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Callus induction in plantain (*Plantago major L.*) for *in vitro* production of mucilage

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Abstract

Plantago major is a medicinal herb belongs to the family Plantaginaceae. The effects of a range of plant growth regulators and different explants on mucilage production were examined. Root and leaf tissues were used as explants. Murashinge and Skoog (MS) medium supplemented with 2,4-D or Kinetin, at 0.01, 0.1 and 0.5 mg l^{-1} alone or in combination with IAA 1 mgl^{-1} for callus induction. Result of analysis of variance showed significant difference between explants for some traits included callus induction rate, fresh weight, and callus diameter. Also, significant differences were observed between media for callus induction, callus diameter, fresh weight, dry weight, relative water content of callus, mucilage harvest index and mucilage yield. The best response was observed from root segments on MS medium containing 0.8 mg/l 2,4,D and 0.1mg l^{-1} kinetin in some traits included callus diameter, fresh and dry weight, mucilage percentage and mucilage yield. Results revealed that the highest relative growth rate of callus was observed on media containing 1 mg l^{-1} 2,4-D and 0.5 mg l^{-1} kinetin. MS medium supplemented with 2.5 mgl^{-1} 2,4-D and 0.01 mg l^{-1} kinetin showed the highest harvest index of mucilage. Optimal medium for mucilage production was found to be MS medium containing 0.8 mgl^{-1} 2,4-D and 1 mg l^{-1} kinetin and root as explants. Results of mean comparison for mucilage production showed that media containing 0.8 mg l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin and leaf as explant was produced higher mucilage than that of control plant.

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Introduction

Medicinal plants have a long history of use by mankind for therapeutic purposes. Since the side effects of chemical drugs became evident, a resurgence of herbal drugs was occurred. This led to a considerable increase in the use of natural drugs. Even today, about 90% of people in developing countries rely on herbal drugs (Omidbeigi, 2001).

Plants are rich sources of chemical compounds which are used as drugs. In fact, secondary metabolites are among the most valuable phytochemicals. It is possible to produce secondary metabolites in *in vitro* as an alternative method. For example, mucilages, which are high molecular weight polysaccharides, are easily extracted from plants and have many applications in folk medicine. Gums and mucilages are classified according to their gummy nature. Mucilages are frequently served in pharmaceutical industries due to their suitable properties such as low cost, availability and non-toxicity. Mucilages are more or less soluble in ware and produce gelatin like substances. Mucilages possess anti-inflammatory, softening and moistening properties (Mirmasoumi, 1993).

P. major has numerous phytochemicals in its leaves, seeds and roots, which apparently have medicinal properties and also can be used as taxonomic markers (Samulsen, 2000). Phytochemical investigations of *P. major* revealed the presence of various chemical constituents such as Flavnoids, Caffeoyl phenylethanoind gulocosides, Iridoid glocosides, Polyphenolic compounds (Zubair *et al.,* 2010). Plantain, as a mucilage containing plant, has a long history of cultivation. The seeds of plant have been used in traditional medicine for their anti-tussive, gastrointestinal anti-inflammatory and laxative properties. Its mucilage is used in cosmetics as an emulsifier agent and its seed husks are served locally for skin irritations. The plant contains iridoid glycosides, mucilage, aucubin, plantagin, succinic acid, adenine and Collin (Razavi *et al.*, 2010). In vitro production of secondary metabolites has many advantages over the conventional systems of production from whole plants. This method provides precise control of different factors resulting in unaffected quality of produced compounds over the time. By contrast, the quality of these compounds in plants growing in natural environments strictly influenced by environmental conditions and pests (Smetanska, 2008). So far, cell suspension cultures have extensively been served for in vitro production of secondary metabolites. The present study was served to investigate the effects of the explant type (leaf and root) and plant growth regulators on callus induction in plantain for in vitro production of mucilage.

Materials and methods

The study was done in the laboratory of the Islamic Azad University of Kermanshah. Leaves and roots of in vitro grown plants were used to obtain calli. Seeds were firstly sterilized by immersing in 75% ethanol for 30 sec and 2.5% sodium hypochlorite for 20 min and then rinsed three times in sterile water before culturing. Sterilized seeds were cultured on the surface of hormone free MS medium containing 0.8% agar. For induction of callus, two types of explants and five different combinations of 2,4-D and IAA (Table 1). Treatments were arranged in a factorial experiment based on the completely randomized design with three replications. For each replication, five explants were placed on the surface of medium. Means were compared according to the Duncan's multiple range test. Petri dishes were then transferred to a growth chamber at 25 °C under a 16/8 h photoperiod. Calli were appeared about 30 days after culture. At this time different traits were measured.

Measured traits

Percentage of callus induction

Percentage of callus induction was calculated according to the following formula:

Percentage of callus induction= (Number of explants formed calli/number of cultured explants) × 100 (Arzani and Mirodjagh, 1999).

Callus diameter

To calculate calli diameters, length and width of each

were measured and the following formula was served:

Callus diameter= $\sqrt{}$ Length × Width(Compton, 1994).

Callus growth rate

To calculate callus growth rate (mm diameter per day), calli diameters before and after 30 days culture were firstly measured and then the following formula was served (Compton, 1994)

CGR= d2-d1/t2-t1

Calli fresh and dry weights

Calli fresh weights were immediately measured after cutting off from explants. To obtain calli dry weights, they were firstly dried in an oven at 72 °C for 48 and then their weights were measured. (Errabi *et al*., 2006)

Relative water content (RWC)

Relative water contents of obtained calli were calculated by using their fresh and dry weights according to the formula (Errabi *et al*., 2006).

RWC= [(fresh weight-dry weight)/dry weight] × 100

Mucilage content

The mucilage content of obtained calli were measured according to the method described by Sharma and Koul (1986). To do this, one gram of callus was powdered in a mortar and mixed with 10 ml 0.1 N HCL. The mixture was boiled for 5 min and filtered. The residue were extracted with 5 ml boiled water two times. Afterwards, obtained filtrate was mixed with 60 ml 96% ethanol and kept in a refrigerator for 5 h. Then, supernatant was removed and precipitated mucilage was dried in an oven at 50 °C for 12 h. The extract was weighed and considered as the amount of mucilage per 1 gram callus.

Harvesting index

Harvesting index was calculated by dividing the amount of obtained mucilage by the dry weight of callus.

Mucilage yield

Mucilage yield was calculated by the following formula:

Mucilage yield= mucilage content × dry weight

Results and discussion

According to the results of ANOVA, there were significant differences among hormonal combinations and all measured traits except callus growth rate and mucilage content. On the other hand, the was a significant difference between the type of explant used on frequency of callus induction, final diameter and fresh weight were significant and on primary diameter, average diameter, relative water content and mucilage content, harvest index and yield were not significant. The interaction between the explant types and hormonal combinations were also significant in terms of percentage of callus induction, primary diameter, final diameter, average diameter, fresh weight, dry weight and callus growth rate. In agreement to our results, a previous study by Sarihan *et al*, (2005) on shoot regeneration in *Plantago afra* showed that explant type and different media had significant effects on all measured traits. Similarly, Mirmasoumi *et al*,(1993) also reported significant differences among different media and productivity of mucilage in *Plantaginace*.

Table 1. Hormonal combinations used in calli induction of *Plantago major*.

Media code	Hormone (mg/l)		
	2,4-D	IAA	Kin
A	1	0	0.1
B	2.5	0	0.01
C	0.8	0	0.1
D	1	0	0.5
E	0.5	1	0

Mean comparisons

Frequency of callus induction

In this study, the highest (100%) and the lowest (86%) frequencies of callus induction were observed in hormonal combinations 2,4-D (1)+Kin (0.1) in the leaf and root explants, 2,4-D (2.5)+Kin (0.01) in the leaf and root explants, 2,4-D (0.5)+IAA (1) in the leaf and root explants and 2,4-D (0.8)+Kin (0.1) in the leaf explant and 2,4-D (1)+Kin (0.5) in the root explant, respectively (Table 2).

Table 2. Different media effects on calli characteristics of *Plantago major*.

Medium (mg/l)	Calli induction (%)	Initial diameter (mm)	Final diameter (mm)	Mean diameter (mm)	Fresh weight (g)	Dry weight (g)	Mean calli growth	RWC (%)	Mucilage harvest index	Mucilage content	Mucilage yield
2.4-D (1)+Kin (0.1)	100^{a}	12.7^{b}	13.85^{b}	13.01^{b}	1.743^{b}	0.557^{b}	0.056^{ab}	6.34^{b}	2.60^{b}	20.91^{ab}	10.08^{b}
2.4-D (2.5)+Kin (0.01)	100^{a}	13.36^{b}	14.36^{b}	13.86^{b}	0.573^{c}	0.043^{c}	0.034^{b}	13.48^{a}	2.83^{a}	14.06^{ab}	0.77^{b}
2.4-D (0.8)+Kin (0.1)	98^{a}	16.14^{a}	17.42^{a}	16.78^{a}	3.583^{a}	1.187^{a}	0.043^{b}	2.68^{b}	0.25^{b}	27.23^{a}	32.29^{a}
2.4-D (1)+Kin (0.5)	93^{b}	9.28^{c}	11.58^{c}	10.43^{c}	2.493^{b}	0.79^{a}	0.076^{a}	5.92^{b}	0.25^{b}	8.42^{b}	6.76^{b}
IAA (1)+ 2.4-D (0.5)	100^{a}	9.03^{c}	10.70^{c}	9.87^{c}	1.868^{b}	0.698^{b}	0.056^{ab}	1.83^{b}	0.41^{b}	17.53^{ab}	10.26^{b}

Mean values in each column with same letter did not differ significantly at $p<0.05$ according to Duncan Multiple range test.

Primary, final and average diameters

While the hormonal combination 2,4-D (0.8)+Kin (0.1) in the root explant has been resulted in the highest callus primary diameter (17.65), the lowest ones (6.43) was observed in the hormonal combination 2,4-D (0.5)+IAA (1) in the root explant. Also, calli in media containing 2,4-D (0.8)+Kin (0.1) in the root explant and 2,4-D (0.5)+IAA (1) in the root explants had the highest (18.88) and lowest (7.87) final diameters, respectively. On the other hand, the highest (18.27) and the lowest (7.15) values of callus average diameter were observed in media supplemented by 2,4-D (0.8)+Kin (0.1) in the root explant (Table 2).

Table 3. Different explant (leaf and root) effects on calli characteristics of *Plantago major*.

Calli characteristics	Leaf	Root
Calli induction (%)	100	96.66
Initial diameter (mm)	12.45	11.54
Final diameter (mm)	14.23	12.93
Mean diameter (mm)	13.34	12.24
Fresh weight (g)	2.40	1.70
Dry weight (g)	0.706	0.606
Mean calli growth	0.059	0.046
RWC (%)	6.57	5.53
Mucilage harvest index	0.641	1.901
Mucilage content	15.51	19.75
Mucilage yield	12.129	11.934

Fresh and dry weights

The highest (3.64) and the lowest (0.55) means for the callus fresh weight was obtained in media containing 2,4-D (0.8) + Kin (0.1) in the root explant and 2,4-D (2.5) + Kin (0.01) in the leaf explant, respectively. Also, calli in media containing 2,4-D (0.8) + Kin (0.1) in the root explant and 2,4-D (2.5) + Kin (0.01) in the leaf explant possessed the highest (1.47) and the lowest (0.033) dry weights, respectively (Table 2).

Callus growth rates

While the hormonal combination 2,4-D (1) + Kin (0.05) in the leaf explant has been resulted in the highest callus growth rate (0.108), the lowest value (0.024) was observed in the hormonal combination 2,4-D (2.5) + Kin (0.01) in the root explant (Table 2).

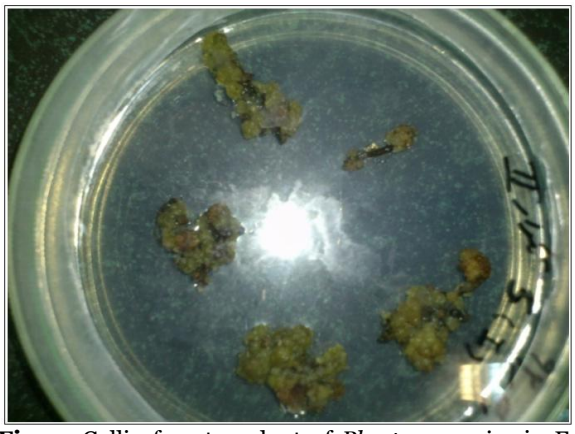

Fig. 1. Calli of root explant of *Plantago major* in E medium.

Relative water content

In this study, the highest (13.48) and the lowest (1.84) values of relative water content were obtained in media containing 2,4-D (2.5) + Kin (0.01) and 2,4-D (0.5) + IAA (1), respectively (Table 2). Also, relative water content values for calli from the leaf and root explants were 6.763 and 5.536, respectively (Table 2).
Mucilage percentage.

According to results of mean comparison, calli from media supplemented with 2,4-D (0.8) + Kin (0.1) and 2,4-D (1) + Kin (0.5) contained the highest (27.24) and the lowest (8.42) mucilage percentage, respectively (Table 2). While calli originated from the leaf explant possessed 15.51% mucilage, those from the root explant contained 19.75% (Table 2). The callus growth on media supplemented with 2,4-D (0.8) + Kin (0.1) had nearly 3 times more mucilage than callus obtained from media containing 2,4-D (1) + Kin (0.5). it represented that the optimized concentrations of Plant Growth Regulators Can Improve mucilage production in tissue culture.

Mucilage yield

In the present investigation, the highest (32.29) and the lowest (0.77) levels of mucilage yield was obtained from calli cultured in media containing 2,4-D (0.8) + Kin (0.1) and 2,4-D (2.5) + Kin (0.01), respectively. On the other hand, calli of leaf and root origin yielded 12.129 and 11.934 mucilage, respectively (Table 2). Considering the ability of callus tissue to produce mucilage, tissue culture methods can be used as a suitable way for mucilage production under controlled conditions.

Mucilage harvest index

This study revealed that calli in media containing 2,4-D (2.5) + Kin (0.01) and 2,4-D (1) + Kin (0.5) had the highest (2.83) and the lowest (0.252) mucilage harvest index, respectively. Also, mucilage harvest index values for the leaf and root explants were 0.641 and 1.901, respectively (Table 2).

Comparison with control plant

In this study, a comparison was performed among the levels of mucilage from induced calli and that from the control plant. As can be seen in Fig. 1, calli induced on the leaf explant in medium supplemented by 2,4-D (0.8) + Kin (0.1) gave the more mucilage than control plant. By contrast, calli from the root explant in medium containing 2,4-D (1) + Kin (0.1) yielded the same level of mucilage as the control plant (Table 3).

mucilage is regarded as normal physiological product of metabolism formed within the cell or deposited on it in layers. Mucilages in plants are thought to aid in water storage and seed germination, and to act as a membrane thickener and food reserve. Natural gums and mucilages have been widely explored as pharmaceutical excipients. (Bhardwag *et al.,* 2000).

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