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EXPERIMENTAL PAPER

In vitro synthesis of mucilage in *Plantago ovata* Forsk affected by genotypes and culture media

POORAN GOLKAR[1*], FARZAD AMOOSHAHI[2], AHMAD ARZANI[2]

[1]Research Institute for Biotechnology and Bioengineering
Isfahan University of Technology
Isfahan 8415683111, Iran

[2]Department of Agronomy and Plant Breeding
Faculty of Agriculture
Isfahan University of Technology
Isfahan 8415683111, Iran

[*]corresponding author: golkar@cc.iut.ac.ir

Summary

Introduction: Psyllium (*Plantago ovata* Forsk) is medicinally used mainly for its mucilage content. **Objective:** In the present study, an attempt was made to improve mucilage yield under *in vitro* callus culture using different genotypes, explants and culture media. **Methods:** The effects of a range of concentrations of plant growth regulators including 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kin) were evaluated on mucilage synthesis under *in vitro* culture using cotyledon, hypocotyl and seed explants. Fourteen genotypes originating from different geographical regions of Iran were used to evaluate their response to *in vitro* mucilage synthesis. **Results**: The highest rate of callus induction (76%) and callus growth rate CGR (0.38 mm/day) were induced on MS medium supplemented with 0.5 mg/l 2,4-D and 1 mg/l Kin and the hypocotyl explant. The results of analysis of variance showed significant genotypic differences for callus induction, CGR and mucilage content of callus and seeds. The mucilage content ranged from 0.38 to 0.08 (g/g *DW*) and 0.13 to 0.042 (g/g *DW*) for callus and seed, respectively. The superior callus induction (73%), CGR (0.45 mm/day) and mucilage content of callus (0.38 g/g *DW*) was denoted to Po_1 genotype. The callus produced nearly three times more mucilage than the seeds using superior genotype (Po_1). **Conclusion**: The results of this study revealed that high efficiency of callus culture of *P. ovata* using hypocotyl explant accompanied by the exploration of genetic diversity are important to improve the yield of mucilage synthesis by *in vitro* callus culture.

Key words: *callus, mucilage production, hypocotyl explants, polysaccharide*

INTRODUCTION

Medicinal plants are rich in secondary metabolites and their biosynthesis could be improved by using *in vitro* cultures for their commercial exploitation in different uses including medicinal and industrial applications [1, 2]. In recent years, plant-derived polymers have evoked tremendous interest due to their diverse pharmaceutical applications such as gelling agents in gels and protective colloids in suspensions [2]. The natural mucilage polymers are cheap and biocompatible [3], and represent an easy access to the stock of polysaccharides. These types of polymers are important in food formulation and studies in plant-water relations [1], medicinal purposes that is used to treat hypertension, high cholesterol level, diabetes, hemorrhoids and bladder problems [4], with wide range of applications such as thickening, binding, disintegrating, suspending, stabilizing and gelling agents [3-5]. Two polysaccharide fractions have been separated from the mucilage component. One fraction is soluble in cold water and on hydrolysis yields d-xylose (46%), aldobiouronic acid (40%), I-arabinose (7%), and an insoluble residue (2%); the other fraction is soluble in hot water, forming a highly viscous solution which sets to a gel on cooling and yields on hydrolysis d-xylose (80%), I-arabinose (14%), aldobiouronic acid (0.3%), and a trace of D-galactose [6].

Plant-originated mucilage is in high demand nowadays, and it is very difficult to fulfill the entire demand by field grown plants only [7]. For this purpose, a striking and very promising alternative system for commercial exploitation is the plant tissue culture by using cell suspension culture systems [8]. To achieve this goal, exploitation of plant variability for mucilage production should be accompanied by manipulation of culture conditions and metabolic engineering.

Isabgol (*Plantago ovata* Forsk) is an annual herb that belongs to *Plantaginaceae* and is indigenous to Asia (India, Pakistan and Iran), mediterranean region of Europe and North Africa [5, 9]. The seed husks of this plant are composed of mucilaginous polysaccharides, with a highly branched acidic arabinoxylan containing a xylan backbone [10] that gel over a wide range of concentrations.

The seeds of *Plantago* have been used for many years in traditional Iranian medicinal prescriptions to be soaked in water and swallowed at once [5]. The seed husks are commonly called 'Psyllium' component harboring a unique importance in pharmaceutical industry because of its suspension, emulsifying and sustainable properties in different drug doses [11,12]. Phytochemical investigations of *Plantago* species revealed the presence of various chemical constituents i.e. alkaloids, flavonoids and mucilage [12].

P. ovata has gained an important attention in recent years as a reservoir for bioactive components, especially mucilage, which makes it useful in pharmaceutical, agricultural and food industries [13]. The amount of mucilage produced by *P. ovata* is about 25% (by weight) of the total seed yield [5].

Plant cell culture has emerged as a potential source of secondary metabolites, which are used as pharmaceuticals, agrochemicals and food additives [14, 15]. The *in vitro* cell culture provides an efficient mean for the production of mucilage at cellular

level [15]. It has been reported that callus culture from higher plants with relatively rich in mucilage commonly makes up between 8–10% [11]. Such differences in levels of secondary metabolites between whole plants and cell or tissue cultures have often been described in *P. ovata* [16]. Notably, higher levels of flavonoids were recorded by *in vitro* culture and undifferentiated tissues contained in *P. ovata* [17]. Out crossing, morphological characteristics, seed shattering, cultivation on broader scale, and environmental limitations are the major confronting challenges of *P. ovata* aside from its narrow genetic base [18]. With the above mentioned difficulties, callus culture has been an alternative and efficient source for the production of higher secondary metabolites using of somaclonal variation [18]. Different explants including shoot buds [19], hypocotyl [20], leaf [7] and root [21] have been used to induce callus in *P. ovata*. The optimization of medium ingredients, especially plant growth hormones, was the basic approaches in bioreactor system of the plant for mucilage production. Some explants of *Plantago* species (leaf and root) were used for mucilage extraction from callus [22], but the explants of hypocotyl and cotyledon, has not been studied for their capability in mucilage synthesis in *P. ovata*. The main aims of this study were to 1) optimize mucilage production in different growth tissue culture media by hypocotyl culture 2) from the theoretical aspect it was interesting to know, whether the mucilage formation in the callus of hypocotyl explants is stimulated by plant growth regulators or not 3) to assess *in vitro* mucilage synthesis of different *P. ovata* genotypes.

MATERIAL AND METHODS

The experiment was conducted at Tissue Culture Laboratory of Research Institute for Biotechnology and Bioengineering at Isfahan University of Technology at 2015. Three sequential experiments were carried out to assess explant and the plant growth regulators in the culture medium as well as to assess the genotypic effects on the *in vitro* mucilage production. A Completely Randomized Design (CRD) with five replications was used for each experiment.

Explant assay

Different explants including cotyledon, hypocotyl and seed were evaluated, preliminarily, for callus induction and callus growth rate. Seeds of *Plantago ovata*, the Po_{12} genotype were sterilized first with 70% ethanol for 1 min, then with 5% (w/v) sodium-hypochlorite solution for 20 min, finally washed three times with sterile distilled water. The sterilized seeds were aseptically germinated on agar-solidified Murashige and Skoog (MS) [23] (Duchefa, the Netherlands) basal medium at a temperature of 25±1°C and relative humidity of 50±5% with 16–8 h light-dark cycle in a laboratory germinator. After the emergence of the seedlings, the cotyledons were excised from seeds and divided in to 3–4 mm sections and cultured on MS medium with adaxial

surface. Hypocotyls were also excised from 10 day-old seedlings, cut into 2–3 mm sections with sterile scalpels and used as another source of explants for callus induction. The Petri dishes were then sealed with parafilm. All types of explants were cultured on MS medium supplemented with 0.5 mg/l 2,4-D and 1 mg/l Kin.

Plant growth regulators assay

Different concentrations of 2,4-D (2,4-dichlorophenoxyacetic acid) (Sigma Inc), Kin (kinetin) (Sigma Inc), NAA (naphtalen acetic acid) (Sigma Inc.) and BAP (benzyl amino purine) (Sigma Inc.) were used to identify the best callus induction, callus growth rate and mucilage synthesis by hypocotyl culture (tab. 2). A total of 30 g/l sucrose was added and the pH of the medium was adjusted with 0.1 mol/l NaOH or 0.1 mol/l HCl to 5.7±0.1. The medium was then solidified with 0.8% (w/v) agar and autoclaved at a pressure of 1.06 kg/cm^2 for 20 min. Then, approximately 25 ml medium was poured into Petri dishes. The Petri dishes were incubated at culture room in dark at 25±1°C. Cultures were observed daily for four weeks. The effects of plant growth regulators on callus development were evaluated based on percentage of callus formation, callus growth rate and the morphology, texture and color of the callus formed.

The ratio of callogenesis was calculated as follows:

$$[(n/N],$$

where n – total number of callused explants and N – total number of cultured explants [24].

The callus growth rate (CGR) was calculated by the mean of callus growth rate (mm/day) at 4, 8, 12 and 16 days after callus induction according to following formulae [24]:

$$cgr_1 = \frac{d4}{4} \quad cgr_2 = \frac{d8}{4} \quad cgr_3 = \frac{d12}{4} \quad cgr_4 = \frac{d16}{4}$$

The callus diameter (d_i) was calculated by root square of (callus length × callus width) [24]. It should be noted that callus diameter in each time (d_4, d_8, d_{12} and d_{16}) was calculated by the difference between callus diameter at each time from previous time.

Genotypic assay for mucilage

Fourteen different genotype of *P. ovata* collected from 12 provinces distributed across North East (Mashhad), North West (Ghazvin), South West (Behbahan, Ahvaz) and central (Isfahan, Aran o Bidgol, Khor o Biabanak, Shiraz) regions of Iran were used in this study. The exotic genotypes (India and Pakistan) were obtained from Seed and Plant Improvement Institute in Tehran, Iran. The genotypes were evaluated

for callus growth rate, callus induction and mucilage content. For each replication, five hypocotyl explants were transferred into a Petri dish that was supplemented by 0.5 mg/l 2,4-D + 1 mg/l Kin (as a superior medium at first experiment).

The frequency of callus induction and callus growth were measured at 4, 8, 12 and 16 days after callus induction. The means of callus induction (%) and callus growth rate was calculated after one month growth of callus. In a final stage, the mucilage content of the *in vitro* induced calli and seeds were calculated in each experimental unit as described below.

Mucilage extraction

Mucilage was extracted from callus according to Sharma and Koul [25] with minor modifications as follows 10 ml of 0.1 N HCL was heated to boiling point in a 100-ml corning flask. The flask was removed from the flame and 1 g of fresh callus was added and heating was resumed. After 5 min the solutions was filtered through clean muslin cloth, while still hot. In order to separate residual traces of mucilage, the mucilage were washed twice in 5 ml of hot water and the solution obtained each time was filtered. 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 refrigerator at 4°C. Finally, the supernatant liquid was decanted off and the beaker containing the precipitate was dried an oven maintained at 50°C for 12 h to calculate mucilage content. Mucilage extracted from the seeds was carried out based on Sharma and Koul [25] protocol, using 1 g dried seeds of *P. ovata*.

Statistical analysis

The data were subjected to analysis of variance using PROC GLM of SAS version 9.3 [26]. Mean comparisons were conducted using least significant difference ($LSD_{0.05}$). A polynomial regression analyses were also performed using PROC REG (method = reg) of SAS version 9.3 (SAS Institute, 2011). Principal component analysis (PCA) was performed using PROC FACTOR (method = prin) in SAS 9.3 [26] Then, a genotype-by-trait biplot was constructed by using the first two traits focused scaling principal components (PC_1 and PC_2) derived from PCA of a genotype-by-trait matrix containing standardized trait data.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS

Results of our preliminary experiment using different explants including hypocotyl, cotyledon and seed cultured on MS medium supplemented with 0.5 mg/l

2,4-D and 1 mg/l Kin, showed that hypocotyl is the most suitable explant for both the callus induction and callus growth rate. The highest rate of callus induction (70%) and callus growth rate (0.33 mm/day) were observed when hypocotyl explants were used (data not shown).

Plant growth regulators

Results of analysis of variance showed that callogenesis, callus growth rate and callus mucilage content were significantly influenced at various concentrations of PGRs (tab. 1). Different plant growth regulators induced different morphology in the calli in view point of their color and type (tab. 2). The PGRs had significant effect on callus initiation of *P. ovata* genotypes. The rate of callus induction varied from 0.76 (0.5 mg/l 2,4-D + 1 mg/l Kin) to 0.53 (2 mg/l 2,4-D + 1 mg/l Kin) (tab. 2). However, the results indicated no significant difference between a wide range of growth regulator concentrations including [(2,4-D (2.5 mg/l) + Kin (1 mg/l)], [2,4-D (0.5 mg/l) + Kin (1 mg/l)], [2,4-D (1 mg/l) + Kin (1 mg/l)] and [2,4-D (1 mg/l) + Kin (0.5 mg/l)] for callus induction (tab. 2). The results of mean comparison for callus growth rate (GCR) is presented in table 2. The highest (0.38 mm/day) and the lowest (0.16 mm/day) CGR obtained in MS medium supplemented with 0.5 mg/l 2,4-D +1 mg/l Kin and 2 mg/l 2,4-D + 1 mg/l Kin, respectively (tab. 2). The relationship between CGR and incubation period at four sequential period after callus induction well fitted by a polynomial function ($Y= -0.25x^2+ 1.21x – 0.86$, fig. 1), and that they were strongly correlated ($r = 0.76$). According to figure 1, the highest CGR (0.86 mm/day) denoted to second period. The callus growth rate increased ascending until the second period (0.86 mm/day), and then continued to decrease significantly up to last period (0.07 mm/day). In the current study, the effects of a wide range of PGRs on mucilage accumulation in callus culture of *P. ovata* were studied as shown in table 2. The highest mucilage production 0.49 (g/g *DW*) was obtained in 0.5 mg/l 2,4-D + 1 mg/l Kin that it was approximately three times more than extracted mucilage by seeds.

Table 1.

Results of analysis of variance of studied traits in the calli originated from different plant growth hormones in *P. ovata*

Source of variation	df¥	Mean squares		
		Callus induction	Callus growth rate	Callus mucilage content
Plant growth regulators	10	0.036**	0.016**	17.17**
Residual	44	0.0035	0.0023	0.0005

**: significant at the 1% of probability level
¥: Degree of freedom

Table 2.

Effects different plant growth regulators at various concentrations on callus – related traits and mucilage content from hypocotyl explants of *P. ovata*

	PGRs [mg/l]*	Callus induction	Callus growth rate [mm/day]	Mucilage [g/g *DW*]	Morphology of callus
1	2,4-D (0.5)+Kin (1)	0.76^{a}	0.38^{a}	0.49^{a}	Compact, yellow dark
2	2,4-D (1)+ Kin (1)	0.73^{a}	0.33^{ab}	0.15^{d}	Compact, yellow
3	2,4-D (1.5)+Kin (1)	0.54^{c}	0.23^{d}	0.14^{de}	Friable, pale yellow
4	2,4-D (2)+Kin (1)	0.53^{c}	0.16^{e}	0.10^{f}	Friable, creamish white
5	2,4 D (2.5)+Kin (1)	0.75^{a}	0.26^{cd}	0.08^{f}	Friable, white
6	2,4-D (1)+Kin (0.5)	0.72^{a}	0.29^{bcd}	0.16^{d}	Friable, white
7	2,4-D (1.5)+Kin (0.5)	0.67^{ab}	0.30^{bcd}	0.14^{d}	Friable, white
8	2,4-D (2) +Kin (0.5)	0.69^{ab}	0.25^{cd}	0.11^{ef}	Friable, white
9	NAA (1)+ BAP (0.5)	0.64^{b}	0.31^{bc}	0.39^{b}	Soft friable, white
10	2,4-D (2.5)	0.54^{c}	0.33^{ab}	0.36^{b}	Semi-compact, yellow
11	2,4-D (3)	0.70^{ab}	0.29^{bcd}	0.30^{c}	Friable, yellow

* - plant growth regulators

Means in each column, followed by the same letters are not significantly different at 1% of probability level

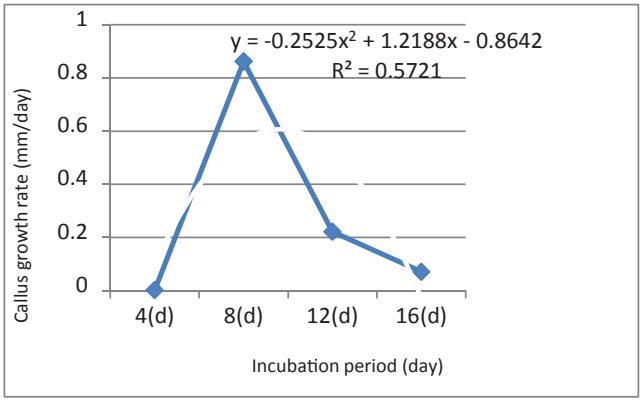

Figure 1.

The polynomial regression line for callus growth rate (mm/day) during four sequential periods of inoculation in *P. ovata*

Mucilage assay in studied genotypes

Analysis of variance showed that there were significant differences ($p<0.05$) among the 14 studied genotypes for the callus initiation, callus growth rate and mucilage content produced either by the seeds or the calli (tab. 3). Mean comparisons of callus induction, CGR, mucilage content of callus and seed are presented in table

4. The highest (0.73) and the lowest (0.55) value of callus induction were observed in genotypes of Po_1 and Po_6, respectively (tab. 4). The results also showed that the combination of 2,4-D (0.5 mg/l) and Kin (1 mg/l) increased the mean of callus induction rate up to 50% overall the genotypes tested.

According to table 4, the highest of CGR (0.45 mm/day) was observed in Po_1 and Po_{11} genotypes. The least value of CGR (0.21 mm/day) was observed in Po_9. In the current study the mucilage extracted from seeds was lower than the mucilage extracted from callus and ranged from 0.13 (g/g *DW*) in Po_9 to 0.042 (g/g *DW*) in Po_5 (tab. 4). The genotypes showed significant differences for mucilage content produced from both callus and seed (tab. 4). The highest mucilage content from callus (0.45 g/g *DW*) and seed (0.130 g/g *DW*) were observed to Po_1 and Po_9 genotypes, respectively (tab. 4). All the genotypes except produced higher mucilage extracted from callus than the seeds, except Po_{14}, Po_8 and Po_{11}. The mucilage content of callus and seeds were nearly identical in Po_9 genotype (tab. 4).

Table 3.

Results of analysis of variance of studied traits in different genotypes of *P. ovata* at *in vitro* callus culture

Source of variation	df¥	Mean square			
		Callus induction	Callus growth rate	Mucilage in callus	Mucilage in seed
Genotype	13	0.017*	0.027**	0.025**	0.003**
Residual	56	0.008	0.002	0.0002	0.00003

**,* Significant differences at $p<0.01$ and $p<0.05$, respectively
¥: df: degree of freedom

Table 4.

The mean comparison of different studied traits in various genotypes of *P. ovata* by *in vitro* callus culture

Genotype Code	Geographical origin	Latitude and longitude	Callus induction	CGR¥ [mm/day]	Callus mucilage [g/g *DW*]	Seed mucilage [g/g *DW*]
ⱠPo_1	Daran, Isfahan	32°59'N ,50°24'E	0.73^{a*}	0.45^{a}	0.38^{a}	0.103 ^{cd}
Po_2	Mashhad, Iran	36°20'N, 59°35'E	0.58^{b}	0.26^{cde}	0.12^{def}	0.096^{de}
Po_3	Shiraz, Iran	29°39'N, 52°35'E	0.70^{a}	0.24^{de}	0.10^{gh}	0.056^{g}
Po_4	Ghazvin, Iran	36°15'N, 50°0'E	0.72^{a}	0.37^{b}	0.17^{b}	0.092^{e}
Po_5	Aran-Bidgol, Isfahan, Iran	34°14'N, 51°29'E	0.62^{ab}	0.27^{cd}	0.09 ^{hi}	0.042^{h}
Po_6	Khor- Biabanak, Isfahan, Iran	34°46'N, 55°05'E	0.55^{b}	0.23^{de}	0.15^{c}	0.108^{bc}
Po_7	Ahvaz, Khuzestan, Iran	31°20'N, 48°40'E	0.64^{ab}	0.30^{c}	0.10^{fgh}	0.058^{g}
Po_8	Behbahan, Khozestan, Iran	30°36'N, 50°14'E	0.58^{b}	0.31^{c}	0.12^{d-g}	0.128^{a}
Po_9	Farsan, Chahrmahal Bakhtiary, Iran	32°16'N, 50°35'E	0.64^{ab}	0.21^{e}	0.13^{d}	0.130^{a}
Po_{10}	Khurasgan, Ishfahan, Iran	32°44'N, 51°46'E	0.66^{ab}	0.28^{cd}	0.11^{efg}	0.080^{f}
Po_{11}	Ishfahan, Iran	32°50'N, 51°50'E	0.63^{ab}	0.45^{a}	0.09^{hi}	0.093^{e}
Po_{12}	Tiran, Isfahan, Iran	32°45'N, 51°8'E	0.71^{a}	0.28^{cd}	0.08^{i}	0.078^{f}
Po_{13}	Pakistan	-	0.55^{b}	0.29^{cd}	0.12^{de}	0.098^{de}
Po_{14}	India	-	0.65^{ab}	0.32^{bc}	0.10^{fgh}	0.115^{b}

* Means in each column followed by the same letters are not significantly different at $p<0.01$
Ⱡ: *Plantago ovata* ; ¥: CGR: Callus Growth Rate

Relationships between traits

An ordination of both the genotypes and studied traits (callus induction, callus growth rate, mucilage in callus and mucilage in seed was computed by principal component analysis (PCA) and then a genotype-by-trait biplot was constructed and presented in figure 2. The cosine of the angle between the vectors of any two traits approximates the correlation coefficient. Therefore, traits that were positive and significantly correlated were generally co-located on the biplot. At this study, the vector of mucilage in callus positioned with angle of around 30 to the vector of callus growth rate and therefore, callus in mucilage was strongly positively correlated with callus growth rate. The genotype × trait biplot also indicated that the genotype Po_1 with the closest distance to CGR and mucilage in callus vectors ranked as the superior genotype for mentioned traits (fig. 1). With considering the position of Po_8 and Po_9 genotypes to mucilage in seed vector, they were considered as superior genotypes for mucilage content in seed.

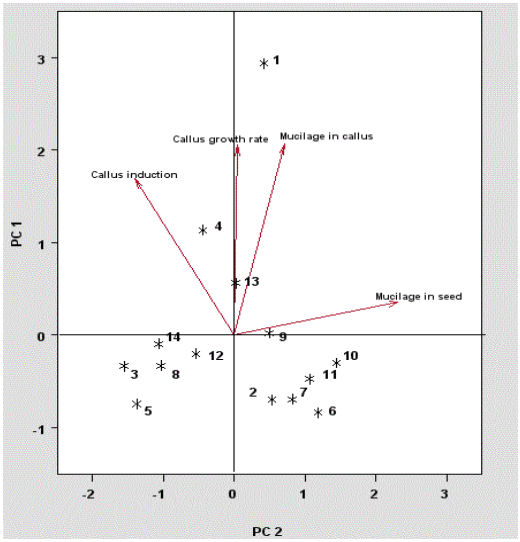

Figure 2.

Biplot presentation for the 1st and 2nd principal components used for studied traits in different genotypes of *P. ovata* at *in vitro* callus culture

DISCUSSION

The explants of seed produced calli silently after four month. Nonetheless, the hypocotyls explants found to be the best ones and were selected for further experiments. These novel explants (hypocotyls, seed and cotyledon) were not been evaluated by previous studies for callus induction, callus growth rate

and mucilage synthesis abilities in *P. ovata*. At different plant growth regulators, callus initiation was observed mostly from the cut surfaces of the hypocotyls. Typically, this was a wound reaction where mitosis was induced in the cells from the cut surface leading to callus formation [27]. Our results demonstrated the effective concentration of 2,4-D and Kin on the increase of callus initiation and callus growth rate in *P. ovata*. The differences between these callus induction rates could depend on the differences between assayed genotypes in response to callus initiation and different explants that were used in this study. The findings of the current study are in agreement with those of Gupta *et al.* [7] who reported the highest rate of callus induction (89%) in *P. ovata* on MS medium containing 0.5 mg/l 2,4-D and 1 mg/l Kin on the leaf explants. Wakhlu and Barna [20] reported that the best initiation and growth of callus (fresh weight of callus) in *P. ovata* was achieved on MS medium containing 1 mg/l 2,4-D and 1 mg/l Kin with hypocotyl culture. Moreover, the combination of low concentration of 2,4-D with higher concentration of kinetin was more efficient in callus induction among concentrations tested on hypocotyl explants. A possible explanation for this result is that the high concentration of 2,4-D, singularly, in the medium may inhibit callus growth from hypocotyl explants. Das and Raychaudhury [19] reported that combination of 4.5 μm 2,4-D and 2.3 μM Kin were more effective for callus initiation from shoot buds of *P. ovata*. The highest callus diameter (2.83 mm) in *P. ovata* was reported in B_5 tissue culture media [28], but according to these findings, the MS medium with combination of 2,4-D and Kin was strongly affected on increasing callus diameter (4.25 mm) in *P. ovata*. Mahmood *et al.* [18] reported that the highest callus growth (mg/fresh weight) in *P. ovata* was obtained in MS medium containing 4 mg/l 2,4-D concentration for shoots, 0.5 mg/l for seeds and 2 mg/l for roots. Different growth media culture (MS, MSH, NT and B_5) studied on callus growth rate of *P. lanceolata* and highest rate of callus growth rate was obtained at 0.8 mg/l 2,4-D and 0.1 mg/l Kin in MSH (Hildebrandt and Schenk Media) [22]. However, it is defined as the use of high doses of 2,4-D, solely dose not leads to increase in cell division and cell elongation, which is consistent with our results showing the requirement of both 2,4-D with Kin for rapid callus growth rate. Literature review showed that callus growth has been studied in a base of callus weight changes in *P. ovata* [19, 21]. This novel equation could discriminate the reaction of callus growth rate (mm/day) at different times after callus induction in *P. ovata*. This study demonstrated explants and plant growth-regulator concentrations at high mucilage synthesis by employment of desirable genotype. Our findings are consistent with those of Gupta *et al.*[7], Mirmasumi *et al*. [22] and Afshar and Golkar [29] who have obtained relatively large amount of mucilage compound as a secondary metabolite in undifferentiated calli of *P. ovata*, *P. lanceolata* and *Alyssum* species, than the seeds obtained by field grown plants, respectively.

Gupta *et al*. [7] reported that 0.25 mg/l 2,4-D and 0.25 mg/l TDZ (thidiazuron) produced the most mucilage content with callus culture (51.3% g/g *DW*) in *P. ovata*.

The highest mucilage content from callus extraction in *P. lanceolata* (14.73% g/g *DW*) was obtained in MSH media culture containing 0.8 2,4-D (mg/l) and 0.1 Kin (mg/l) [22]. Farzan *et al.* [30] reported that optimal medium for mucilage production was found to be MS medium containing 0.8 mg/l 2,4-D and 1 mg/l Kin and root as explants in *P. major*. The significant genetic variation observed for callus-related traits. The genotypic dependence of callus growth rate were supported by previous studies on *P. ovata* [18] and *P. major* [29]. Moreover, CGR has been shown to be a strong genetic component in the callus cultures [30]. Exploration of genetic diversity for *in vitro* culture to gain higher secondary metabolites is one of crucial components in the improvement of mucilage yield. This result illustrated the significant positive effect of PGRs on increasing the mucilage synthesis under *in vitro* callus. In Po_1 (Isfahan origin) genotype, the mucilage content of callus was nearby three times greater than mucilage content of the seeds, that demonstrated at high cellular mechanisms of this genotype to exclude synthesized mucilage from intercellular space into outside of the cells. The accumulation of secondary metabolites in cultured cells at a higher level than those in native plants through optimization of cultural conditions has been observed for production of shikonin in *Lithospermum erythrorhizon* [31] diosgenin in *Dioscorea* [32], jaceosidin and syringing in *Saussurea medusa* [33].

CONCLUSIONS

The evolving commercial importance of secondary metabolites has led to a great demand in the pharmaceutical industry in recent years. The results of *in vitro* culture of *P. ovata* revealed that the enhancement of mucilage production at the cellular levels is not only genotypic-dependent but also depends on the explant type (hypocotyl, based on our findings) as well as the plant growth regulators. Considering relatively low yield of mucilage compounds in the seeds of *P. ovata*, high-yield production of mucilage by callus culture would be feasible at commercial scale using amenable genotypes. In a final note, an important functional aspect of this research was selecting genotypes with high potential for mucilage production for medicinal use. Perhaps the most intriguing aspect of this research as it unravels in future works will be the determination of how each of the plant growth-condition factors as well as *in vitro* culture manipulations contributes to a greater production of mucilage.

Conflict of interest: Authors declare no conflict of interest.

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WPŁYW GENOTYPU I POŻYWKI NA SYNTEZĘ *IN VITRO* ŚLUZÓW PRZEZ KULTURY *PLANTAGO OVATA* FORSK

POORAN GOLKAR[1*], FARZAD AMOOSHAHI[2], AHMAD ARZANI[2]

[1]Research Institute for Biotechnology and Bioengineering
Isfahan University of Technology
Isfahan 8415683111, Iran

[2]Department of Agronomy and Plant Breeding
Faculty of Agriculture
Isfahan University of Technology
Isfahan 8415683111, Iran

[*]autor, do którego należy kierować korespondencję: golkar@cc.iut.ac.ir

Streszczenie

Wstęp: Babka płesznik (*Plantago ovata* Forsk) jest stosowana w fitoterapii głównie ze względu na zawartość śluzów. **Cel:** Prowadzone badania miały na celu zwiększenie wydajności produkcji śluzów przez kultury kalusa w warunkach *in vitro* przy wykorzystaniu różnych genotypów, eksplantatów oraz pożywek. **Metody:** Badano wpływ różnych stężeń regulatorów wzrostu, w tym kwasu 2,4-dichlorofenoksyoctowego (2,4-D) i kinetyny (Kin), na syntezę śluzów w warunkach *in vitro* stosując jako eksplantaty liścienie, hypokotyl i nasio-

na. Wykorzystano14 genotypów pochodzących z różnych regionów geograficznych Iranu w celu określenia ich odpowiedzi na syntezę *in vitro* śluzów. **Wyniki:** Najwyższy współczynnik indukcji kalusa (76%) i współczynnik wzrostu kalusa CGR (0,38 mm/dzień) uzyskano na pożywce MS z dodatkiem 0,5 mg/l 2,4-D i 1 mg/l Kin przy zastosowaniu hypokotyla jako eksplantatu. Wyniki analizy wariancji wskazują na znaczące zróżnicowanie genotypów pod względem indukcji kalusa, wartości CGR oraz zawartości śluzów w kalusie i nasionach. Zawartość śluzów wahała się od 0,38 do 0,08 (g/g s.m.) i od 0,13 do 0,042 (g/g s.m.) odpowiednio dla kalusa i dla nasion. Lepszą indukcję kalusa (73%), wartości współczynnika wzrostu kalusa CGR (0,45 mm/dzień) i zwartości śluzów (0,38 g/g s.m.) oznaczono dla genotypu Po_1. Kalus tego genotypu produkował blisko trzy razy więcej śluzów niż nasiona. **Wnioski:** Wyniki badań wskazują, że wysoka wydajność kultur kalusowych *P. ovata* otrzymanych przy zastosowaniu hypokotyla w połączeniu z wykorzystaniem zróżnicowania genetycznego jest ważna dla zwiększenia poziomu syntezy śluzów w kulturach *in vitro*.

Słowa kluczowe: *kalus, produkcja śluzów, eksplantaty z hypokotyla, polisacharydy*