



# A highly efficient method for somatic embryogenesis of *Kelussia odoratissima* Mozaff., an endangered medicinal plant

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## Abstract

*Kelussia odoratissima* Mozaff. (or Kelus) is a medicinal plant native to the Zagros Mountains in Iran. This plant is widely used as a food flavoring and for its health-promoting properties. It has been considered an endangered species by the United Nations Development Programme. In this study, a somatic embryogenesis (SE) method was developed for mass propagation of Kelus. The green globular embryogenic callus was induced on cotyledonary leaves using the Murashige and Skoog (MS) medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25 mg/l Kinetin. Different treatments were assayed for proliferation of the embryogenic callus. The calli remained embryogenic in an MS medium containing 2,4-D (1 mg/l). The light treatments and carbon source showed significant effects ( $P \leq 0.05$ ) on the proliferation and development of somatic embryos. These treatments improved the conversion rate of the cotyledonary-stage embryos by 100%. The average numbers of embryos in the globular, heart, torpedo, and cotyledonary stages decreased by the addition of 3 g/l case in hydrolisate. The genetic stability among tissue culture-derived plants and the mother plant were assessed using the amplification fragment length polymorphism. No polymorphic band was observed among all the plants, exhibiting the genetic stability during in vitro multiplication. This research provides a promising approach for true-to-type plant multiplication of *K. odoratissima* through SE.

**Keywords** *Kelussia odoratissima* Mozaff. · Somatic embryogenesis · 2,4-D · Carbon source · Light treatment · AFLP

## Introduction

A quarter of new medicines in the market are based on plant-originated molecules (Raskin et al. 2002; Terry et al. 2006). The plant-derived medicine industry is estimated to grow to around 35.4 billion USD between 2013 and 2020 (Giri and Zaheer 2016). However, catastrophic environmental conditions following climate change and anthropogenic activities such as overgrazing, landslides, and indiscriminate

harvesting for food and medicinal purposes are the main reasons for the depletion of medicinal plant resources. Up to 80–90% of medicinal plants are harvested from their natural habitats (Nosov 2012). Around 4000–10,000 medicinal plant species are categorized as endangered (Canter et al. 2005). For example, *Dioscorea balcanica*, *Podophyllum hexandrum*, and *Pilocarpus jaborandis* are now regarded as extinct species and *Panax ginseng* has nearly disappeared in its natural growing areas (Nosov 2012).

*Kelussia odoratissima* Mozaff., belonging to the *Apiaceae* family, is found only in Iran and is locally known as “Karafse-koohi” or “Kelus” (Sajjadi et al. 2012). The natural habitats of this perennial medicinal plant is limited to heights of 2500 m above the sea in the Zagros Mountains, with at least 4–5 months of freezing temperature (Askari-Khorasgani et al. 2013; Razeghi et al. 2016). The aerial parts of *K. odoratissima* contain important secondary metabolites such as Z-Lingustilide and 3-e-butyl phthalide. Positive influences of Z-ligustilide on the nervous system, blood pressure, and cholesterol have been reported by Shojaei et al. (2011). These compounds are considered as

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anti-inflammatory, anti-cancer, neuroprotective, anti-hepatotoxic and anti-cardiovascular agents (Wu and Hsieh 2011; Chao and Lin 2012). Kelus is also used in salads, soups and as a seasoning powder in soups and dairy (Raiesi et al. 2013). It has been considered an endangered species by the United Nations Development Programme (UNDP) (Ahmadi et al. 2007). A long-term seed dormancy, slow growth rate, insufficient plant production in limited local habitats (Askari-Khorasgani et al. 2013) and over-harvesting in early growth stages (Raiesi et al. 2013) have decreased the population of Kelus in recent years. Hence, the adoption decisions such as domestication and cultivation can prevent its extinction and support a sustainable production of *K. odoratissima*.

Somatic embryogenesis (SE) is a tissue-culture technique with a strong potential to speed up the propagation rate in plants. By far, there is no scientific report on micropropagation of Kelus using SE. In vitro studies related to the breaking of the seed dormancy and organogenesis (Askari-Khorasgani et al. 2013; Abdollahi Sahlabadi et al. 2012), callus induction, and the production of secondary metabolites through cell-suspension culture (Razeghi et al. 2015, 2016) of *K. odoratissima* had been previously reported. SE is the development of embryo-like structures from somatic cells without any gametic fusion (Haque and Ghosh 2016), having three main advantages concerning medicinal plants: mass propagation of selected materials (Kumar and Sheela 2014), generation of diversity and genetic transformation (Von Arlond 2008; Vázquez-Flota et al. 2016). It has been shown that, 2,4-D at 0.5 or 1 mg/l are among the most convenient PGRs and concentrations for the induction and proliferation of embryogenic callus (Kato 1996; Pedroso and Pais 1999; Yantcheva et al. 1998; Li et al. 2006; Naing et al. 2013). Early studies, indicating the presence or absence of 2,4-D, is necessary to induce an altered genetic program in somatic cells (Fujimura and Komamine 1979; Choi and Sung 1984). The transcriptome analysis of the early stages of SE and leaf tissues in *Arabidopsis* indicated that the expression of more than 2500 genes changed in immature somatic embryos induced by 2,4-D (Wickramasuriya and Dunwell 2015). Besides, 2,4-D, other factors, too, have been shown to determine induction, frequency, and the development of somatic embryos in plants. For example, a combination of the cytokinins with auxin can improve the frequency of SE. Berthouly and Michaux-Ferrière (1996) produced a highly embryogenic callus in leaf explants of *Coffea canephora* using a combination of 2,4-D, IBA, and 2iP. Neuenschwander and Baumann (1992) showed that 4.5  $\mu\text{M}$  2,4-D with 18.4  $\mu\text{M}$  kinetin improved SE in *C. Arabica*. Nitrogen and carbon sources are also among a set of factors determining the fate of embryogenic response of tissues, although there are controversies over the type and concentrations of these compounds (Loyola-Vargas 2016).

Plant regeneration from relatively undifferentiated callus cells induces different types of genetic changes including DNA methylations, chromosome rearrangements, and single-gene mutations, generally termed ‘somaclonal variation’ (Phillips et al. 1994). The somaclonal variation (Larkin and Scowcroft 1981) can result in useful traits (Hwang and Ko 2004; Khan et al. 2014; Slazak et al. 2015) or unwanted characteristics that limit the mass micropropagation of similar plants (Basavaraj et al. 2016). Several molecular markers such as Methylation Sensitive Amplified Polymorphism (MSAP) (Peraza-Echeverría et al. 2001), Random Amplification of Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Inter Simple Sequence Repeats (ISSR), Amplified Fragment Length Polymorphism (AFLP) and Start Codon Targeted (SCoT) have been used for the detection of tissue-culture-induced mutations (Martins et al. 2004; Rathore et al. 2014; Sebastiani and Ficcadenti 2016; Slazak et al. 2015; Vroh-Bi et al. 2011).

Here, we developed, for the first time, an efficient protocol for the production of true-to-type plantlets in *K. odoratissima* Mozaff via SE. This study followed the most usual procedures for the induction of SE using 2,4-D. Moreover, the effects of the carbon source and casein hydrolysate were investigated in the proliferation of embryogenic callus. To analyze the genetic stability of in vitro regenerated plantlets, the AFLP marker was used, which produced a large number of reproducible bands (Powell et al. 1996).

## Materials and methods

### Plant materials

The seeds of *K. odoratissima* Mozaff were collected from its natural habitat of “Chaharmahal and Bakhtiari” province in Iran. The seeds were treated with 70% ethanol for 1 min, and washed immediately in sterile distilled water, followed by dipping in freshly prepared 1% (w/v) hypochlorite sodium and a few droplets of tween-20 for 15 min. Then, the seeds were rinsed three times with sterile distilled water and cultured in a hormone-free (HF) MS basal medium (Murashige and Skoog 1962) containing 3.0% (w/v) sucrose (pH 5.8) and incubated in a dark condition for 2 months at 4 °C for germination.

### Embryogenic callus induction and proliferation

The hypocotyls and cotyledonary leaves were removed from in vitro germinated seeds for use as explants for callus induction. The cotyledonary leaves were cut in half and cultured with the adaxial side in the medium. For the induction of embryogenic callus, explants were cultured in an MS medium supplemented with four combinations of growth

regulators 2,4-D (0.5 and 1 mg/l) + Kin (0 and 0.25 mg/l). A few replicates were used as a control in a HF-MS medium. At least 4 replications with 4 petri dishes/replicate were used for each treatment. Each petri dish separately contained 25 ml of culture medium with 10 samples from each explant type. The cultures were incubated at  $25 \pm 1$  °C in a dark condition. The percentage of embryogenic calli was recorded after 4 weeks. The proliferation of embryogenic calli was assayed by applying two different concentrations of 2,4-D (0.5 and 1 mg/l) supplemented with Kin (0.25 mg/l) with 0 and 3 g/l casein hydrolysate (pancreatic hydrolysate of casein-Duchefa/C1301) in MS basal salts. Three types of carbon source including sucrose (0 g/l) + maltose (30 g/l), sucrose (15 g/l) + maltose (15 g/l) and sucrose (30 g/l) + maltose (0 g/l) and two different light conditions (0 and 20  $\mu\text{M}/\text{m}^2/\text{s}$  photosynthetic photon flux density (PPFD) emitted from cool fluorescent tubes; 16/8 light/dark regime) were also tested for all treatments. The cultures were kept at  $25 \pm 1$  °C and, after 4 weeks, the percentage of calli that remained embryogenic and the average number of embryos at different developmental stages were recorded.

### Somatic embryo development

At least 50 embryos in the globular, heart, and torpedo stages from the last step were subcultured in petri dishes containing 25 ml of the HF-MS medium supplemented with sucrose (15 g/l) + maltose (15 g/l) 'according to the best medium composition from the proliferation stage', separately. Each petri dish was considered as a replicate and the assay was repeated thrice. The cultured media were incubated in the dark or 20  $\mu\text{M}/\text{m}^2/\text{s}$  PPFD; 16/8 light/dark regime at  $25 \pm 1$  °C. The effects on embryo at various developmental stages and the light treatment on development of the embryos were evaluated after 4 weeks.

### Maturation and germination

The fully developed cotyledonary embryos were subjected to desiccation to stimulate the maturation process. The embryos were transferred to empty petri dishes containing 2 ml of the MS medium without plant growth regulators (PGRs) dropped at the corner to avoid over desiccation. Each petri dish had at least 100 embryos and was kept in a laminar flow at  $25 \pm 1$  °C in a dark condition for 2, 4, 6, 8 h without sealing by parafilm. After the expiration of the dedicated durations, the embryos were transferred to a ½ macro HF-MS medium to evaluate the effect of desiccation times on embryo germination. The same number of embryos was used as control (0 h) without any desiccation treatment and subjected directly to the ½ macro HF-MS medium. The cultures were incubated at  $25 \pm 1$  °C under a 16/8 light/dark regime with 40  $\mu\text{M}/\text{m}^2/\text{s}$  light intensity of a fluorescent lamp.

The percentage of germination of the embryos under different treatment was recorded after 4 weeks. This experiment was performed with 3 replicates. The germinated embryos were transferred to the soil after 2 weeks of acclimatization at  $20 \pm 1$  °C and 85% relative humidity in the phytotron (100  $\mu\text{M}/\text{m}^2/\text{s}$  PPFD; 16/8 light/dark regime) (Fig. H).

### DNA extraction and AFLP assays

The genomic DNA from hypocotyl of a single seed as a mother plant and leaves of 12 regenerated plantlets from cotyledonary leaves of the same seed were extracted using *DNeasy Plant Mini Kit* (Qiagen, USA) and 200 ng of DNA was subjected to a digestion step using *EcoRI* and *MseI* restriction enzymes (Thermo Fisher Scientific, USA). The ligations of adapters, preselective and selective amplification, were performed, according to the standard AFLP procedures (Vos et al. 1995). In the selective amplification step, 2  $\mu\text{l}$  of 1:30 diluted pre-amplified products were added to the PCR tube with 12  $\mu\text{l}$  of the final volume reaction of the PCR mix containing 0.5 unit *Taq* DNA polymerase (Thermo Fisher Scientific, USA), 2 mM  $\text{MgCl}_2$ , 0.2 mM each dNTPs, and 0.5  $\mu\text{M}$  each of reverse and forward primers. Amplification products were analyzed by electrophoresis in a 7% polyacrylamide gel and visualized using a silver staining method (Blum et al. 1987). The gel images were scanned with 300 dpi resolution using ImageScanner III (GE Healthcare, Sweden). The search for polymorph bands was performed visually.

### Statistical analysis

All the experiments were performed in factorial using a CRD basal design with at least 3 replicates. The SAS ver. 9.2 was used for the analysis of variance. Comparisons of the mean values were made by Duncan's multiple range test ( $P \leq 0.05$ ).

## Results and discussion

### Effect of explant type and PGRs on induction of embryogenic callus

The callus induction was begun after 12–14 days of the culture of both explant types. No callus was observed on explants incubated in the HF-MS medium; so this treatment was removed for further investigation and data analysis. According to ANOVA, significant differences ( $P \leq 0.05$ ) were observed among the different treatments for the induction of embryogenic callus on explants originating from in vitro germinated seeds. The maximum frequency of embryogenesis was observed on cotyledonary leaves incubated in the

MS medium supplemented with 1 mg/l of 2,4-D + 0.25 mg/l Kin (Table 1; Fig. 1a). In this treatment, 17.67% of embryogenic callus was produced on the cotyledonary leaves (Table 1). Despite the callus formation on hypocotyls using these treatments, these explants proved to be incompetent to induce somatic embryos. The embryogenic callus generally showed a green, globular color and texture. The formation of embryogenic callus depended on the explant type and composition of culture media. The in vitro cultured somatic cells were able to differentiate into embryos using appropriate PGRs (Hand et al. 2016). The 2,4-D has been recorded as one of the most convenient treatments for the induction of SE on competent cells and tissues, partly through the induction of DNA hypermethylation (Altamura et al. 2016). The single-effect study of PGR indicated that 1 mg/l of 2,4-D + 0.25 mg/l of Kin produced 7.25% and 7.83% embryogenic callus, respectively. This concentration was significantly different ( $P \leq 0.05$ ) from 2,4-D (0.5 mg/l) + Kin (0.0 mg/l). Our findings are in agreement with other previous studies of 2,4-D mediated SE in medicinal plants (Martin 2003; Beena and Martin 2003; Dhir et al. 2014). Martin (2003) showed that 1 mg/l 2,4-D was the most effective growth regulator for the induction of embryogenic callus in *Holostemma adakodien*. The internode and leaf explants of *Ceropegia Candelabrum* L. showed SE on the MS medium containing 4.52  $\mu\text{M}$  (~1 mg/l) of 2,4-D (Beena and Martin 2003). Same results have been reported on the induction of SE in *Anethum graveolens* L., a medicinal herb, using 2,4-D with a lower concentration (Dhir et al. 2014). A combination of cytokinin with auxin was shown to improve SE in some plants. A successful induction of SE was reported by the culture of immature zygotic embryos of *Acacia farnesiana* and *A. schaffnerii* in an MS medium supplemented with 9.05  $\mu\text{M}$  (~2 mg/l) 2,4-D and 4.65  $\mu\text{M}$  (1 mg/l) Kin (Ortiz et al. 2000). Similarly, the application of 8.8  $\mu\text{M}$  (~2 mg/l)

BA and 4.0  $\mu\text{M}$  (~0.9 mg/l) 2,4-D was highly efficient for the induction of SE and embryogenic callus in *Phellodendron amurense* Rupr. (Azad et al. 2009).

### Proliferation of embryogenic callus

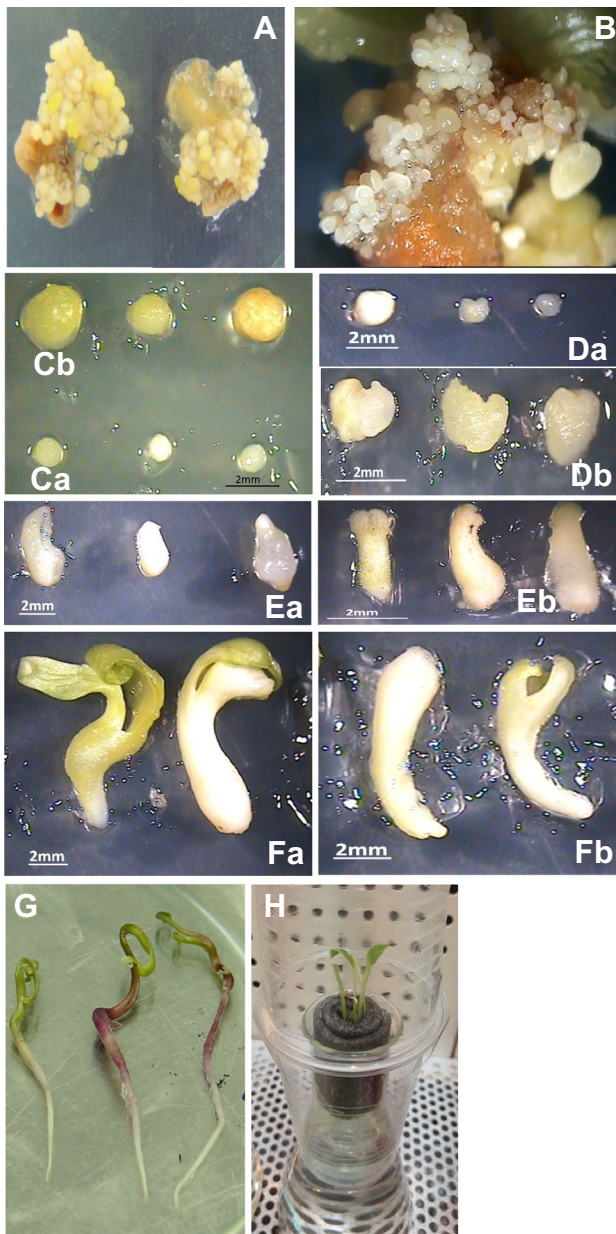
The proliferation of embryogenic callus was significantly ( $P \leq 0.05$ ) affected by different concentrations of 2,4-D, casein hydrolizate, carbon source, and light treatment. A single-effect study of 2,4-D on the proliferation of embryogenic callus indicated that in 1 mg/l of 2,4-D, 70.32% of calli remained embryogenic (Table 2). This was higher than 60.8% for 0.5 mg/l of 2,4-D. As demonstrated in Tables 2 and 3, following the application of casein hydrolizate (3 g/l), the average numbers of embryos in the globular, heart, torpedo and cotyledonary stages decreased significantly ( $P \leq 0.05$ ). This finding was in contradiction with the results of some of the other researchers such as Ageel and Elmeer (2011), who argued that the use of 3 g/l of casein hydrolizate improved the somatic embryogenesis in date palm. Although the casein hydrolizate was shown to provide a favorable nitrogen source during SE (Rybczynski and Zdunczyk 1986; Halperin 1995; Ageel and Elmeer 2011; Khierallah and Hussein 2013), its role remained controversial. It seems that the amount of nitrogen is more important than its type and form for the induction of SE (Reinert et al. 1967; Marques 1987; Fuentes-Cerda et al. 2001). As Ageel and Elmeer (2011) showed, the best result was obtained in ½ strength of the MS macro nutrients and 3 g/l of casein hydrolizate; so it is assumed that, in our experiment, the amount of nitrogen in the full MS macro nutrients was adequate to meet the requirement of the embryogenic tissues and the excessive nitrogen source had a deleterious effect on their proliferation.

**Table 1** The Effect of 2,4-D + Kin and explant type on the induction of embryogenic callus in *K. odoratissima* Mozaff

Explant type	Growth regulators (mg/l)		Number of explant	Callus color texture	% of embryogenic callus Mean $\pm$ SD
	2,4-D	Kin			
Cotyledonary leaf	0.5	0.0	40	White friable	7.33 $\pm$ 2.04 <sup>c</sup>
	0.5	0.25	40	Green globular	13.67 $\pm$ 3.21 <sup>b</sup>
	1.0	0.0	40	Green globular	11.33 $\pm$ 3.05 <sup>b</sup>
	1.0	0.25	40	Green globular	17.67 $\pm$ 2.52 <sup>a</sup>
Hypocotyl	0.5	0.0	40	White friable	0.0 $\pm$ 0.0 <sup>d</sup>
	0.5	0.25	40	White friable	0.0 $\pm$ 0.0 <sup>d</sup>
	1.0	0.0	40	White globular	0.0 $\pm$ 0.0 <sup>d</sup>
	1.0	0.25	40	Brown compact	0.0 $\pm$ 0.0 <sup>d</sup>

Data was recorded after 4 weeks in 4 replications (4 petri dishes/replicate and 10 sample/petri dish)  
SD standard deviation

Each value in the column represents the mean  $\pm$  standard deviation. The means with the same letters in each column are not significantly different according to Duncan's multiple range test ( $P \leq 0.05$ )



**Fig. 1** Somatic embryogenesis in *K. odoratissima* Mozaff. **a** induction of embryogenic callus on cotyledonary leaves, **b** embryogenic callus in proliferation step, **c** comparison of somatic embryo at globular stage in embryo development treatment; **a** dark and **b**: light conditions, **d** comparison of somatic embryo at heart stage in embryo development treatment; **a**: dark and **b**: light conditions, **e** comparison of somatic embryo at torpedo stage in embryo development treatment; **a**: dark and **b**: light conditions, **f** comparison of somatic embryo at cotyledonary stage in embryo development treatment; **a**: dark and **b**: light conditions, **g** germinated somatic embryos, **h** acclimatized plant from germinated somatic embryo 4 weeks after hardening

The carbon source proved to be another determining factor in the maintenance of embryogenic callus. The dedicated parameters were significantly ( $P \leq 0.05$ ) higher in a

medium containing a combination of 15 g/l of both sucrose and maltose (Tables 2, 3). The percent of embryogenic callus in this treatment was significantly higher (85.8%) than in the two other treatments with a carbon source. The source is a conventional carbon source for tissue culture of several plant species. The SE is suppressed with high concentrations of sucrose. The sucrose and nutrient starvation can induce embryogenic responses (Nic-Can et al. 2016). This stress can mediate for the induction of some stress related regulatory pathways involved in SE. Although the stress signal recognition and transduction pathway in explants to obtain the SE competency are not fully understood, the new findings indicate that these processes are controlled genetically. For example, the *RWP-RK DOMAIN CONTAINING (RKD)* and *AGAMOUS-15 (AGL15)* are two transcription factors with regulatory effects on the induction of cellular competency during SE. (Közsegi et al. 2011; Zheng and Perry 2014). The function of *SERK1 (SOMATIC EMBRYOGENESIS RECEPTOR KINASE1)* (Hecht et al. 2001) and *LEAFY COTYLEDON (LEC1, LEC2)* (Braybrook et al. 2006) have also been studied extensively for their involvement in the enhancement of cell competency in explants. Expression of these genes is correlated with the nutrient induced stresses under the in vitro condition (Nic-Can et al. 2016). Maltose is a reducing sugar with the same value or sometimes exceeding the sucrose in the SE of a number of species, such as carrot, alfalfa, wild cherry, Malus etc. This sugar is used as both osmoticum and a carbon source (George et al. 2008). Fuentes et al. (2000) showed that the substitution of fructose or maltose with sucrose increased the SE in *Coffea canephora* (Fuentes et al. 2000).

In this study, light treatment had a positive effect on the growth of embryos (Fig. 1c–f). Although the percent of embryogenic callus and the average number of embryos at the heart stage were not affected by the light treatment, the average number of embryos at torpedo and cotyledonary stages were influenced (Table 2). The embryos at different developmental stages had a bigger and greener appearance under the light treatment compared to dark conditions. Light treatment promoted the development of embryos in the torpedo and cotyledonary stages (Table 3). It seems that the dark condition is preferable for the maintenance of embryogenic callus in globular stage. All of the calli remained embryogenic in the MS medium with 1 mg/l 2,4-D and 15 g/l of both sucrose and maltose under light and dark conditions, but the dark condition was preferable for its ability to produce 114 embryos in the globular stage per explant compared to light conditions with 77 embryos in the globular stage (Table 3). Light is one of the main physical parameters determining the fate of an embryogenic tissue. Meneses et al. (2005) indicated that one week of dark treatment of embryogenic calli in the pre-regeneration step of indica rice (*Oryza sativa*) followed by light can improve the

**Table 2** The single effect of 2,4-D, casein hydrolyzate, carbon source and light on proliferation of embryogenic callus of *Kelussia odorotissima* Mozaff. (X1) % of embryogenic calli, (X2) average number of

embryos in the globular stage, (X3) average number of embryos in the heart stage, (X4) average number of embryos in the torpedo stage, (X5) average number of embryos in the cotyledonary stage

Variable	Different treatments									
	2,4-D (mg/l)		Casein hydrolyzate (g/l)		Carbon source (g/l)			Light ( $\mu\text{M}/\text{m}^2/\text{s}$ )		
	0.5	1	0	3	0 Suc.+30 Malt.	15 Suc.+15 Malt.	30 Suc.+0 Malt.	0	20	
	Mean $\pm$ SD*	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	
X1	60.83 $\pm$ 1.91b	70.32 $\pm$ 2.54a	66.6 $\pm$ 2.53a	64.3 $\pm$ 2.05a	37 $\pm$ 6.18c	85.8 $\pm$ 1.07a	74.2 $\pm$ 9.7b	65 $\pm$ 2.37a	65.9 $\pm$ 2.24a	
X2	12.19 $\pm$ 4.89b	33.03 $\pm$ 3.2a	29.5 $\pm$ 3.34a	15.58 $\pm$ 8.7b	10 $\pm$ 2.9c	38.08 $\pm$ 3.6a	19.17 $\pm$ 1.5b	25.3 $\pm$ 3.01a	19.6 $\pm$ 1.93b	
X3	3.1 $\pm$ 2.29b	10.06 $\pm$ 8.5a	7.9 $\pm$ 3.558a	5.17 $\pm$ 4.5b	3.5 $\pm$ 1.9b	9.04 $\pm$ 6.7a	7.04 $\pm$ 5.44a	6.94 $\pm$ 3.25a	6.08 $\pm$ 2.37a	
X4	3.58 $\pm$ 2.3b	6.9 $\pm$ 5.03a	6.34 $\pm$ 2.25a	4.13 $\pm$ 2.7b	3.67 $\pm$ 2.3c	6.62 $\pm$ 3.2a	5.39 $\pm$ 1.94b	3.86 $\pm$ 1.77b	6.63 $\pm$ 3.2a	
X5	2.06 $\pm$ 1.5b	5.0 $\pm$ 2.4a	4.3 $\pm$ 1.78a	2.7 $\pm$ 1.8b	1.5 $\pm$ 0.9b	4.79 $\pm$ 2.22a	4.26 $\pm$ 2.03a	1.2 $\pm$ 0.8b	5.86 $\pm$ 1.79a	

Data was recorded after 4 weeks in 4 replications (4 petri-dishes/replicate and 10 sample/petri dish)

SD standard deviation

Each value in the column represents the mean  $\pm$  standard deviation. The means with the same letters in each column are not significantly different according to Duncan's multiple range test ( $P \leq 0.05$ ).

regeneration of somatic embryos. In another attempt, Elmeer and Hennerty (2008) reported that five times more SEs were induced on calli in cucumber using a medium containing 2 mg/l of 2,4-D under dark conditions compared to a light regime (*Cucumis sativus*) (Elmeer and Hennerty 2008). This experiment indicated that the proliferation of embryogenic callus in *K. odorotissima* Mozaff. may not require light treatment, while further development of somatic embryos was enhanced by light.

### Somatic embryo development

Although the general belief is that auxin removal is essential to allow the induction and further development of somatic embryos (Halperin 1964), here, we assayed the possibility that the somatic embryo development and maturation in *K. odorotissima* is influenced by light. To study the conversion rate of somatic embryo to the cotyledonary stage, the effects on embryo at different developmental stages and light conditions were evaluated. According to the single-effect analysis, the highest conversion rate (100%) was observed among embryos in the torpedo stage, which was significantly ( $P \leq 0.05$ ) more than the globular (15.5%) and heart (20.8%) stages. Light or dark conditions had a positive impact on the developmental behavior of embryos. The best conversion rate to the cotyledonary stage, which occurred under the light condition (51.4%), was significantly ( $P \leq 0.05$ ) higher than the dark treatment (39.4%). The Fig. 2 indicates the interaction effects of the embryo developmental stage and light treatment (0 and 20  $\mu\text{M}/\text{m}^2/\text{s}$ ) on the development of embryos to the cotyledonary stage. As shown in the Fig. 2, light treatment improved the development of embryos at both the globular and heart stages. The morphological

changes of embryos at different stages under light and dark regimes are presented in Fig. 1c–f. As mentioned earlier, the development of embryos in the torpedo and cotyledonary stages was promoted under light condition, and the embryos in different developmental stages were bigger and greener in light condition compared to the dark regime. The efficacy of light and dark conditions on the induction, development, and maturation of somatic embryos of various plant species were investigated. Our finding is consistent with Oh et al. (2013), demonstrating that light is an essential factor for the conversion of the globular embryos to the cotyledonary stage in *Houttuyniacordata*. Around 15% of the embryos at the globular stage successfully converted to the cotyledonary stage in the presence of light (Oh et al. 2013). The induction and development of somatic embryos in other plant species like squash (*Cucurbita pepo* L.), melon (*Cucumis melo* L.), pepper (*Capsicum annum* L.), gardenia (*Gardenia jasminoides* L.) and rose (*Rosa hybrida* L.) have been studied under different light intensity and duration (Kintzios et al. 1998). Although more somatic proembryos and globular embryos were induced on explants initially incubated in the dark or under a low PPFD, the development of embryo to the cotyledonary stage and the embryo maturation were significantly affected by the exposure of the cultures to light (Kintzios et al. 1998).

### Effect of desiccation on maturation and germination of somatic embryos

Most of the medicinal plants are difficult to propagate through SE. They are mostly recalcitrant plants because of poor embryo maturation and conversion (Facchini et al. 2008). Treatment of somatic embryos with ABA and GA

**Table 3** Interaction of 2,4-D, casein hydrolyzate, carbon source and light on the proliferation of embryogenic callus of *Kelussia odorotissima* Mozaff. (X1) % of embryogenic calli, (X2) average number of

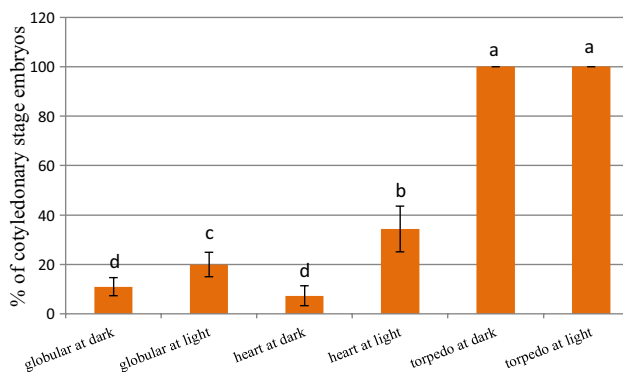
embryos in the globular stage, (X3) average number of embryos in the heart stage, (X4) average number of embryos in the torpedo stage, (X5) average number of embryos in the cotyledonary stage

Treatment					Mean ± SD				
2,4-D (mg/l)	Casein hydrolysate (g/l)	Carbon source (g/l)		Illumination (µM/m <sup>2</sup> /s)	X1	X2	X3	X4	X5
		Sucrose	Maltose						
0.5	0	0	30	0	31.67 ± 7.63 <sup>g</sup>	6.33 ± 1.52 <sup>lm</sup>	6.33 ± 1.5 <sup>ef</sup>	7.00 ± 1.0 <sup>bc</sup>	0.66 ± 0.15 <sup>gh</sup>
0.5	0	0	30	20	35.00 ± 5.00 <sup>g</sup>	10.00 ± 1.0 <sup>kl</sup>	4.33 ± 0.5 <sup>fgh</sup>	4.33 ± 1.15 <sup>cdef</sup>	1.33 ± 0.2 <sup>fgh</sup>
0.5	0	15	15	0	76.67 ± 2.89 <sup>def</sup>	12.00 ± 2.64 <sup>hijk</sup>	2.33 ± 0.5 <sup>ghij</sup>	2.33 ± 0.5 <sup>fghi</sup>	1.00 ± 0.2 <sup>gh</sup>
0.5	0	15	15	20	71.67 ± 2.88 <sup>def</sup>	19.33 ± 4.04 <sup>fgh</sup>	1.00 ± 0.2 <sup>hij</sup>	7.00 ± 1.0 <sup>bc</sup>	9.00 ± 1.0 <sup>bc</sup>
0.5	0	30	0	0	73.33 ± 5.77 <sup>def</sup>	12.67 ± 1.15 <sup>hijk</sup>	3.66 ± 1.1 <sup>fghi</sup>	0.66 ± 0.4 <sup>i</sup>	0.00 ± 0.0 <sup>h</sup>
0.5	0	30	0	20	69.33 ± 5.13 <sup>ef</sup>	18.33 ± 1.52 <sup>fgh</sup>	3.33 ± 0.5 <sup>fghi</sup>	2.33 ± 0.5 <sup>fghi</sup>	2.66 ± 1.1 <sup>efg</sup>
0.5	3	0	30	0	35.00 ± 8.6 <sup>g</sup>	10.67 ± 1.15 <sup>ijkl</sup>	2.66 ± 0.5 <sup>fghij</sup>	1.33 ± 0.5 <sup>hi</sup>	0.66 ± 0.2 <sup>gh</sup>
0.5	3	0	30	20	40.00 ± 5.00 <sup>g</sup>	10.00 ± 1.0 <sup>kl</sup>	2.67 ± 1.5 <sup>fghij</sup>	1.33 ± 0.7 <sup>hi</sup>	0.66 ± 0.3 <sup>gh</sup>
0.5	3	15	15	0	78.33 ± 2.8 <sup>de</sup>	14.33 ± 0.57 <sup>fghij</sup>	0.66 ± 0.1 <sup>ij</sup>	2.33 ± 0.8 <sup>fghi</sup>	0.66 ± 0.21 <sup>gh</sup>
0.5	3	15	15	20	80.00 ± 5.0 <sup>cd</sup>	10.00 ± 2.0 <sup>ijkl</sup>	0.00 ± 0.0 <sup>j</sup>	4.66 ± 0.5 <sup>cdef</sup>	3.33 ± 1.5 <sup>ef</sup>
0.5	3	30	0	0	67.67 ± 6.43 <sup>f</sup>	17.67 ± 2.5 <sup>fghi</sup>	6.66 ± 2.8 <sup>ef</sup>	6.33 ± 2.1 <sup>bcd</sup>	1.33 ± 0.4 <sup>fgh</sup>
0.5	3	30	0	20	71.33 ± 2.31 <sup>def</sup>	5.00 ± 2.0 <sup>m</sup>	3.33 ± 1.15 <sup>fghi</sup>	3.33 ± 0.6 <sup>efgh</sup>	3.33 ± 1.2 <sup>ef</sup>
1	0	0	30	0	40.00 ± 5 <sup>g</sup>	8.67 ± 0.57 <sup>ijklm</sup>	1.67 ± 0.5 <sup>ghij</sup>	3.33 ± 2.0 <sup>efgh</sup>	0.66 ± 0.3 <sup>gh</sup>
1	0	0	30	20	36.67 ± 7.6 <sup>g</sup>	7.33 ± 2.5 <sup>klm</sup>	2.0 ± 1.2 <sup>ghij</sup>	4.66 ± 0.6 <sup>cdef</sup>	4.00 ± 2.66 <sup>de</sup>
1	0	15	15	0	100.00 ± 0.0 <sup>a</sup>	114.0 ± 18.5 <sup>a</sup>	18.0 ± 2.1 <sup>ab</sup>	8.33 ± 1.5 <sup>b</sup>	0.66 ± 0.12 <sup>gh</sup>
1	0	15	15	20	100.00 ± 0.0 <sup>a</sup>	77.00 ± 8.5 <sup>b</sup>	26.0 ± 3.6 <sup>a</sup>	15.66 ± 4.04 <sup>a</sup>	15.66 ± 4.04 <sup>a</sup>
1	0	30	0	0	89.00 ± 1.41 <sup>ab</sup>	35.67 ± 3.32 <sup>c</sup>	14.6 ± 1.78 <sup>bc</sup>	5.66 ± 2.2 <sup>bcd</sup>	6.00 ± 4.3 <sup>cd</sup>
1	0	30	0	20	87.50 ± 3.53 <sup>bc</sup>	35.00 ± 9.2 <sup>de</sup>	13.50 ± 2.14 <sup>cd</sup>	19.00 ± 1.41 <sup>a</sup>	13.00 ± 4.24 <sup>a</sup>
1	3	0	30	0	38.33 ± 7.6 <sup>g</sup>	13.33 ± 4.16 <sup>hijk</sup>	5.33 ± 0.5 <sup>efg</sup>	1.66 ± 0.6 <sup>ghi</sup>	0.66 ± 0.23 <sup>gh</sup>
1	3	0	30	20	39.67 ± 4.5 <sup>g</sup>	13.67 ± 1.52 <sup>hij</sup>	3.0 ± 1.0 <sup>fghij</sup>	5.66 ± 2.5 <sup>bcd</sup>	3.33 ± 1.5 <sup>ef</sup>
1	3	15	15	0	91.67 ± 2.8 <sup>ab</sup>	35.33 ± 7.02 <sup>d</sup>	14.6 ± 6.4 <sup>bc</sup>	4.00 ± 1.7 <sup>defg</sup>	1.66 ± 0.6 <sup>efgh</sup>
1	3	15	15	20	88.33 ± 5.7 <sup>b</sup>	22.67 ± 7 <sup>ef</sup>	9.6 ± 1.52 <sup>de</sup>	8.66 ± 3.5 <sup>b</sup>	6.33 ± 1.5 <sup>bc</sup>
1	3	30	0	0	73.33 ± 7.6 <sup>def</sup>	22.33 ± 3.88 <sup>efg</sup>	6.67 ± 2.8 <sup>ef</sup>	3.33 ± 1.52 <sup>efgh</sup>	0.66 ± 0.2 <sup>gh</sup>
1	3	30	0	20	68.33 ± 2.89 <sup>ef</sup>	12.00 ± 3 <sup>hijk</sup>	6.6 ± 3.2 <sup>ef</sup>	7.00 ± 1.7 <sup>bc</sup>	10.00 ± 1.73 <sup>b</sup>

Data was recorded after 4 weeks in 4 replications (4 petri dishes/replicate and 10 sample/petri dish)

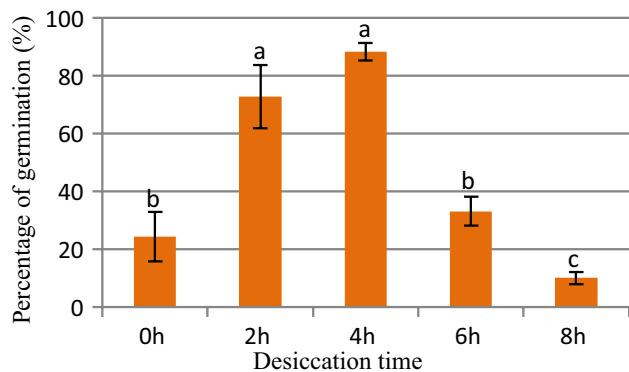
SD standard deviation

Each value in the column represents the mean ± standard deviation. The means with the same letters in each column are not significantly different according to Duncan’s multiple range test (P ≤ 0.05).



**Fig. 2** Interaction effects of embryo developmental stage and light treatment (0 and 20 µM/m<sup>2</sup>/s) (the x axis) on development of embryos to cotyledonary stage the y axis is the percentage of cotyledonary stage embryos

(0.1 and 0.2 mg/L) can promote the maturation process (Pathak et al. 2012). Partial desiccation is another practical treatment for the improvement of subsequent plant germination from somatic embryos (Othmani et al. 2009). In this experiment, the effects of four desiccation times (2, 4, 6 and 8 h) on the percentage of germination were examined and compared with control (0 h). The best result was observed by 4 hours’ treatment (Fig. 3) but was not significantly different from 2 h (P ≤ 0.05). These treatments significantly improved the percentage of germination compared to control (0 h). As shown in Fig. 3, the lowest percentage of germination occurred in the 8-hour desiccation treatment. The best germination rate of somatic embryos in our study occurred after 4 or 2 h of desiccation time. This finding is consistent with maturation of somatic embryos in date palm reported



**Fig. 3** The effect of desiccation time on percentage of germination (%) of fully developed cotyledonary somatic embryos on 1/2MS culture medium. The assay was performed with three replicates and at least 100 embryos/replicate

by Shareef et al. (2016). The maximum germination percentage was achieved when embryos were desiccated for 3 h. In the case of date palm, partial desiccation (0, 1, 2, 3 and 4 h) treatments were applied to boost the in vitro germination of somatic embryos. Like their zygotic counterparts, somatic embryos can remain dormant after development, and this can reduce embryo germination. It has been reported that the regulation of late embryogenesis abundant (LEA) proteins and the genes involved in biosynthesis and deposition of storage proteins are regulated by water stress and ABA treatment (Dodeman et al. 1997). These treatments can stimulate some important events such as starch depletion, raffinose occurrence, and sucrose and dehydrin accumulation to promote embryo maturation (Bomal and Tremblay 1999; Bomal et al. 2002). It is assumed that 2–4 h of desiccation treatment was enough for the maturation of somatic embryos in *K. odoratissima*, possibly through the inducement of these events.

### AFLP assays

Among molecular markers, AFLP produces much more reproducible bands, making it a good option for detection of genetic variability of plants regenerated by in vitro culture and somaclonal variations (Aversano et al. 2011; Mehta et al. 2011; Mo et al. 2009; Prado et al. 2007). The genetic stability of the mother plant and 12 regenerated plantlets from the best treatment (MS + 1 mg/l 2,4-D + 15 g/l maltose + 15 g/l sucrose + 20  $\mu$ M/m<sup>2</sup>/s illumination) was assayed using Ten selective primer combinations of AFLP (Table 4). Totally, 312 clear bands were amplified in the range of 100–600 bp (Fig. 4) all of which were monomorphic. This data shows that the propagation of *K. odoratissima* using in vitro embryogenesis produces true-to-type plantlets, which is important for mass

**Table 4** Number of bands in each primer combination among parental sample and 12 regenerated plantlets of *K. odoratissima* using in vitro embryogenesis

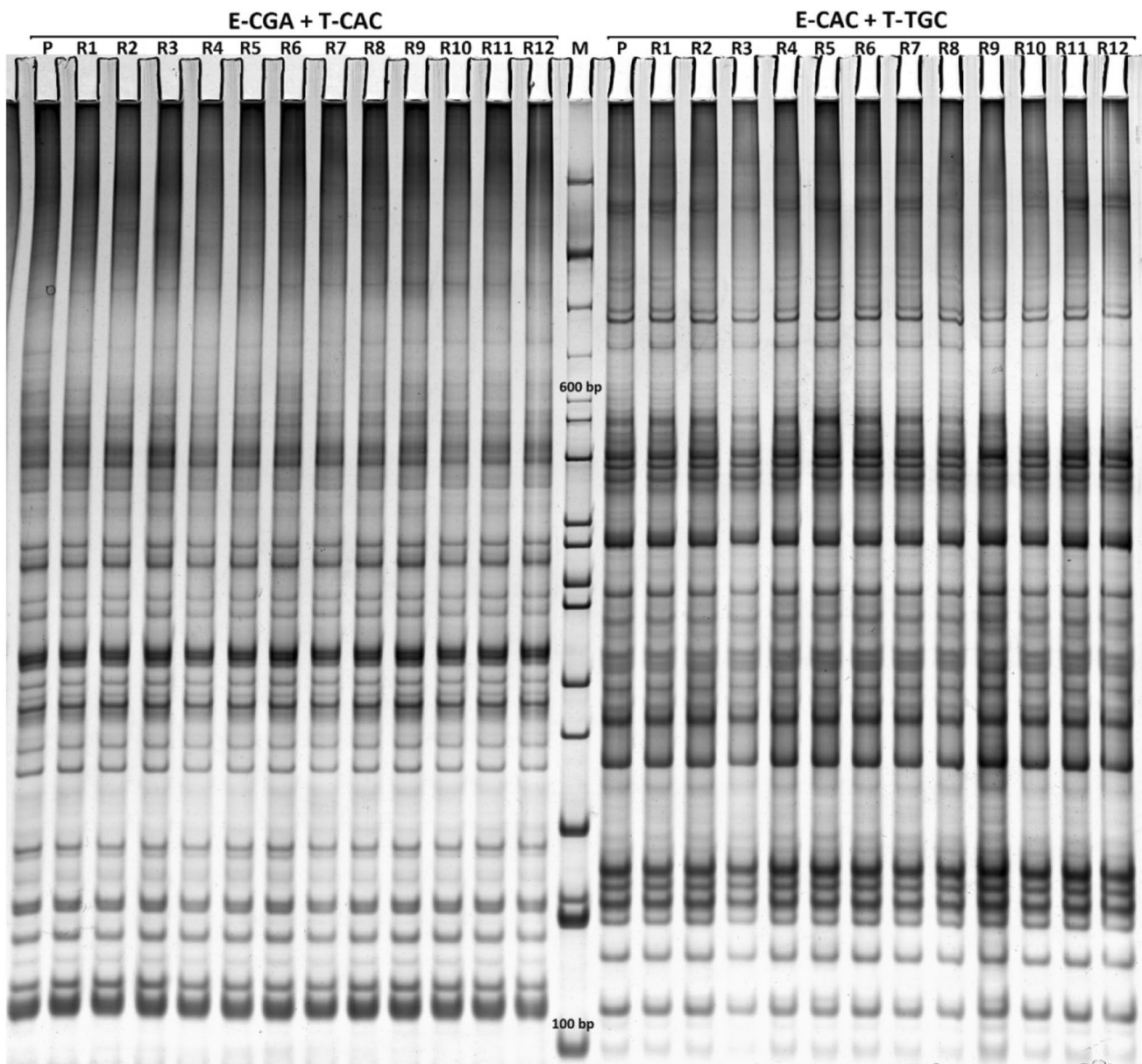
	Primer combinations		No. of bands	No. of polymorph bands
1	E-AAG	T-CAC	25	0
2	E-AAG	T-TGC	36	0
3	E-ACA	T-CAC	28	0
4	E-ACA	T-TGC	31	0
5	E-ACG	T-GAT	29	0
6	E-AGC	T-GGA	35	0
7	E-CAC	T-TGC	37	0
8	E-CAT	T-GGA	24	0
9	E-CGA	T-CAC-	28	0
10	E-CGA	T-CAA	39	0
	Total bands		347	0

production and conservation of this species. Depending on the species and genotype, in vitro regeneration of plants leads to genetic variation (Prado et al. 2007) or production of true-to-type regenerants (Mehta et al. 2011; Sebastiani and Ficcadenti 2016; Yadav et al. 2013). The effect of hormones and other treatments on the stimulation of somaclonal variation is crucial. The AFLP analysis indicates that the method can be used for the protection and commercial true-to-type multiplication of *Kelus*.

### Conclusion

Micropropagation of elite materials and genetic engineering-mediated improvements are the two main advantages of somatic embryogenesis in medicinal plants. The recent advances in transcriptome analysis have led to the elucidation of critical steps and regulatory mechanisms involved in secondary metabolism. However, the genetic manipulation of medicinal plants has been hampered by the fact that most of these plants are recalcitrant in matters of embryogenesis and transformation. Despite a successful primary embryogenesis, it is believed that embryo maturation and conversion are two limiting steps in the development of somatic embryo. In the present research, we developed a highly efficient protocol for the true-to-type micropropagation of an endangered medicinal plant through somatic embryogenesis. Some factors affecting the induction, proliferation, maturation, and conversion of somatic embryos in *K. odoratissima* have been studied. This method can be useful for germplasm conservation, mass propagation, and genetic engineering process in *K. odoratissima* Mozaff.





**Fig. 4** Poly acrylamide gel electrophoresis of the AFLP reaction in selective step with two primer combinations (E-CGA+T-CAC and E-CAC+T-TGC) in parental sample (P) and regenerated plants (R1–R12). The length of size marker (M) bands 100 and 600 bp are indicated

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