



Mucilage synthesis by *in vitro* cell culture in different species of *Alyssum*

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Abstract

Mucilage is a class of polysaccharides found in some plants that have pharmaceutical effects as anti-hemorrhoids. *Alyssum* is one such species; its seeds produce mucilage that possesses pharmaceutical properties. The aim of this study was to optimize the conditions for callus production and mucilage synthesis in *Alyssum* species in a tissue culture procedure. In the study presented here, callus initiation in different genotypes of *Alyssum* species (*A. inflatum*, *A. lepidium*, and *A. strigosum*) has been investigated for the first time. Different combinations of 2,4 Dichlorophenoxy acetic acid (2,4 D), Kinetin (Kin) and Benzyl amino purin (BAP) were used to optimize callus initiation frequency and callus growth rates (CGR) in hypocotyl explants. The highest rates of callus induction (%) and callus growth rates (CGR) were achieved with $2.5 \text{ mg} \cdot \text{l}^{-1}$ 2,4 D + $0.1 \text{ mg} \cdot \text{l}^{-1}$ Kin and $3 \text{ mg} \cdot \text{l}^{-1}$ 2,4 D + $0.1 \text{ mg} \cdot \text{l}^{-1}$ Kin, respectively. The highest callus induction was recorded for *A. inflatum*. The mucilage content was estimated in callus cultures of different explants. The novel finding of the study is the superiority of the mucilage extracted from the callus rather than the seed, which is about ten times higher. The highest mucilage production (g/g dry weight) was obtained with Murashige and Skoog (MS) medium supplemented with $2 \text{ mg} \cdot \text{l}^{-1}$ 2,4 D and $0.1 \text{ mg} \cdot \text{l}^{-1}$ Kin. Among the genotypes investigated, Kerman (*A. lepidium*) was found to produce the most mucilage (0.51 g/g d.w.) in its callus. The proposed method is beneficial for mucilage production from *Alyssum* sp. using *in vitro* cell culture.

Key words: callus, explant, growth, induction, medicinal plants

Introduction

Polysaccharide hydrocolloids including mucilage are abundant in nature and commonly found in many higher plants (Whistler and BeMiller, 1993; Malviya et al., 2011). Mucilage exudates provide an easy and cheap access to the stock of polysaccharides, most of which are important in food formulations, because of their ability to modify the functional properties of food systems (Koocheki et al., 2009). Mucilage is a natural polymer and is widely used in food processes as a thickener, gelling agent, or stabilizer (Koocheki et al., 2010; Malviya et al., 2011). Moreover, plant-based mucilage has wide pharmaceutical and medicinal applications (Gupta et al., 2015). Plants containing mucilage are used to treat high blood pressure, high cholesterol, diabetes, hemorrhoids and bladder problems (Malviya et al., 2011).

Alyssum is one of the six genera belonging to the *Brassicaceae* family, with species endemic to Irano-Turanian, Mediterranean, and SaharoSindian regions (Hedge, 1976). The genus comprises annual and perennial herbaceous plants that grow 10-100 cm tall (Al-Shehbaze and Beilstein, 2006). The species endemic to Iran include *A. homolocarpum*, locally called "Qudume" in Persian (or Qudume Shirazi in the local Persian), *A. lepidium* (or Qudume Shahri in the local Persian), *A. inflatum* and *A. strigosum* (Amin, 2005). These species are of great interest, mainly because of their phytoremediation potential via nickel hyperaccumulation (Vinterhalter and Vinterhalter, 2005; Ghasemi et al., 2009) and medicinal properties (Topcu et al., 2009; Koocheki et al., 2010).

The seeds of *Qudume* are distributed in different parts of Iran, especially in western, central, and sou-

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thern regions, and are used as herbs (Koocheki et al., 2010). *A. lepidium* has round, pale brown seeds (1.5-2.5 mm in diameter) that are endemic to serpentine soils of Iran (Ghaderian et al., 2007). Those of *Qudume Shirazi* are flat and round (1.5-2.5 mm) in shape and reddish brown in color with dry fruits narrowly winged all around (Koocheki et al., 2009). They absorb water quickly when soaked in water and contain a large amount of mucilaginous substances (Koocheki et al., 2009).

A number of *Alyssum* species are commonly used in traditional herbal medicine; its seeds, in particular, are known to contain large amounts of bioactive substances (Topcu et al., 2009). For centuries, *Qudume* seeds have been prescribed by herbalists for their mucilage content, especially as remedies, such as demulcents for dry coughs, asthma, lung infections and kidney stones (Amin, 2005; Koocheki et al., 2009).

While many parts of Iran, especially the central regions, offer favorable agro-climatic conditions for the production of *Alyssum*, the availability of *Alyssum sp.* is becoming scarce due to its narrow genetic diversity. It is anticipated that some species of *Alyssum*, such as *A. inflatum* and *A. strigum*, will face extinction and severe genetic loss if necessary cultivation steps are not taken in time.

Plant cell culture has emerged as a potential source of genetic preservation, while it is also a method of interest for the production of secondary metabolites which are used as pharmaceuticals, agrochemicals, and food additives (Raoa and Ravishankar, 2002; Namedo, 2007; Sree et al., 2010). Moreover, research into various plant cells, tissues, and organ cultures has enabled an evaluation of the capability of these cultures to synthesize secondary metabolites (Bourgand et al., 2001; Rajikumar et al., 2010). Plant cell cultures are currently used in medicinal industries as a source of high value secondary metabolites (Raoa and Ravishankar, 2002).

Indeed, these cultures serve as alternatives for obtaining products that are otherwise difficult to obtain by conventional methods or whose production is not economically viable (Rajikumar et al., 2010). It has been reported that callus cultures are relatively rich in mucilage, commonly making up between 8-10% of their weights (Kokate and Radwan, 1978). Even though *Qudume* seeds produce a desirable amount of mucilaginous substances when wetted under optimized protocols (Koocheki et al., 2009), no study has been conducted on its extraction

from callus cultures. *in vitro* cell cultures provide efficient means for the production of mucilage at the cellular level (Gupta et al., 2015). Moreover, the system of cell suspension cultures is a very promising method that can be exploited for the commercial production of mucilage. Mucilage synthesis has been reportedly accomplished by callus cultures of *Plantago lanceolata* (Mirmasumi et al., 2001), *Plantago major* (Mirmasumi et al., 2001), and *Plantago ovata* (Gupta et al., 2015). A successful mucilage synthesis by *in vitro* culture, however, requires callus production to be initially optimized in *Alyssum sp.* Our literature review showed no published reports on callus induction of Iranian and exotic genotypes of *A. lepidium* or *A. Strigosum*. Paunescu (2008) reported that different combinations of auxins (2,4D, IAA, and NAA) and cytokinins (BAP and Kin) led to no callus induction on *Alyssum borzaenum*, but rather only to direct shoot regeneration. Ghasemi and Samie (2013) investigated callus induction in *A. inflatum* by applying a combination of $1.5 \text{ mg} \cdot \text{l}^{-1}$ 2,4D and $0.1 \text{ mg} \cdot \text{l}^{-1}$ Kin.

Considering the wide-ranging applications of mucilage and the ecological importance of the *Alyssum* plant species as a new source of mucilage, the present study was designed and implemented to investigate the composition of its constituent monosaccharides. As a second objective, callus production and mucilage synthesis of the three species of *Alyssum* (*lepidium*, *strigosum*, and *inflatum*) were optimized for the first time. The developed protocol may not only serve as a valuable alternative to the *ex-situ* conservation of this endangered species with its very narrow genetic diversity, but may also be employed to extract and preserve bioactive substances from these species of *Alyssum*.

Materials and methods

Explant preparation and callus induction

This experiment was conducted at the Tissue Culture Laboratory of the Institute of Biotechnology and Bioengineering at Isfahan University of Technology in 2015 using a completely randomized factorial split plot design with five replications. The combinations of plant growth regulators and genotypes were considered as the main plots, while time (in months) formed the subplots. The seeds of five accessions of Iranian *Alyssum* genotypes were collected from different geographical regions of Iran; these included *A. lepidium* (collected from Isfa-

han, Jiroft, and Yazd), *A. inflatum* (collected from Kerman), and *A. strigosum* (collected from Qom). The seeds of *Alyssum* sp. were optimized using different treatments to enhance their germination percentage. For this purpose, they were initially sterilized in a 5% w/v sodium hypochlorite solution for 3 min before being washed three times with sterile distilled water. The sterilized seeds were aseptically germinated on agar-solidified Murashige and Skoog (MS) (1962) basal medium at a temperature of $24 \pm 1^\circ\text{C}$ and a relative humidity of $50 \pm 5\%$ in 16-8 h (light-dark) cycles using a lab germinator.

Table 1. Different types and concentrations of plant growth regulators used for callus induction from hypocotyl explants of *Alyssum* sp. on MS medium

Number	PGRs ($\text{mg} \cdot \text{l}^{-1}$)
1	2,4 D (1.5) + Kin (0.5)
2	2,4 D (2) + Kin (0.5)
3	2,4 D (2.5) + Kin (0.5)
4	2,4 D (3) + Kin (0.5)
5	2,4 D (1.5) + Kin (0.1)
6	2,4 D (1.5) + BAP (0.1)
7	2,4 D (2) + BAP (0.5)

Hypocotyl segments (2-3 mm) were excised from 14-day-old seedlings of all the genotypes investigated to be used as explants for callus induction. The hypocotyl pieces were cultured on the MS medium (Duchefa) supplemented with different concentrations of 2,4 D, Kinetin (Sigma), NAA (Naphthalene acetic acid) (Sigma), and BAP (Benzyl amino purine) (Sigma) in 30 g l^{-1} sucrose, and 0.1 mg l^{-1} myoinositol for callus initiation (Table 1). The pH of the media was adjusted to 5.8 and solidified with 0.8% (w/v) agar (Sigma) before autoclaving for 20 min at a pressure of 1.06 kg/cm^2 . Petri dishes were incubated in the dark at $25 \pm 1^\circ\text{C}$ (six explants cultured in each dish). For callus induction, the hypocotyl cultures were maintained in a dark incubator (Binder Model, Bd-115) at $24 \pm 1^\circ\text{C}$ for 1 month before they were transferred to fluorescent light over a 16 h photoperiod with a photon flux density of $40 \mu\text{mol/m}^2$ at $25 \pm 1^\circ\text{C}$. Sub-culturing of the callused explants was performed twice a month.

The percentage of callogenesis was calculated using the following equation: $[(n/N) \times 100]$, in which n is the

total number of callused explants and N represents the total number of cultured explants (Wakhlu and Barna, 1989).

The callus growth rate (CGR) was calculated based on the means of callus growth rates (mm/day) at 30, 60, 90, and 120 days after callus induction using the following equation (Compton, 1994):

$$cgr = \frac{cgr_1 + cgr_2 + cgr_3 + cgr_4}{4}$$

$$cgr_1 = \frac{d_{30}}{30}, \quad cgr_2 = \frac{d_{60}}{30}, \quad cgr_3 = \frac{d_{90}}{30}, \quad cgr_4 = \frac{d_{120}}{30}$$

Callus diameter (d_i) was calculated as the square root of the product of callus length and callus width. It should be noted that callus diameter for each of the experimental ages (i.e., d_{30} , d_{60} , d_{90} , and d_{120}) was calculated as the difference between callus diameter at a given age and that at the preceding age.

Mucilage extraction

The method described in Sharma and Koul (1986) was used, with minor modifications, to extract mucilage from *Alyssum* sp. calluses. In this procedure, ten milliliters of 0.1 N HCl was heated to boiling point in a 100-ml corning flask. After removing the flask from the flame, 1 g of fresh callus was added to it and heating was resumed. After 5 min, the solution was filtered through a piece of clean muslin cloth while still hot. In order to separate residual traces of mucilage, the mucilage was washed twice in 5 ml of hot water and the solution thus obtained was filtered each time. The combined filtrate solution containing the dissolved mucilage was mixed with 60 ml of 95% ethyl alcohol, stirred, and allowed to stand for 5 h in a refrigerator at 4°C . Finally, the supernatant was decanted and the beaker containing the precipitate was dried in an oven maintained at 50°C for 12 h to calculate the mucilage content in dry weight.

Statistical analysis

The data were subjected to an analysis of variance using PROC ANOVA in the SAS statistical package (Ver. 9.1). Mean comparisons were conducted using the least significant difference ($\text{LSD}_{0.05}$) method.

Results and discussion

Results of the preliminary experiment using different explants including hypocotyls, cotyledons, and

Table 2. Analysis of variance (ANOVA) for genotype, hormone, incubation period and their interaction effects for callus induction and callus growth rate in *Alyssum* sp.

Source of variation	df [‡]	Mean squares	
		callus induction	callus growth rate
Genotype	4	0.055**	0.083**
Hormone	4	0.0152**	0.0387**
Genotype × hormone	16	0.0056	0.0038
Error (a)	100	0.0034	0.0083
Incubation period	3	24.88**	0.306**
Genotype × incubation period	12	0.0314**	0.082**
Hormone × incubation period	12	0.015**	0.033**
Hormone × incubation period × genotype	48	0.0036	0.011
Error (b)	300	0.0028	0.009

*, ** are significant at $P < 0.05$ and $P < 0.01$, respectively; [‡] – degree of freedom

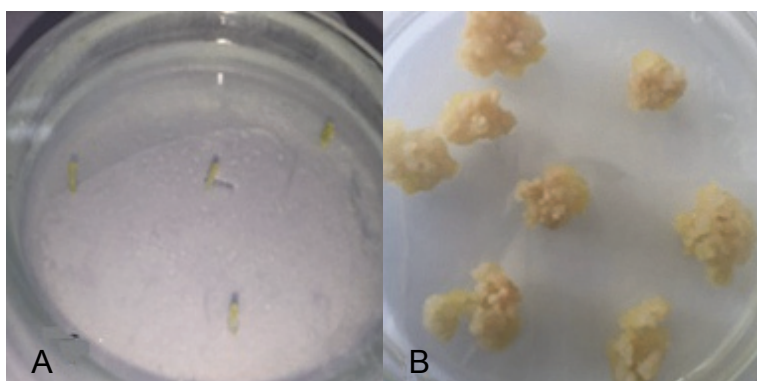


Fig. 1. The callus induction from *A. lepidium* hypocotyls (A), and callus growth explants (B) after four months of induction, on Murashige and Skoog (MS) medium with $2.5 \text{ mg} \cdot \text{l}^{-1}$ 2,4D and $0.1 \text{ mg} \cdot \text{l}^{-1}$ Kin

seeds cultured on the MS medium showed that only hypocotyl explants led to callus induction. They were, therefore, selected for further experiments. The analysis of variance showed that genotypes, hormones, incubation period, genotype × incubation period, and hormone × incubation period showed significant differences with respect to callus induction (%) and callus growth rate (mm day) at $P < 0.05$ (Table 2).

Hormone assay

For callus induction, hypocotyls are normally cultured on a medium containing growth-regulating substances (Fig. 1). The *in vitro* hypocotyl explants of *Alyssum* sp. cultured in the media became swollen after one month of culture. This happened before signs of callus formation were observed, although with a low frequency

of callogenesis at different concentrations of 2,4 D + Kin. Callus initiation was observed after the hypocotyls swelled at their cut surfaces. It has been reported that wounding induces the release of plant hormones that elicit cell dedifferentiation and callus induction (Kahl, 1983). Application of NAA and BAP showed no obvious effects on callus initiation. Explants were observed to swell slowly without any modification of epidermal cells in the treatments containing combinations of 2,4 D + BAP and NAA + Kin. These cultures on fresh media did not lead to any morphological events.

These findings demonstrate the importance of the components of different plant growth regulators and explants for callus initiation and growth. It was found that the combination of the 2,4 D + Kin had the most prominent effect on hypocotyl dedifferentiation and cal-

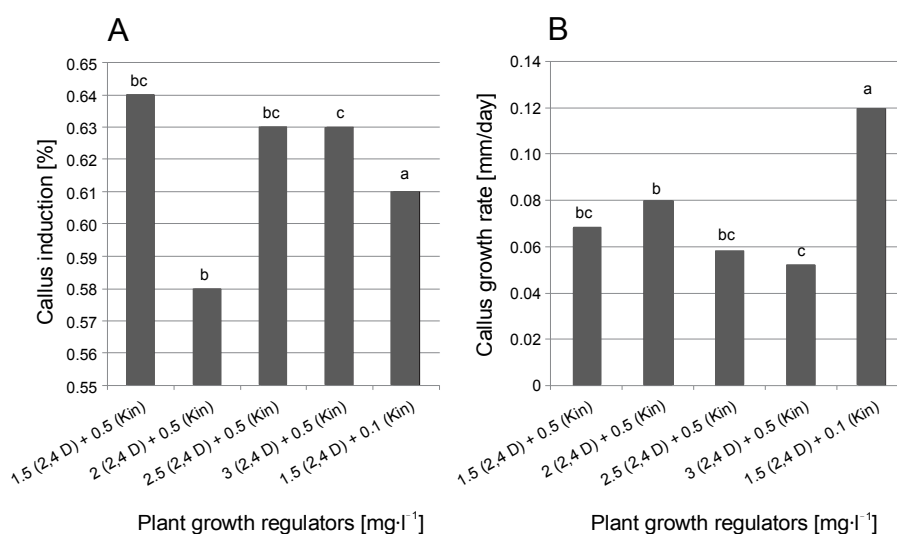


Fig. 2. The effect of different plant growth regulators on A) callus induction (%) and B) callus growth rate in *Alyssum* sp.

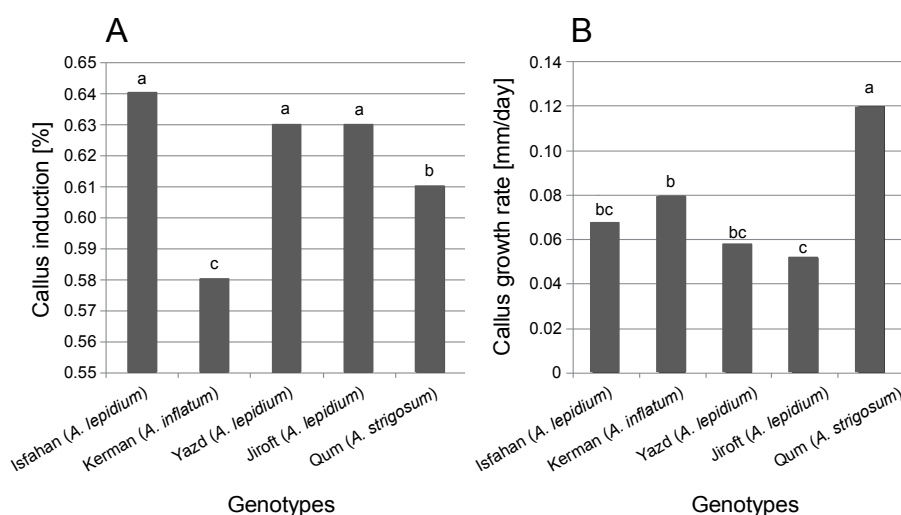


Fig. 3. The response of different *Alyssum* species to A) callus induction (%) and B) callus growth rate in *in vitro* hypocotyl cultures

lus induction in *Alyssum* species, which is in agreement with the findings of Ghasemi and Samie (2013). No callus induction was observed on the MS medium containing 2,4D, NAA + Kin and 2,4D + BAP, but the percentage of callogenesis increased from 58% in medium containing 2 mg·l⁻¹ 2,4D + 0.5 mg·l⁻¹ Kin to 64.4% in medium containing 1.5 mg·l⁻¹ 2,4D + 0.5 mg·l⁻¹ Kin (Fig. 2A). Our findings concerning the nature of the calluses confirm those by Paunescu (2008), who reported that the combination of 1 mg·l⁻¹ of 2,4D and 0.1 mg·l⁻¹ of Kin had the greatest effect on callus induction in *A. borzaenum*, producing embryogenic callus of globular shape. Investigation into the effects of different combina-

tions of hormones on CGR revealed that 1.5 mg·l⁻¹ 2,4D and 0.1 mg·l⁻¹ Kin led to the highest callus growth rate (0.12 mm/day), but 3 mg·l⁻¹ 2,4D + 0.5 mg·l⁻¹ Kin led to the lowest CGR (0.052 mm/day) – Figure 2B.

Genotypic assay

The evaluated genotypes showed significant differences in their callus initiation and callus growth rates. The calluses of *A. inflatum* were pale green to yellow and friable, i.e. easily fell apart, but those of other species were dark to pale yellow with a compact structure.

The mean percentage values for callus induction (%) ranged from 64% in *A. lepidium* (Istfahan) to 58% in Ker-

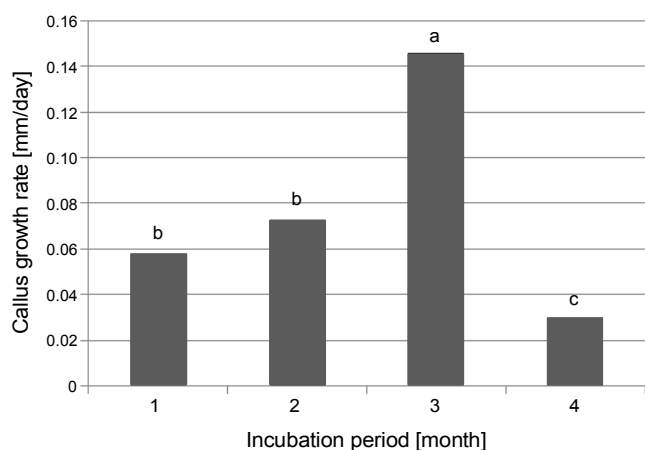


Fig. 4. The callus growth rate (mm/day) of *Alyssum* sp. at four sequential months after callus induction

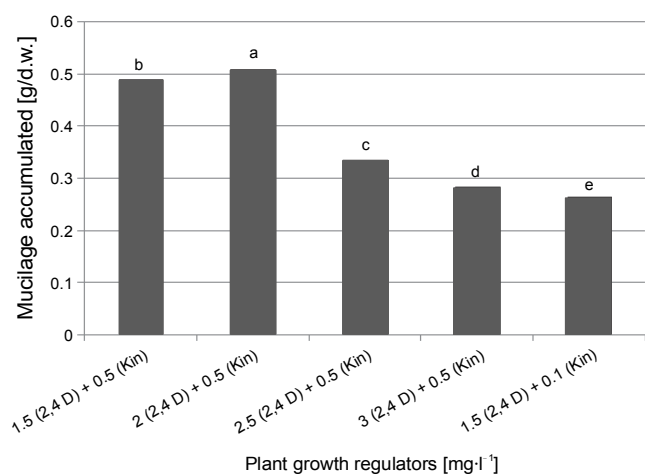


Fig. 5. The effect of different plant growth regulators ($\text{mg} \cdot \text{l}^{-1}$) on mucilage accumulation on callus cultures of *Alyssum* sp.

man accession *A. inflatum* – (Fig. 3A). The comparison of means in the evaluated genotypes showed that the highest (0.12 mm/day) and the lowest (0.052 mm/day) CGR were observed in Qom (*A. strigosum*) and Kerman accessions (*A. inflatum*), respectively (Fig. 3B).

The comparison of callus growth rates at four sequential times – 30, 60, 90, and 120 days after callus initiation – with the plant growth regulators examined showed that the highest (0.146 mm/day) and the lowest (0.03 mm/day) CGR were recorded in the third and fourth months, respectively (Fig. 4).

The comparison of callus induction (%) in the explants at different times showed that callus induction increased slowly from the first (13%) to the fourth months (100%). The comparison of callus induction in *Alyssum* sp. during these periods demonstrated that the rate

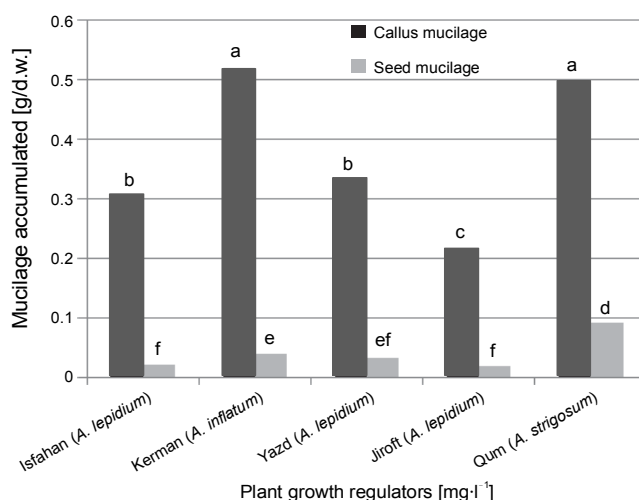


Fig. 6. The accumulated mucilage (g/g d.w.) of different genotypes of *Alyssum* by hypocotyls in *in vitro* culture

of callus induction in these species was slow. Moreover, our results showed that light had a dramatic effect on the differentiation of *Alyssum* explants.

The analysis of variance of callus and seed mucilage contents revealed significant differences among the genotypes and hormone treatments (data not shown). The highest mucilage content in dry weight (0.501 g/g d.w.) was achieved in the *in vitro* cell culture at a hormone combination of $2 \text{ mg} \cdot \text{l}^{-1}$ 2,4 D + 0.1 Kin, while the lowest (0.265 g/g d.w.) was obtained with $1.5 \text{ mg} \cdot \text{l}^{-1}$ 2,4 D + 0.1 $\text{mg} \cdot \text{l}^{-1}$ Kin (Fig. 5).

The highest (0.520 g/g d.w.) and the lowest (0.218 g/g d.w.) callus mucilage quantities extracted were recorded for Kerman (*A. inflatum*) and Jiroft (*A. lepidium*), respectively (Fig. 6). The seed mucilage content varied from 0.021 (g/g d.w.) in Jiroft (*A. lepidium*) to 0.093 (g/g d.w.) in Qum (*A. strigosum*). Clearly, Jiroft (*A. lepidium*) recorded the least mucilage content under both extraction methods. This indicates the superiority of the Kerman *A. inflatum* var for traditional mucilage production. The best finding of the present study was that the amount of mucilage extracted from callus culture was approximately 10-times higher than the mucilage extracted from seeds. This is similar to reports on callus mucilage extracted in *P. lanceolata* (Mimasoomi et al., 2001) and *P. ovata* (Gupta et al., 2015).

Similar results have been reported for the increased extraction of solasodine in *Solanum nigrum* (Yogananth et al., 2009), taxol in *Taxus baccata* (Khosroushahi et al., 2005), artemisinin in *artemisia annua* (Pu et al.,

2009), nitidine in *Toddalia asiatica* (Rajkumar et al., 2010), stevioside in *stevia rebaudiana* (Janarthanam et al., 2010), ajmalicine and indole alkaloids in *Catharanthus roseus* (Zhao et al., 2001; Namdeo, 2002), jaceosidin and syringin in *Saussurea medusa* (Yu et al., 2006), and artemisinin in *Artemisia annua* (Pu et al., 2009) from *in vitro* cell cultures, all of which were much higher than the extracts obtained from field grown plants. The chemical structures of secondary metabolites are usually complex and their production is costly, but plant cell tissue cultures could help overcome these limitations.

Conclusions

In this paper, we have described a newly developed procedure for callus initiation of *A. lepidium*, *A. strigosum*, and *A. inflatum*. This report provides a new and efficient method for the production of mucilage by a cell culture of *Alyssum* species. We managed to stimulate the formation and accumulation of mucilage by hypocotyl culture of *Alyssum* species. The significant features of this study include the production of mucilage from seeds of *A. inflatum* and *A. strigosum* as well as a significant increase in mucilage production within a relatively short time span in callus cultures of *Alyssum sp.* For future research, attempts should be made to produce cell suspension cultures from callus collections and to investigate the production of mucilage in liquid media to which both biotic and abiotic elicitors are added for enhanced synthesis. These results suggest that enhancement of mucilage by elicitation and precursor feeding of a cell suspension culture of *Alyssum* in a bioreactor can be targeted in the future.

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