

SUPPLEMENTARY MATERIAL

Production of New Mucilage Compound in *Lepidium sativum* Callus by Optimization of *in vitro* Growth Conditions

Pooran Golkar^{*1}, Fatemeh Hadian² and Mehrana Koochi Dehkordi³

1- Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology, Isfahan, Iran.

2- Department of Agriculture, Payame Noor University (PNU), P.O. Box, 19395-3697, Tehran, Iran.

3- Department of Agriculture, Payame Noor University (PNU), P.O. Box, 19395-3697, Tehran, Iran.

ABSTRACT

The mucilage in *Lepidium sativum* L. is considered a biologically active compound with diverse medicinal properties. Different explants (i.e., hypocotyls and leaf) were transferred to MS medium supplemented with twelve different plant growth regulator combinations under two different incubations (light and dark). The best mucilage production from callus (36.76% g g⁻¹ dry weight) was obtained in the MS medium supplemented with 1 mg L⁻¹ of 2, 4-D and 2 mg L⁻¹ of BAP under the light condition. The mucilage produced by callus culture was nearly three times more than the mucilage yield of the seeds. The glucose, arabinose + mannose and galactose were 43.4 (mg g⁻¹ DW), 195.3 (mg g⁻¹ DW) and 86.2 (mg g⁻¹ DW) in the mucilage originated from seed, callus leaf and callus hypocotyl, respectively. The present study proposes an efficient method for producing large scales of mucilage with a favorable sugar aimed at food or pharmaceutical industries.

Keywords: callus, medicinal, mucilage, plant growth regulators.

Experimental

3.1 Explant training

The GC seeds (the native genotype "Isfahan") were sterilized by 70 % (v/v) ethanol for 1 min and then by 2.5 % w/v NaOCl for 15 min in a laminar air flow cabinet. Afterwards, they were rinsed 3 to 5 times with double distilled water. For germination, the seeds were placed on an agar-solidified MS basal medium (Duchefa Com.) and incubated for a 16/8 (light/dark) photoperiod at the temperature of 25 ± 1 °C and the photon density of 50 μmol m⁻² s⁻¹. As the explant source for

further experiments, leaf (0.5-1 cm) and hypocotyl (3-4 mm) segments were excised from young seedlings (14 day-old). The explants (leaves, hypocotyls and seeds) were transferred to petri dishes (10 mm) and then kept at a growth chamber at 25 ± 2 °C under the fluorescence light intensity of $50 \mu\text{mol m}^{-2}\text{s}^{-1}$ and a 16/8 h (light/dark) regime. Different auxins (2,4-Dichlorophenoxyacetic acid :2,4- D and Naphtalenacetic acid: NAA) and cytokinins (Kinetin: Kin and Benzylaminopurine: BAP) were used at different concentrations (1,2 and 3 mg L⁻¹) for callus induction. All applications of NAA+ Kin, NAA+ BAP and 2,4D+ Kin (contemporary or sequential) showed no obvious callus. The seed explants had no response to callus induction. Thus, the ineffective treatments were not included in subsequent studies. The significant effect of plant growth regulators on callogenesis and callus morphology is presented in Table 1. The callus induction was initiated in the first tissue culture media (MS₁) (Table S1). After two weeks, all the primary small calli were transferred to the second tissue culture media (MS₂) (Table S2) combinations. Data were recorded after two weeks of inoculation in the second new media (MS 2).

3.2. Trait measurement

3.2.1. Callus-related traits

The callus fresh weight (g) was measured after two months from callus culture initiation. Callus induction (%) was calculated as follows: $[(n/N) \times 100]$, where n = the total number of calluses, and N = the total number of cultured explants. The callus growth rate (CGR) was measured as the mean of callus growth rate (mm day⁻¹) 7, 15, 23 and 31 days after callus induction. The CGR at each time was calculated by dividing callus diameter (d_i) (square root of callus length × callus width) to the number of each period. It should be noted that the callus diameter in each period (d₇, d₁₅, d₂₃ and d₃₁) was calculated based on the difference between callus diameters at each time from previous time.

3.2.2 Mucilage assay

Mucilage was identified according to Gupta et al. (2015). The mucilage was hydrolyzed by the acidic method. One (g) of the fresh callus was added to 10 ml of HCL (0.1 N) in boiling phase. After 6 min, the solutions were filtered by a clean muslin cloth. The produced mucilage was observed optically. The combined solution mucilage and other callus components were mixed with 60 ml of ethyl alcohol (95%), stirred, and then maintained in refrigerator (4 °C) for 5 hour. Finally, the liquid supernatant was removed, and the precipitate was dried in an oven at 50 °C for

14 h to obtain pure mucilage [(g g⁻¹ dry weight callus (%)). Mucilage was extracted from seeds based on the method proposed by Gupta et al. (2015).

3.2.3. Sugar quantification in mucilage

To quantify the sugar components of the mucilage produced in the calli, High Performance Liquid Chromatography (HPLC) was used by an RI detector with the flow rate of 0.6 ml/min and the injection volume of 20 µl (Jasco International Co., Tokyo, Japan) at 40 °C. The mucilage samples (100 mg) were dissolved in 100 ml of Trifluoro acetic acid (4M), boiled at 100 °C for 12 min and filtered by a 0.45-µm syringe filter. The separation was done in the fluid phase of deionized water with the flow rate of 0.6 ml/min in Aminex HPX-87P Column #1250098 (Bio Rad, Richmond, CA), 300 × 7.8 mm, 9-µm particle size and pH 5–9. Four standard sugar samples were obtained from the Merck Chemicals Stock solution (50 mg/ml) of sugar that was prepared in deionized water. The injected volume was 20 µl. From each stock solution, dilutions of 2–50 ml were taken and analyzed two times by HPLC. Standard curves were plotted between different concentrations and the peak area for each sugar component. Different concentrations of sugars including glucose, xylose, galactose, arabinose and mannose (0.5, 1, 2 and 4 mg/ml) were used for HPLC calibration of monosaccharide sugars. Based on the retention time of the standard samples, the sugar concentration (mg/ml) in mucilage was determined based on Misra and Shiva Kameshvar (2016) formulae. The computer program T2000 was used for peak integration and calculation.

3.2.4 Statistical analysis

The experiment was carried out as a completely randomized design with five replications (10 explants per replication). The mean of all samples were considered for callus induction. The presented data were analyzed by variance analysis using SAS (version 9.1). The Least Significant Difference (LSD) _(0.05) was determined for mean comparisons.

Table S1. The effect of plant growth regulators, explants and incubation condition (light/ dark) on different evaluated traits of the calli originating from *Lepidium sativum* L.

Source of variation	df	Mean squares			
		CI	CGR	CFW	Mucilage
PGR [¥]	11	3675.50 ^{**}	0.013 ^{**}	0.14 ^{**}	699.6 ^{**}
Explant	1	33373.5 ^{**}	0.621 ^{**}	3.44 ^{**}	5.95 [*]
Light/dark	1	3352.05 ^{**}	0.047 ^{**}	0.29 ^{**}	2613.93 ^{**}
PGR× Explant	11	1507.03 ^{**}	0.022 ^{**}	0.047 ^{**}	1.79 ^{ns}
PGR× light/dark	11	60.32 ^{ns}	0.0003 ^{ns}	0.009 ^{**}	28.68 ^{**}
Explant× light/dark	1	66.71 ^{ns}	0.0001 ^{ns}	0.18 ^{**}	143.05 ^{**}
PGR× Explant ×Light/dark	11	26.43 ^{ns}	0.0001 ^{ns}	0.009 ^{**}	5.49 ^{ns}
Residual	192	77.7	0.008	0.0025	1.52

^{**}, ^{*}, ^{ns}: significant at the 1% and 5% probability levels, non significance, respectively.

[¥]: PGR Plant growth regulators; df: degree of freedom; CI: Callus induction; CGR: Callus growth rate, CFW: Callus fresh weight.

Table S2. The effect of plant growth regulators on callus induction, callus growth rate and callus fresh weight of *Lepidium sativum* on the MS culture medium

No.	MS+ Plant growth regulators (mg L ⁻¹)		CI (%) [¥]		CGR(mm day ⁻¹)		CFW (g)		Callus morphology
	MS(1) [†]	MS (2)	Light	Dark	Light	Dark	Light	Dark	
T ₁	MS+ 2,4 (D) (1)	MS+ 2,4D (1)+BAP (0)	52.00±8.73 †	45.70±7.81	0.123±0.004	0.102±0.003	0.288±0.02	0.246±0.02	Compact
T ₂	MS+ 2,4 (D) (1)	MS+ 2,4 (D) (1)+BAP(1)	51.50±4.24	46.13±3.50	0.135±0.011	0.10±0.03	0.401±0.04	0.298±0.04	Semi soft
T ₃	MS+ 2,4 (D) (1)	MS+ 2,4 (D) (1)+BAP (2)	67.00±6.33	61.42±6.42	0.131±0.009	0.110±0.008	0.336±0.06	0.255±0.05	Soft friable,
T ₄	MS+ 2,4D (1)	MS+ 2,4D (1)+BAP (3)	52.67±7.31	46.54±6.67	0.142±0.004	0.106±0.008	0.446±0.08	0.291±0.05	Semi soft
T ₅	MS+ 2,4D (2)	MS+ 2,4D (2)+BAP(0)	52.17±2.74	46.22±2.16	0.174±0.006	0.137±0.01	0.211±0.02	0.185±0.02	semi- compacts
T ₆	MS+ 2,4D (2)	MS+ 2,4D (2)+BAP (1)	56.50±4.41	51.23±4.46	0.160±0.017	0.123±0.02	0.176±0.04	0.148±0.02	compact, nodular
T ₇	MS+ 2,4D (2)	MS+ 2,4D (2)+BAP(2)	61.50±10.2	54.10±9.42	0.137±0.031	0.115±0.03	0.177±0.04	0.153±0.03	compact nodular
T ₈	MS+ 2,4D(2)	MS+ 2,4D(2)+BAP(3)	56.00±4.94	51.00±4.76	0.169±0.022	0.151±0.02	0.256±0.07	0.158±0.04	Soft friable
T ₉	MS+ 2,4D (3)	MS+ 2,4D (3)+BAP(0)	66.17±2.53	60.80±2.67	0.163±0.022	0.142±0.02	0.104±0.02	0.072±0.01	semi- compacts
T ₁₀	MS+ 2,4D (3)	MS+ 2,4D (3)+BAP(1)	86.50±1.99	77.00±1.85	0.153±0.037	0.127±0.03	0.188±0.04	0.136±0.03	Soft friable,
T ₁₁	MS+ 2,4D (3)	MS+ 2,4D (3)+BAP(2)	84.83±5.65	73.80±4.80	0.196±0.034	0.163±0.03	0.203±0.04	0.135±0.03	Soft friable,
T ₁₂	MS+ 2,4D (3)	MS+ 2,4D (3)+ BAP(3)	93.00±2.21	76.20±2.05	0.217±0.03	0.178±0.02	0.285±0.08	0.153±0.04	Soft friable,
T ₁₃ -T ₂₀	MS+ 2,4D (1,2,3)+ Kin (1,2, 3)		-	-	-	-	-	-	No callus formation
T ₂₁ -T ₂₉	MS+ NAA (1,2,3) +BAP (1,2,3)		-	-	-	-	-	-	No callus formation
T ₃₀ -T ₃₈	MS+NAA (1,2,3)+ Kin (1,2,3)		-	-	-	-	-	-	No callus formation

I: MS (1); Murashige and Skoog (MS) + Plant growth regulators (mg L⁻¹); MS (2): Murashige and Skoog (MS) + Plant growth regulators (mg L⁻¹);
† :Means ± Standard errors; ¥: CI: Callus induction; CGR: Callus growth rate, CFW: Callus fresh weight.

Table S3. Identification of the sugar components by HPLC in mucilage extracted from different callus explants (leaf and hypocotyls) and seed in *Lepidium sativum*.

Sugar name	Mucilage of seed (mg g ⁻¹ DW)	Area (%)	Mucilage of callus (hypocotyls) (mg g ⁻¹ DW)	Area (%)	Mucilage of callus leaf (mg g ⁻¹ DW)	Area (%)
Glucose (RT: 14.2)	43.4± 0.21 [¥]	23.52	53.2±0. 18	34.65	0	0
Xylose (RT:15.4)	0	0	0	0	0	0
Galactose (RT:17.2)	28.4 ± 0.12	15.15	86.2±14.1	56.25	0	0
Arabinose+Mannose (RT:19.1)	28.7± 0. 24	30.68	42.6±0.19	3.82	195.3±2.5	99.1
Others (non- detected)	-	30.65	-	5.28	-	0.9
Total	-	100	-	100	-	100

RT: retention time. ¥: means ± standard errors.