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Full Length Research Paper

Callus induction and plant regeneration from leaf explants of *Falcaria vulgaris* an important medicinal plant

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Falcaria vulgaris* is an important medicinal plant belonging to the family Apiaceae. The leaf of explants of this plant was cultured for callus induction and plant regeneration. The explants of this plant were cultured onto Murashig and Skoog (MS) medium supplemented with different concentrations of (alpha)-naphtalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), thidiazuron (TDZ), alone and in combination with 6- benzyladenine (BA) for callus induction. The highest callus was induced in medium containing (0.5 and 1.0 mg L^{-1}) 2,4-D in combination with BA. These callus and leaf segments were transferred to MS medium supplemented with different combination of NAA and BA for indirect and direct regeneration, respectively. The medium containing (1.0 mg L^{-1}) NAA in combination with (0.5 and 1.0 mg L^{-1}) BA showed the highest number of shoot and root formation in plant regeneration through the callus. In direct regeneration, NAA with 1.0 mg L^{-1} concentration was observed to be more potent than with concentration of 0.5 mg L^{-1} and showed highest root regeneration frequency (15.7%). *In vitro* raised plantlets were acclimatized onto natural condition with 90% survival. These results provide a basis for future studies on genetic improvement and could be applied to production of secondary metabolites through cell culture in *Falcaria vulgaris.

Key words: *Falcaria vulgaris*, plant growth regulators, callus induction, *in vitro* propagation, direct regeneration.

INTRODUCTION

Plants are a valuable source of a wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavors, fragrances, colours, biopesticides and food additives. Over 80% of the approximately 30,000 known natural product are of plant origin (Fowler and Scragy, 1988; Balandrin and Klocke, 1988; Phillipson, 1990). The advent of chemical analyses and the characterization of molecular structures have helped in precisely identifying these plants and correlating them with their activity under controlled experimentation (Ramachandra and Ravishankar, 2002). Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in-vitro* regeneration and genetic transformations (Tripathi and Tripathi, 2003). Plant cell culture has been successfully applied to produce large quantities of secondary metabolites from many plants (Taniguchi et al., 2002).

Propagation of plants holds tremendous potential for the production of high-quality plant-based medicines (Murch et al., 2000). Induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators and the control of conditions in the culture medium (Tripathi and Tripathi, 2003). *Falcaria vulgaris* (locally named ghazzyaghi/poghazeh), a member of Apiaceae family, is consumed as a vegetable in some regions of Iran including Hamedan Province, for healing of skin ulcer, stomach disorders including peptic ulcer, liver diseases and stones of kidney and bladder. Naturally, this plant exists for a short time, therefore, *in*

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Table 1. Effect of different growth regulators combinations on callus induction from leaves of *Falcaria vulgaris.*

Growth regulators (mg L^{-1})				Explant forming callus (%)
BA	2,4-D	NAA	TDZ	
0	0	0	0	0
0	0.5	0	0	8.5
0	1	0	0	1
0	2	0	0	0
0	4	0	0	8.5
0	0	0.5	0	8.5
0	0	1	0	0
0	0	2	0	0
0	0	4	0	0
0	0	0	0.5	0
0	0	0	1	0
0	0	0	2	0
0	0	0	4	0
0.5	0.5	0	0	92.5
1	1	0	0	83.5
2	2	0	0	5.7
4	4	0	0	62.5
0.5	0	0.5	0	20
1	0	1	0	27
2	0	2	0	0
4	0	4	0	17
0.5	0	0	0.5	45
1	0	0	1	28
2	0	0	2	0
4	0	0	4	20

vitro tissue culture technique helps this plant to be more available in all seasons, and improve the leaf explants of wild *F. vulgaris*, produce cultivars with higher yield, tolerance and resistance to diseases. Callus production is also a necessary step for obtaining protoplasts used in protoplast fusion, a useful tool in genetic improvement of vegetatively propagated plants (Yamashita et al., 2002; Liu et al., 2005). Although *F. vulgaris* is a very valuable medicinal plant but to our knowledge, no method has been reported about *in vitro* study such as callus induction and *in vitro* propagation of this plant. Therefore, we conducted this study to assay the effects of plant growth regulators on callus induction, shoot regeneration from callus and direct regeneration from leaf explant in *F. vulgaris*.

MATERIALS AND METHODS

Plant materials

The leaves of *F. vulgaris* were collected from Hamedan Province in the Western part of Iran and identified by the Botanic Laboratory of Bu-Ali Sina University in May, 2010.

Callus induction

The leaves of *F. vulgaris* were washed thoroughly with tap water, then the explants were surface sterilized in 70% (v/v) ethanol for 1 min, subsequently in 2% (w/v) sodium hypochlorite with tween-20 for 10 min. Finally, the explants were washed with sterile deionized water for three times. For callus induction, the sterilized leaves segments of 5 × 5 mm length were placed onto the surface of Murashig and Skoog (MS) medium (Murashig and Skoog, 1962) supplemented with different concentrations of (alpha)-naphtalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), thidiazuron (TDZ), alone and in combination with 6-benzyladenine (BA) (Table 1). The pH was adjusted to 5.8 and autoclaved (121°C, 15 min). All media contained 0.8% agar, 3% sucrose and incubated at 25 ± 2°C, under two conditions of photoperiod (darkness and 16/8 h day/night). Then, the suitable callus was separated and transferred to fresh medium with the same composition. Callus induction rate was calculated after 4 weeks.

Tissue culture and plant propagation

Callus produced from leaf were used for indirect regeneration. After 4 weeks, for shoot regeneration of callus, the callus that have been best grown were dissected into small pieces and transferred to regeneration medium containing of half strength MS medium supplemented with different concentrations (0.5 and 1 mg L^{-1}) of

NAA in combination with different concentrations (0.5, 1, 2, 5, 7 and 10 mg L^{-1}) of BA (Table 2). Regenerated shoots were transferred to rooting medium, which consisted of half strength MS medium supplemented again with different concentration of NAA in combination with BA. Explants were maintained at 25 ± 2°C under a photoperiod of 16 h of cool-white florescent light and subcultured every two weeks.

Direct regeneration

Leaves of *F. vulgaris* were used for direct regeneration without callus formation. Explants were cultured on half-strange MS medium supplemented with the various concentrations and combinations of NAA with BA for shoot and root direct regeneration (Table 2). Regeneration rate was calculated after 2 weeks and explants were maintained at 25 ± 2°C under a photoperiod of 16 h of cool-white florescent light. Shoot regeneration was estimated by percentage of callus forming shoots and number of shoots formed by leaf after 8 weeks of culture.

Acclimatization

After 8 weeks, plantlets with well-developed roots washed in distilled water to remove agar from their roots were transferred to plastic cups containing sand, fertile soil and vermiculite (1:1:1) and growth under greenhouse conditions with 90% humidity. After 2 month, the plants placed in shade under natural conditions.

Statistical analysis

For all experiments there were three repeats and in each repeats five explants. Data were analyzed statistically using SAS 9.1 software. The mean values of different treatments were compared using Duncan's multiple tests (At $p < 0.05$).

RESULTS AND DISCUSSION

Callus induction

The callus was produced from the explants after 4 weeks of culture. The callus induced in the dark condition produce less chlorophyll and these had most growth compared with light condition. The light did not aid callus growth. Thus, darkness was beneficial for callus induction in comparison of light condition. The highest callus induction was achieved when MS medium supplement with 2,4-D in combination with BA (0.5 and 1.0 mg L^{-1}) was used (Figure 4A and B). Also the growth index increased when callus were exposed to MS medium supplemented with 1.0 mg L^{-1} TDZ in combination with 0.5 mg L^{-1} BA (Figure 4C and D). Different hormonal treatments were studied for callus induction of *F. vulgaris* as shown in Table 1 and Figure 1, of which most of them led to callus induction. The results of the present study showed that, auxin alone and in combination with cytokinin can produce callus but 2,4-D was the most effective for callus induction. These results is in line with other research, same as many other plants, because 2,4-D is the primary auxin which is used for the callus induction. TDZ can be substituted with a demine type cytokines in various culture systems, including callus and micro propagation of many plants (Lu, 1993). In the present study, callus induction from leaf explant in medium containing of 2,4-D in combination with BA were better than medium containing TDZ and BA. These results showed that equal concentrations of auxin and cytokinen (especially 0.5 and 1.0 mg L^{-1}), induced large and fragile callus which had better potential for regeneration. Similar result was reported by Pal and Dhar (1985) and Hitmi et al. (1998). Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots, or somatic embryos from which whole plants can subsequently be produced. Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies.

Plant propagation through callus

For plant regeneration from callus, callus was transferred to the development media; MS medium supplemented with different concentration of NAA and BA under light conditions (Table 2). After 2 weeks of culture, most callus started to turn to light green, then they became dark green (Figure 5A and B). The medium containing NAA (1.0 mg L^{-1}) and BA (0.5 and 1.0 mg L^{-1}) showed the highest number of shoot and root formation in this plant (Figure 7A to F). Among the various concentrations of BA (0.5 to 10 mg L^{-1}) tested, 0.5 and 1.0 mg L^{-1} BA showed the highest shoot regeneration from callus (Figures 2, 3 and 7C and D). This rate decreased on media supplemented with higher or lower NAA concentrations. Root formation increased with higher NAA concentration. There are few reports about plant regeneration by callus in Apiaceae family (Tiwari et al., 2000; Makunga et al., 2005).

Transition of callus to regeneration pathway was affected by interaction effect between BA and NAA. This interaction plays a key role in regeneration through indirect organogenesis pathway. Our finding is in agreement with Tiwari et al. (2000) reported micro propagation in *Centella asiatica* in the combination of NAA and BA for leaf and nodel explants. Data did not match those of Ayan et al. (2005) which reported that shoots of *H. perforatum* were rooted very intensively on MS medium supplemented with 1 mg L^{-1} of IAA.

Direct regeneration

Maximum rate of direct regeneration without callus formation was obtained with MS Medium supplemented with different concentrations of NAA and BA. In direct regeneration, NAA with 1.0 mg L^{-1} concentration was observed to be more potent than concentration of 0.5 mg

Table 2. Effects of different growth regulator combinations on regeneration from callus and leaf of *F. vulgaris.*

Growth regulators (mg L^{-1})		Callus explants		Leaf explants	
BA	NAA	Explants forming shoot (%)	Explant forming root (%)	Explants forming shoot (%)	Explant forming root (%)
0	0	0^e	0^c	0	0
0.5	0.5	92.3^b	5.7^b	0	0
1	0.5	100^a	13.1^a	0	0
2	0.5	12.1^c	0^c	0	0
5	0.5	5.9^d	0^c	0	0
7	0.5	0^e	0^c	0	0
10	0.5	0^e	0^c	0	0
0.5	1	94.3^b	48^b	31.8^b	7.33^b
1	1	100^a	88.3^a	0^c	0^c
2	1	16.1^c	5.7^c	56^a	15.9^a
5	1	6.3^d	2.88^d	0^c	0^c
7	1	0^e	0^e	0^c	0^c
10	1	0^e	0^e	0^c	0^c

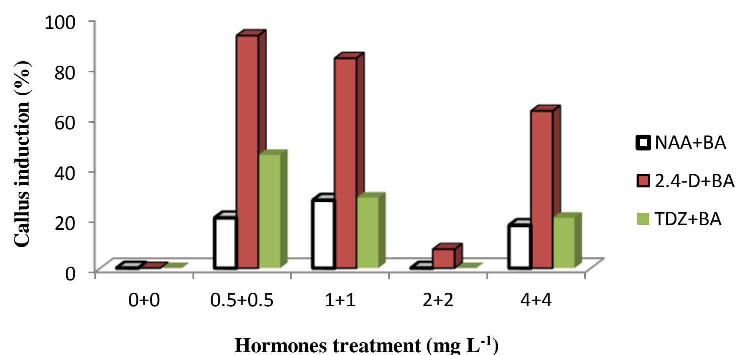

Figure 1. Effect of different growth regulator combinations on callus induction from leaves of *F. vulgaris.*

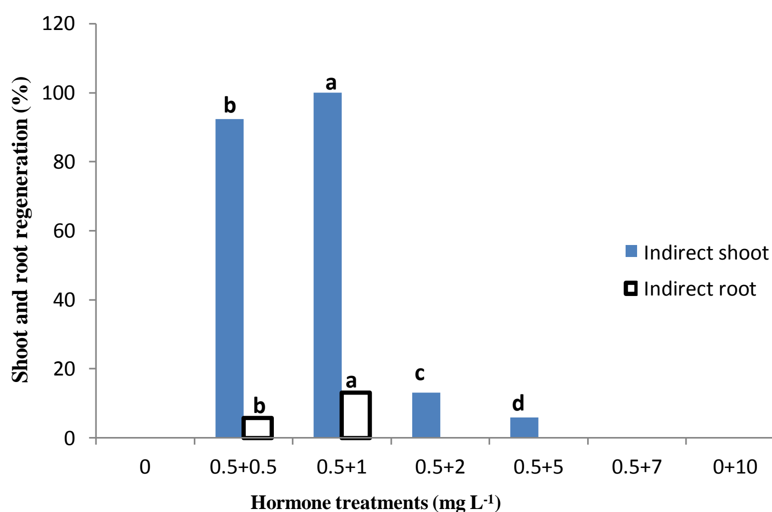

Figure 2. Effects of 0.5 mg L^{-1} NAA in combination with BA on regeneration from callus and leaf of *Falcaria vulgaris.*

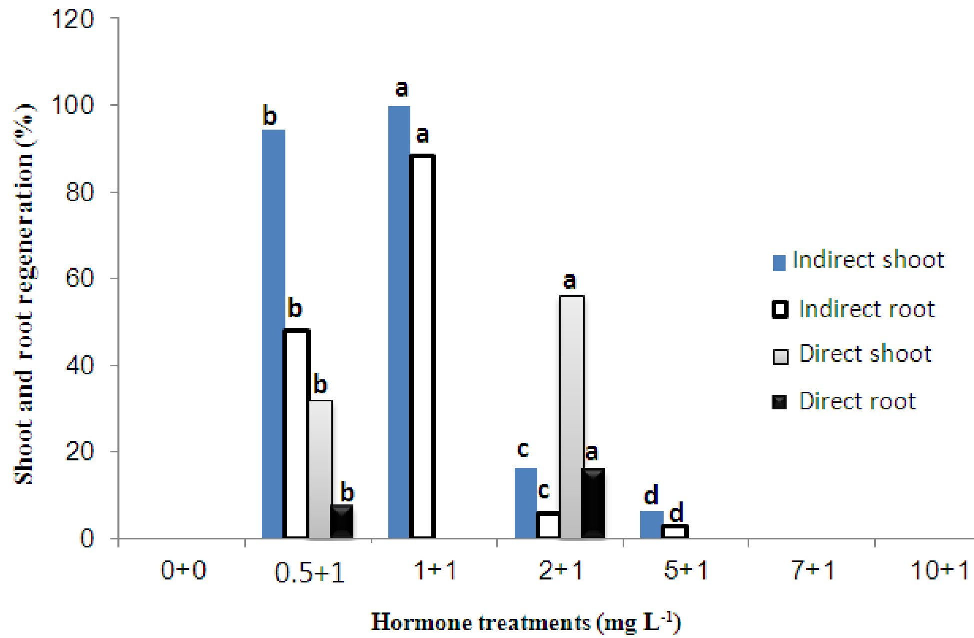

Figure 3. Effects of 1 mg L^{-1} NAA in combination with BA on regeneration from callus and leaf of *F. vulgaris*

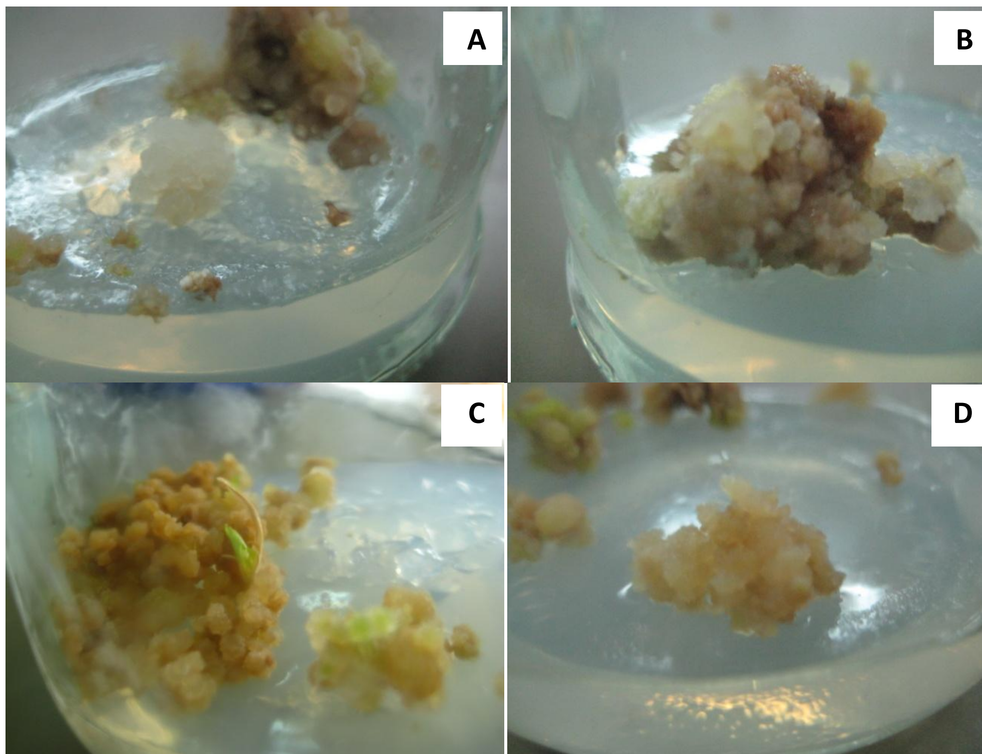

Figure 4. (A and B) Large and white callus induced from leaf under the culture on MS basal medium containing TDZ and BA. (C and D) Yellowish callus induced from leaf under the culture on MS basal medium containing TDZ and BA.

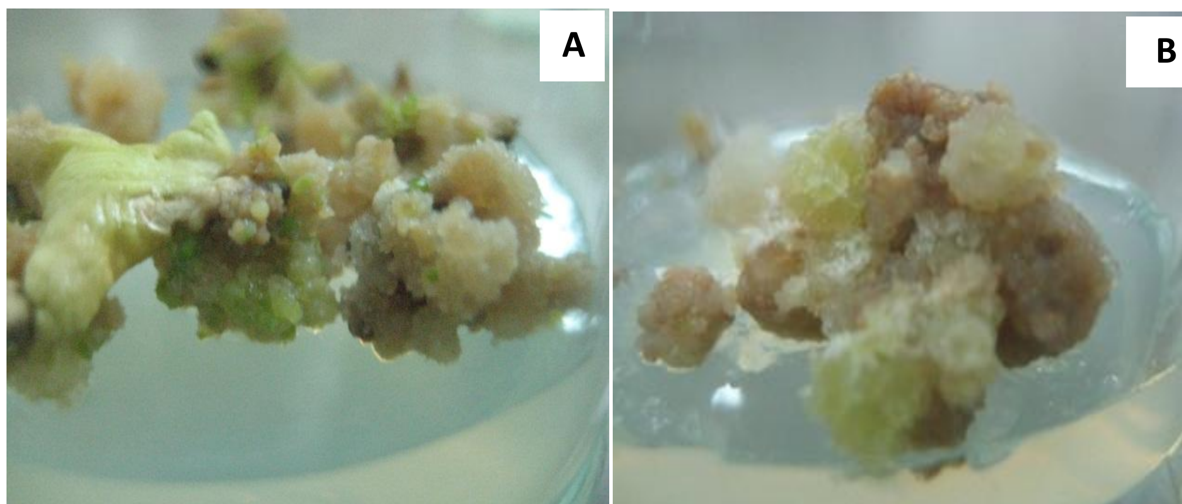

Figure 5. (A and B) Callus started to turn to light green after 2 week of callus culture on regeneration medium.

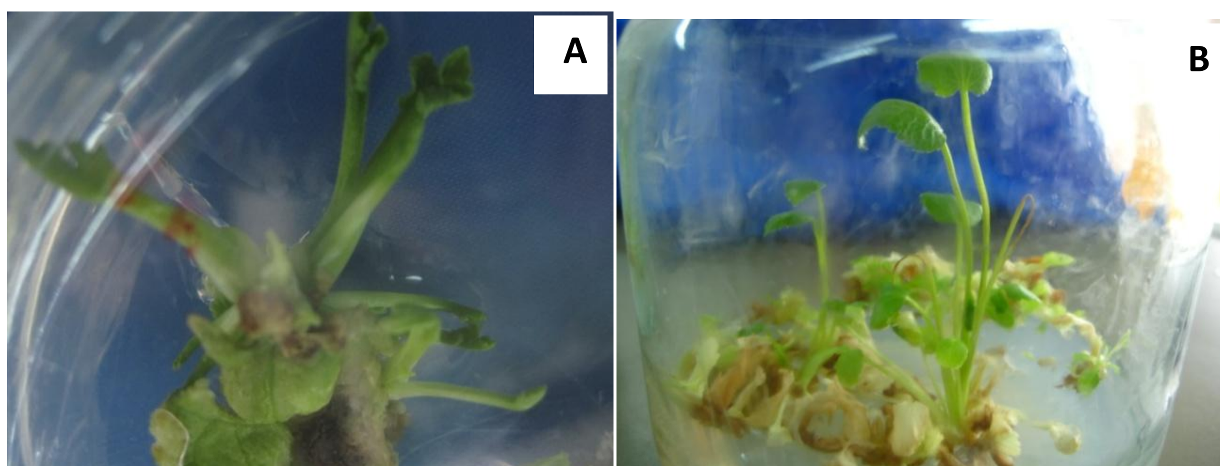

Figure 6. (A and B) Direct regeneration without callus formation from *Falcaria vulgaris* leaves and plantlets development of *F. vulgaris*.

L^{-1} NAA showed the highest root regeneration frequency (15.7%) and number of root (Figure 6A and B). Based on the results, application of NAA causing was producing of root and shoot in *F. vulgaris*. BA was the most important cytokinin used for regeneration of this plant. Because this hormone is effective in increasing of endogenous cytokinin plants. NAA was found to be the best for root induction. The findings are in agreement with those observed in other plant species such as *Caphaelis ipecacuanha* (Jha and Jha, 1989), *Plantago ovata* (Wakhlu and Barna, 1989). Effectiveness of BA + NAA for *in vitro* shoot regeneration and multiplication from shoot tip and leave cultures was reported in several other plants (Conver and Lits, 1987; Tokuhara and Mii, 1993). Our findings are compatible with those of Pretto and Santarem (2000), who reported that in *Hypericum perforatum*, BA was found to be the most efficient in promoting shoot regeneration when leaves were used as the explants.

Acclimatization

When the propagated plantlets were transferred to small plastic cups containing sand, fertile soil and vermiculite (1:1:1), and the humidity was maintained at approximately 90% by covering with plastic. After 2 month the plants were transferred to large pots and after acclimatization, the 100-day-old plants were transferred to field.

Over 90% of the plantlets were successfully acclimatized and were similar to the mother plants.

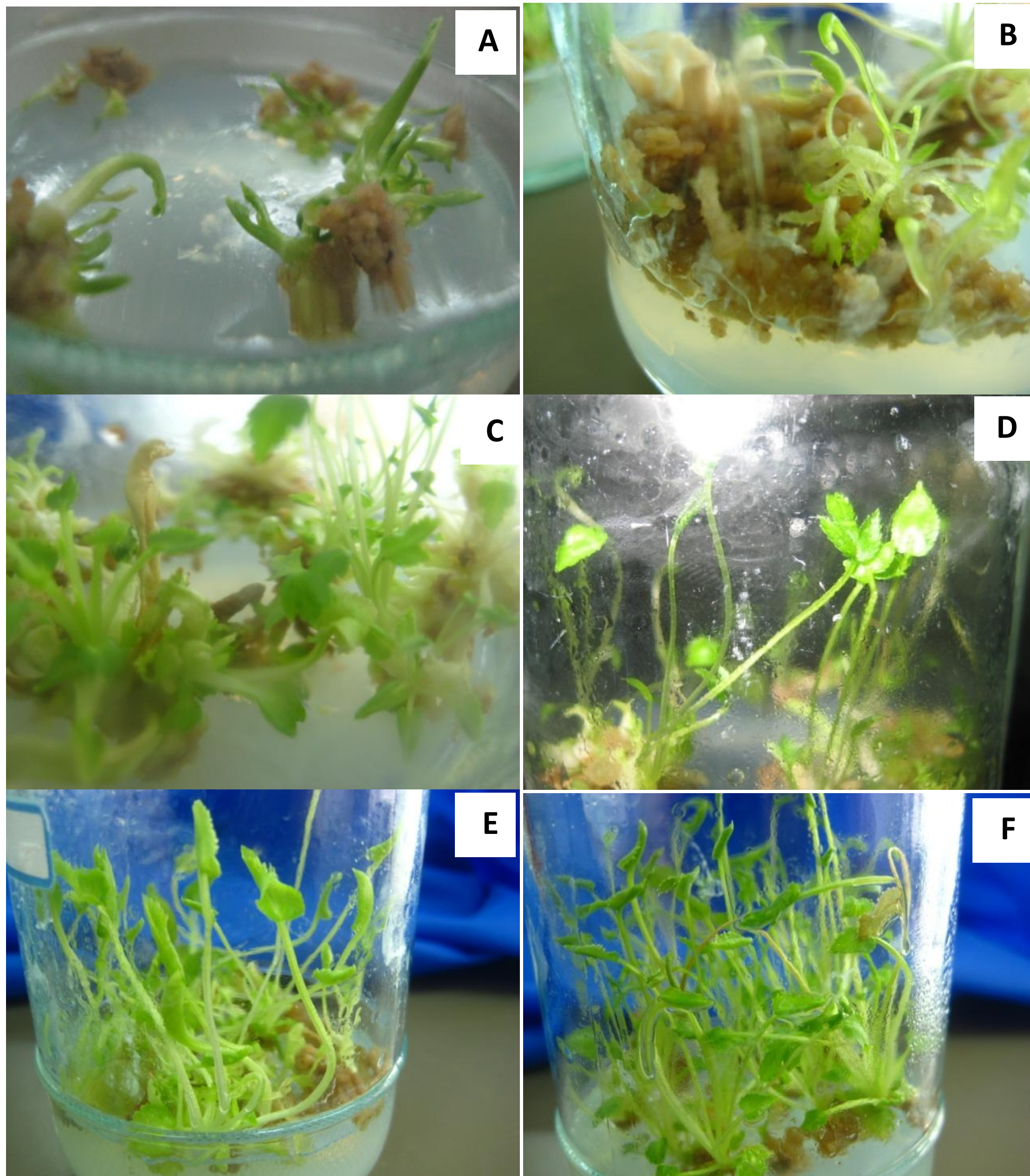

Figure 7. (A and B) Shoot and root regeneration from callus after six weeks of culture. (C and D) Plantlets rooting and shoot regeneration of *Falcaria vulgaris* in half strength MS + NAA and (0.5 and 1) BA after two weeks of subculture. (E and F) Plantlets development of *F. vulgaris*.

Conclusion

In this study, we reported for the first time a protocol for the successful callus induction, direct regeneration from leaf explants and regeneration through callus in *F. vulgaris* which would provide more homogenous source of medicine. The frequency of callus induction and regeneration from leaf explants described here was high enough to encourage us to carry out, protoplast culture genetic transformation and cell suspension culture for improvement of oil and secondary metabolites quality and quantity.

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