	2.6
H_8 (0.5 + 1)	1.57	3.8
H_9 (0.5 + 0.5)	1.42	3.3
H_{10} (0.25 + 1.5)	3.42	4.1
H_{11} (0.25 + 1)	1.28	2.6
H_{12} (0.25 + 0.5)	2.85	2.5

Table 3. Results of data analysis of the produced callus surface in different times (incubate in medium consisted 2, 4-D).

Source of variation	df	Mean square
Time	1	14.93**

**significant at 0.01 level

Table 4. The results of ANOVA for the effects of 2,4-D and BA on regeneration of leaf explants of *Leuzea carthamoides* in first experiment.

Source of variation	df	Mean square
2,4-D (A)	3	2551.48**
BA (B)	2	401.22^{ns}
A×B	6	1718.09**
Experimental error	24	370.35

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

producing average amount of callus) and thereafter code 4 (explants producing much callus) had the maximum percentages among other codes (Figure 1). Based on Table 3, there was significant difference between amounts of callus produced in term of time. In the first experiment, rapid transmission into medium containing caused the explants regenerated rapidly, whereas in second experiment, with longer culturing of explants on medium supplemented with 2, 4-D and BA produced more callus amount and regenerated later than explants in first experiment. Figures 2 and 3 show Callogenesis and regeneration of leaf explants in first experiment and second experiment, respectively.

Regeneration was obtained from leaf explants in first experiment within 18 to 21 days after transfer into medium containing 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} BA. Therefore, in the first experiment, regeneration percent were measured simultaneous with measurement of the rates of explants callogenesis. The effects of 2, 4-D and BA on regeneration of leaf explants was statistically significant (Table 4). Based on result, the maximum regeneration percent was acquired by using 0.25 mg L^{-1} 2, 4-D and 1.5 mg L^{-1} BA (Table 5). Results obtained from the first and second experiment were in 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 and with longer culturing on this medium produced callus tissues incapable of regenerating

Table 5. Effect of different concentration of 2,4-D and BA on regeneration of leaf explants in first experiment.

Treatment (2,4-D+BA) (mg L^{-1})	Shoot regeneration (%)
H_1 (1 + 1.5)	44.44^{bc}
H_2 (1 + 1)	11.11^{dc}
H_3 (1 + 0.5)	55.56^{ab}
H_4 (0.75 + 1.5)	66.67^{ab}
H5 (0.75 + 1)	44.44^{bc}
H6 (0.75 + 0.5)	44.44^{bc}
H7 (0.5 + 1.5)	0^{d}
H8 (0.5 + 1)	55.56^{ab}
H9 (0.5 + 0.5)	44.44^{bc}
H10 (0.25 + 1.5)	88.89^{a}
H11 (0.25 + 1)	55.56^{ab}
H12 (0.25 + 0.5)	66.67^{ab}

Means in columns with different letters are significantly different at (P ≤ 0.05).

Table 6. Effect of different concentration of IAA and BA on regeneration percent of root explants and Length of greatest leaf of plantlets produced from root explants.

Treatment (BA+IAA) (mg L^{-1})	Mean ± SE	
	Regeneration percent	Length of greatest leaf (cm)
1 (1.5 + 1.5)	$25±25^{b}$	$0.63±0.63^{c}$
2(1.5 + 1)	$85.42±8.58^{a}$	$1.93±0.08^{ab}$
3 (1.5 + 0.5)	$91.67±8.32^{a}$	$1.01±0.14^{bc}$
4 (1 + 1.5)	$100±0^{a}$	$1.57±0.23^{abc}$
5 (1 + 1)	$75±25^{a}$	$0.57±0.09^{c}$
6 (1 + 0.5)	$100±0^{a}$	$1.38±0.08^{abc}$
7 (0.5 + 1.5)	$91.67±8.32^{a}$	$1.37±0.25^{abc}$
8 (0.5 + 1)	$100±0^{a}$	$2.32±0.26^{a}$
9 (0.5 + 0.5)	$91.67±8.32^{a}$	$0.96±0.31b^{c}$

Means in columns with different letters are significantly different at (P ≤ 0.05).

plants. Shoot propagation of plantlets made on MS medium supplemented with 0.5 mg L^{-1} IBA and 0.2 mg L^{-1} BA. 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. The rooted plantlets were optimized within one month, and then were planted in Zardband research garden (Figure 5).

In the the third experiment, root explants had direct regeneration (Figure 4). The effect of hormone was statistically significant in the case of the regeneration percent and length of greatest leaf per explants, but the hormone treatment did not have any statistically significant effect on the number of leaf produced per explants (Table 6). The medium containing 1 mg L^{-1} BA + 1.5 mg L^{-1} IAA, 1 mg L^{-1} BA + 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} BA + 1 mg L^{-1} IAA, produced the maximum regeneration percent (100%). Longest leaf (2.32 cm) was obtained when 0.5 mg L^{-1} BA + 1 mg L^{-1} IAA was employed (Table 6). Therefore, MS medium with 0.5 mg L^{-1} BA + 1 mg L^{-1} IAA can be a suitable medium for direct regeneration of root explants. Table 7 shows main effect

Table 7. Main effect of BA on leaf number.

BA (mg l^{-1})	Leaf number
1.5	18.94^{b}
1	28.55^{ab}
0.5	35.29^{a}

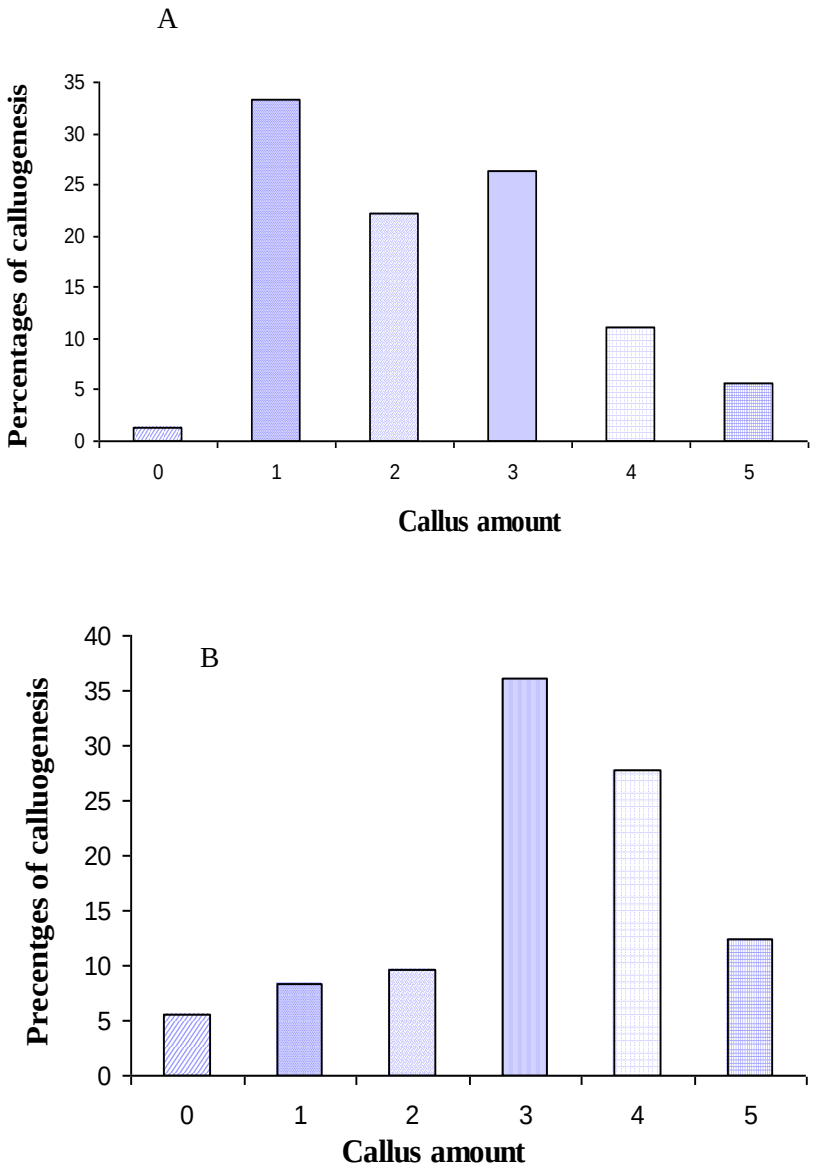

Figure 1. Study of rates of leaf explants callogenesis. A: in first experiment, B: in second experiment.
(Code 0: explants producing no calli or gone black; Code 1: explants producing little callus (< 50 mm^2); Code 2: explants producing a little callus (50 to 100 mm^2); Code 3: explants producing average amount of callus (100 to 200 mm^2); Code 4: explants producing much callus (200 to 300 mm^2); and Code 5: explants producing too much callus (300<).

of BA on leaf number. The concentration of 0.5 mg L^{-1} BA resulted in the most leaf number (35.29).

Conclusion

In summary, present experiment showed that use of leaf and root explants for micropropagation is beneficial. It also can be useful in conservation and genetic transformation studies aimed at improving this plant. In *L. 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

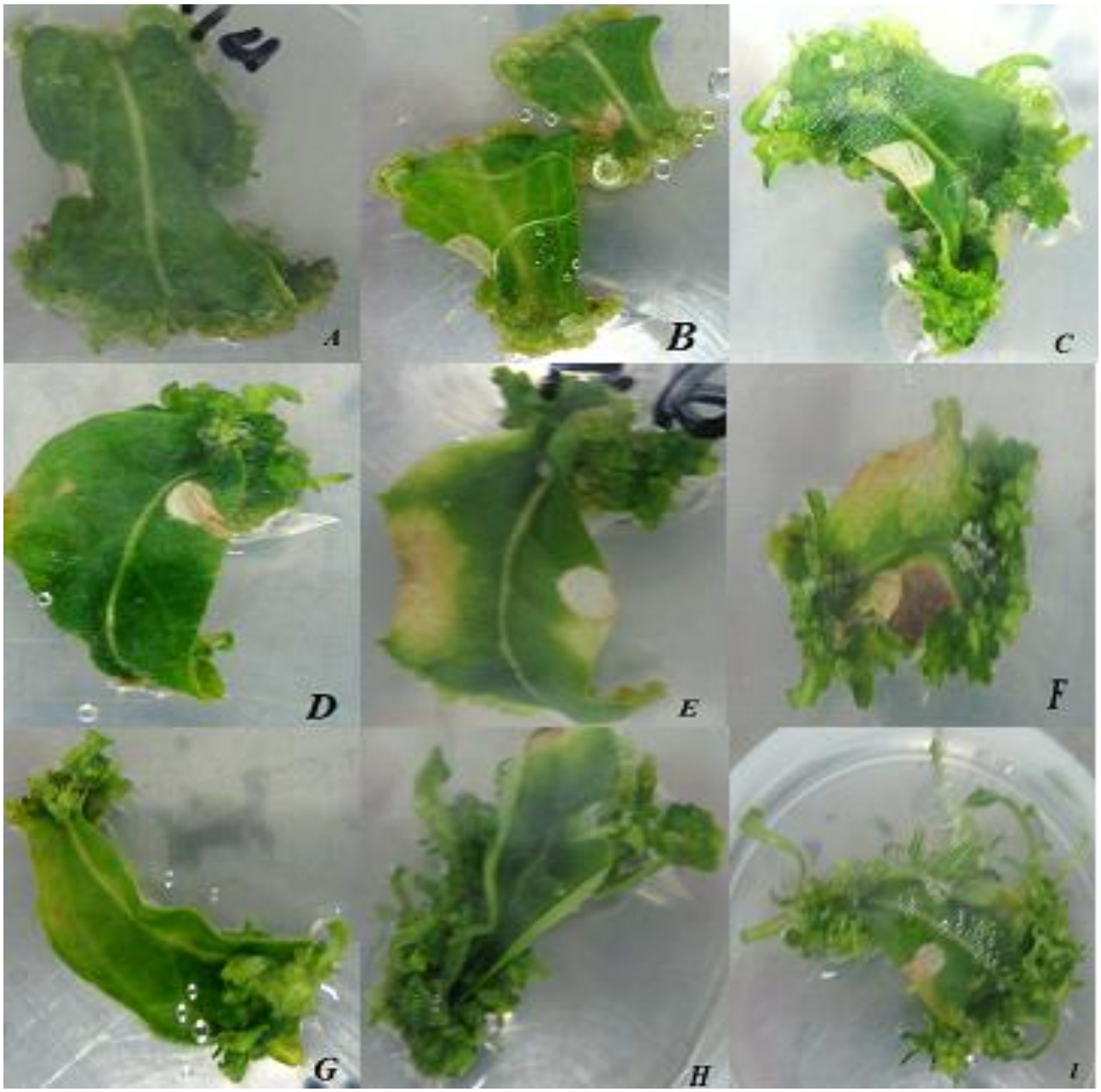

Figure 2. Callogenesis and regeneration of leaf explants in first experiment.
A) 2,4-D: 1 mg/L, BA: 1/5 mg/L; B) 2,4-D: 1 mg/L, BA: 1 mg/L; C) 2,4-D: 0/75 mg/L, BA: 1/5 mg/L; D) 2,4-D: 0/75 mg/L, BA: 1 mg/L; E) 2,4-D: 0/75 mg/L, BA: 0/5 mg/L; F) 2,4-D: 0/5 mg/L, BA: 1 mg/L; G) 2,4-D: 0/5 mg/L, BA: 0/5 mg/L; H) 2,4-D: 0/25 mg/L, BA: 1/5 mg/L; I) 2,4-D: 0/25 mg/L, BA: 0/5 mg/L.

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. In the third experiment, root explants had direct regeneration. MS medium with 0.5 mg L^{-1} BA + 1 mg L^{-1} IAA can be suitable medium for direct regeneration of root explants. Regeneration of plantlet from underground stem and leaf explants of *Curculigo orchioides*

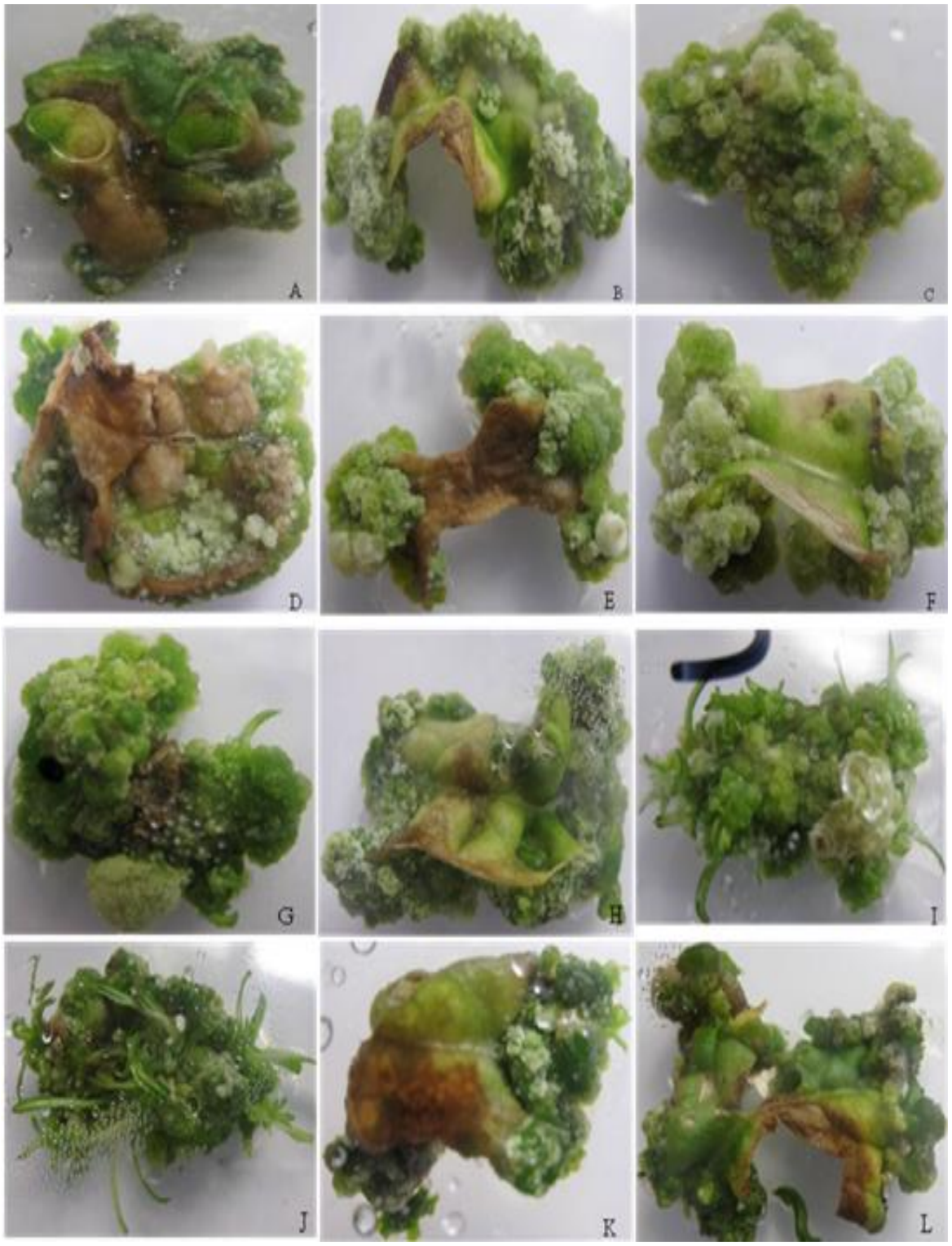

Figure 3. Callogenesis of leaf explants in second experiment.
A) 2,4-D: 1 mg/L, BA: 1/5 mg/L; B) 2,4-D: 1 mg/L, BA: 1 mg/L; C) 2,4-D: 1 mg/L, BA: 0/5 mg/L; D) 2,4-D: 0/75 mg/L, BA: 1/5 mg/L; E) 2,4-D: 0/75 mg/L, BA: 1 mg/L; F) 2,4-D: 0/75 mg/L, BA: 0/5 mg/L; G) 2,4-D: 0/5 mg/L, BA: 1/5 mg/L; H) 2,4-D: 0/5 mg/L, BA: 1 mg/L; I) 2,4-D: 0/5 mg/L, BA: 0/5 mg/L; J) 2,4-D: 0/25 mg/L, BA: 1/5 mg/L; K) 2,4-D: 0/25 mg/L, BA: 1 mg/L; L) 2,4-D: 0/25 mg/L, BA: 0/5 mg/L.

Figure 4. Direct regeneration from root *explants* of *Leuzea carthamoides* on different hormonal combinations.
A) BA: 1.5 mg/L, IAA: 1.5 mg/L; B) BA: 1.5 mg/L, IAA: 1 mg/L; C) BA: 1.5 mg/L, IAA: 0.5 mg/L; D) BA: 1 mg/L, IAA: 1.5 mg/L; E) BA: 1 mg/L, IAA: 1 mg/L; F) BA: 1 mg/L, IAA: 0.5 mg/L; G) BA: 0.5 mg/L, IAA: 1.5 mg/L; H) BA: 0.5 mg/L, IAA: 1 mg/L; I) BA: 0.5 mg/L, IAA: 0.5 mg/L.

Figure 5. Hardened plant and transferring to the filed.

without intervening callus can be efficiently used to preserve true-to-type traits in the propagules and direct regeneration of plantlets from the explant facilitates rapid multiplication (Suri et al., 1999).

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