Linseed mucilage – optimized extraction procedure for genomic research and nutraceutical applications

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Since the mucilage is released by specialized cells on the surface of the seed coat of the flax seed, some of the DNA from these cells is transferred into the mucilaginous mass. The isolation of DNA from the mucilage is greatly influenced by the conditions of mucilage extraction as well as the conditions of DNA extraction. When extracting the mucilage, it is necessary to separate it from the seeds by centrifugation, as pressing in gauze is likely to cause mechanical damage to the cells. The temperature of the mucilage extraction is also an important factor, 40 °C was ideal for our studies, as higher temperatures release additional metabolites into the mucilage and thus make it more challenging to isolate DNA. For the actual extraction of DNA from the mucilage, a low temperature is essential as the mucilage has a higher solubility with increasing temperature. Thus, the lower the temperature, the easier it is to separate the mucilage polysaccharides from the nucleic acids. DNA purification is key for the amplification efficiency of the DNA isolated from the mucilaginous mass of flax seeds. The initial consistency of the mucilage before the isolation itself does not affect this result, although it should be noted that it acquires a consistency similar to that after the lyophilization process even by the action of liquid nitrogen on the extracted mucilage. We demonstrated the amplification efficiency of the extracted DNA using the random (RAPD) and sequence-specific (microRNAs) primers.

Keywords: *Linum usitatissimum* L., mucilaginous substances extraction, DNA extraction, DNA amplification, miRNAs

1   Introduction

Linum usitatissimum L. seeds are capable of differentiating the seed coat into a specialized cell layer producing hydrophilic mucilage. The mucilage has several functions for the plant, such as seed hydration, spatial fixation, protection, stimulation of metabolic activity and seed development (Kučka et al., 2022). In addition, the consumption of mucilage has many beneficial effects such as weight reduction, treatment of gastric lesions induced by ethanol, has prebiotic properties, antioxidant properties, anti-ulcer activity, and is a great source of dietary fiber (HadiNezhad et al., 2013, Bongartz et al., 2022; Safdar et al., 2019; Dugani et al., 2008; Ding et al., 2014). Mucilages can also be used as natural stabilizers in dairy products such as yoghurt, increasing viscosity, and reducing syneresis (Basiri et al., 2018; Sungatullina et al., 2023). In addition to its use as a stabilizer in foods, it can be used as a fat replacer or plasticizer (Tee et al., 2016; Akl et al., 2020). Several methodologies of mucilage extraction have been described. Most methodologies agree that the composition of the mucilage and thus its properties are primarily dependent on the extraction temperature, extraction time and seed/solvent ratio. Higher extraction temperatures increase the content of phenolic compounds and thus improve the antioxidant properties and reduce the extraction time. On the other hand, they reduce the rheological properties of the mucilage. Therefore, it is necessary to adjust all parameters to make the mucilage as pure as possible while achieving high yields (Vieira et al., 2019; Mehtre et al., 2017; Kaur et al., 2018; Rocha et al., 2021; Barbary et al., 2009; Ziolkovska, 2012; Kaewmanee et al., 2014).

The aim of this work was to establish a protocol for the extraction of DNA from the mucilage matter and test

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the DNA amplification efficacy for the downstream molecular markers analyses of the genotype-dependent variability in the properties of linseed mucilage. We aimed to optimize the mucilage extraction protocol in order to minimize its degradation for further putative nutraceutical applications.

2 Material and methods

2.1 Biological material

Altogether, eight registered linseed (*Linum usitatissimum* L.) genotypes of European origin; Agram, Agriol, Astella, Raciol (Czech Republic), Flanders, Libra, Lola (Netherland), Natural (France) were selected for the mucilage analyses, including the genotype of Canadian origin (CDC Bethune) due to the availability of bioinformatic data for the downstream molecular analyses. The genotypes differ in seed color (brown seeds; Agram, Astella, CDC Bethune, Flanders, Libra, Lola, Natural and yellow seeds; Agriol, Raciol), food applications (Agram, Agriol, Astella, CDC Bethune, Lola, Raciol), industrial applications (Flanders, Libra) and dual applications (Natural). The biological seed material was provided by Agritec Plant Research, Ltd., Šumperk, Czech Republic.

2.2 Extraction of mucilage

An optimized protocol, elaborated based on several protocols (Barbary et al., 2009; Ziolkovska, 2012; Kaewmanee et al., 2013), was used for mucilage extraction (Figure 1). The procedure is as follows: the flax seeds were first washed shortly three times with running water to remove the surface dust. Ten grams of cleaned flaxseed of each genotype were transferred to sterile falcon conical centrifuge tubes (50 mL). Subsequently, 40 ml of distilled water (1 : 5 ratio; seeds : water) was added and each labelled falcon tube was placed in a water bath at 40 °C for 5 hours (without stirring). The pH was 7.0 ±1.0. After incubation, the seed-mucilage matter was either pressed in gauze to separate the mucilage, or the falcon tubes were centrifuged for 15 minutes at 4 °C, and 2710 rcf. The supernatant was transferred into new sterile labeled falcon tubes (50 mL) and the mucilage was precipitated by adding 80% ethanol (p. a.) at a 1 : 4 v/v ratio (mucilage : ethanol). The precipitate was allowed to settle overnight at 4 °C and the mucilage was recovered by centrifugation at 2,710 rcf for 35 minutes on the next day. After determining the mucilage extraction methodology, this procedure was applied to all flax genotypes in three replications in order to determine the volume of produced mucilage.

The subsequent analyses of isolated DNA have shown that the preferred mucilage mass is obtained

by centrifugation and not by pressing in gauze. The established extraction protocol is accurate, short, reproducible and mucilage protective.
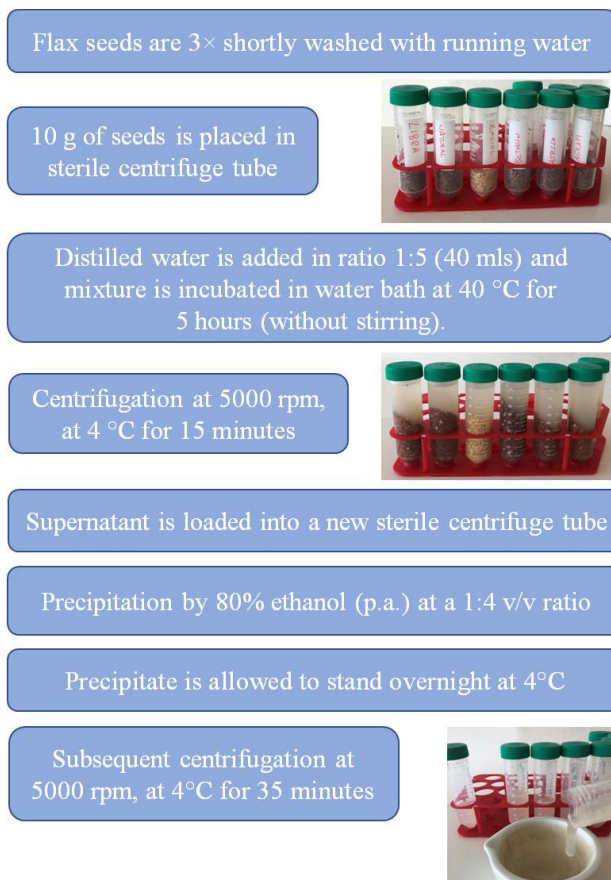

Figure 1 The optimized protocol for the extraction of the linseed mucilage

2.3 DNA isolation from the extracted mucilage matrix

As mentioned above, the separation procedure for mucilage matter was tested on the mucilage collected from the seeds suitable for the extraction of nucleic acids. Once the seeds were incubated in distilled water for 5 hours at 40 °C, the seed-mucilage matter was either pressed in gauze or centrifuged in falcon tubes at 4 °C (2710 rcf). Consequently, four variants were tested for DNA isolation: pressed mucilage (1), pressed mucilage pulverized in nitrogen (2), centrifuged mucilage (3), and centrifuged mucilage pulverized in nitrogen (4). DNA isolation was performed using the ISOLATE II Plant DNA Kit (Bioline™). Both types of included lysis buffers were tested. As the PA2 lysis buffer involved incubation at 65 °C to dissolve the mucilage and additional incubation on ice when the mucilage was precipitated again and could be filtered, this lysis buffer was proved to be more suitable for the isolation of DNA from mucilage. The quality and quantity of the isolated DNA was

measured by the Implen NanoPhotometer P360® spectrophotometer.

2.4 Purification of isolated DNA

The isolated DNA was purified using the Qiagen's DNeasy PowerClean Pro Cleanup Kit (Qiagen™) to remove the carbohydrates and other contaminants from the DNA according to the manufacturer's requirements.

2.5 Lyophilization of extracted mucilage

The mucilage obtained by the above-mentioned optimized procedure was poured into labelled sterile plastic Petri dishes and lyophilized by the Alpha 2–4 LSCplus (CHRIST™) lyophilizer with the following parameters: freezing at -30 °C for 20 minutes; warm-up at -30 °C for 20 minutes; main drying at -30 °C for 10 hours, vacuum 0.1 mbar and final drying at 35 °C for 18 hours, a vacuum of 0.01 mbar.

The lyophilization produced thin and dry layers of mucilage (Figure 2), which were processed into powder using liquid nitrogen. Subsequently, the DNA isolation was carried out using the ISOLATE II Plant DNA Kit from Bioline™, applying the PA2 lysis buffer. The isolated DNA was purified by the DNeasy PowerClean Pro Cleanup Kit (Qiagen™).

Figure 2   Lyophilized linseed mucilage

2.6 Testing the efficiency of DNA amplification

To test the suitability of the isolated DNA for its amplification potential, several PCR reactions using both random (RAPD markers) (Williams et al., 1990) and sequence-specific primers (miRNA-based molecular markers) (Fu et al., 2013; Ražná et al., 2020) were established (Table 1).

Table 1   Primers sequences for DNA amplification

Primer identification	Sequences (5′–3′)
lus-miR168_F	CACGCATCGCTTGGTGCAGGT
lus-miR168_R	CCAGTGCAGGGTCCGAGGTA
lus-miR408_F	GGCTGGGAACAGACAGAGCATGGA
lus-miR408_R	GGGAAAAAGGCCAGGGAAGAGG
OPB-11	GTAGACCCGT
OPB-18	CCACAGCAGT

F – forward primer, R – reverse primer

The RAPD reactions were performed in a total volume of 20 μL, which contained 1 × FIREPol PCR buffer (Solis BioDyne®); $MgCl_2$ (1.5 mmol·dm^{-3}); dNTP (200 μmol·dm^{-3}); OPB11 and OPB18 primers (1 μmol·dm^{-3}), one unit of the FIREPol DNA polymerase and DNA (25 ng). The temperature and time profile of amplification was as follows: 95 °C for 2 min; 44 cycles of 60 sec at 94 °C; 60 sec at 36 °C; 2 min at 72 °C and a final extension of 7 min at 72 °C.

The miRNA-based markers were amplified in a 20 μL reaction mixture, which contained 2 units of the DreamTaq DNA polymerase (Thermo Scientific™), 0.8 mmol·dm^{-3} dNTPs, 10 pmol·dm^{-3} of each primer and 1 × DreamTaq PCR buffer (KCl; $(NH_4)_2SO_4$; 20 mmol·dm^{-3} $MgCl_2$). The PCR amplification used the 'touchdown' method with the following steps: initial denaturation at 94 °C for 5 min; 5 cycles of 30 s at 94 °C; 45 s at 64 °C (the temperature was decreased by 1 °C per cycle), and 60 s at 72 °C; 30 cycles of 30 s at 94 °C; 45 s at 60 °C, and 60 s at 72 °C; and the final extension at 72 °C for 10 min.

The PCR products were separated on 1% (RAPD) and/or 3% agarose gel (miRNA assay) in the 1 × TBE Running Buffer at a constant power of 90 V, 120 mA for 60 minutes. The size of DNA fragments was compared to the 100 bp Gene Ruler (ThermoScientific™) and O'RangeRuler 10 bp DNA Ladder (ThermoScientific™), respectively. The gels were visualized under UV by the G-Box electrophoresis documentation system (Syngene®).

3   Results and discussion

3.1 DNA isolation from the mucilage matter and DNA purification

Mucilage can be found in the seed coat and mucilaginous epidermis, which overgrows with the seed's endosperm

(Ziolkovska, 2012; Soto-Cerda et al., 2018). The seed coat epidermis is differentiated into a layer of mucilage secretory cells (MSCs) (Arsovski et al., 2009; Soto-Cerda et al., 2018; Francoz et al., 2022). The epidermal cells release mucilage when the seed is moistened, and mucilage forms a gelatinous layer around the seed (Naran et al., 2008). Cytological analyses show that MSCs are structured in four superimposed layers where flax mucilage is released from inside the MSC (between the upper and lower layers) (Miart et al., 2019).

The mucilage obtained by our established procedure (Figure 1) was transformed into a powdery mass in liquid nitrogen, as the effect of the low temperature on the mucilaginous mass has proved to be an important step (Figure 3). The DNA was isolated using a commercially available kit designed for the isolation of DNA from plant tissues, which offers the option of choosing from two types of lysis solutions that have been tested. So, we chose a lysis solution whose application requires incubation of the sample at 65 °C to dissolve the mucilage. This step has been shown to be important during cell lysis. This step was followed by incubation on ice and the mucilage could be precipitated again, which it turned out to be more suitable for DNA isolation from mucilage. Subsequent spectrophotometric quality control revealed the presence of secondary metabolites. Therefore, the isolated DNA was purified using another commercially available kit to remove carbohydrates and other contaminants. The quality and quantity of isolated DNA was sufficiently improved for PCR analyses.

Ramos et al. (2014) tested several protocols for the extraction of DNA from mucilaginous seeds, including the commercial kits, with no success. Consequently, they modified the protocol initially intended to extract RNA samples. Other studies focused on the regulation of mucilage synthesis, collection of DNA or RNA from seed tissues at different developmental stages (Li et al., 2009; Venglat et al., 2011), treatment of tissues with polyphenol and polysaccharide precipitation prior to the extraction of nucleic acid (Turbant et al., 2016),

or extraction of DNA from young leaf tissues of flax Soto-Cerda et al., 2018). The commercially available kit was used to extract RNA from dissected Arabidopsis seeds in the developmental stage of 6 to 12 days after pollination to study the mucilage biosynthesis (Parra-Rojas et al., 2019). The Cetyltrimethyl Ammonium Bromide (CTAB) method was used for the extraction of RNA from Arabidopsis siliques and seeds to study the regulation of demethylesterification of homogalacturonan in seed mucilage (Xu et al., 2020).

3.2 Testing the DNA amplification effectivity

The amplification potential of DNA for further molecular analyses was tested by random (RAPD) and sequence-specific (miRNA) primers. It was shown that the mucilage obtained by centrifugation and not by pressing in gauze is suitable for these types of analyses. It is probably the mechanical effects of pressure on the viscous seed epidermis cells that cause their damage and the damage of biological molecules. The effects of low temperatures of liquid nitrogen on the mucilaginous mass were proven to be another important factor (Figure 3).
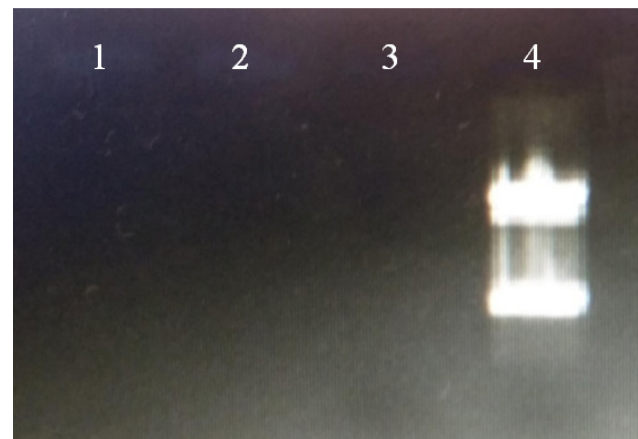

Figure 3 DNA amplification from lyophilized linseed mucilage by OPB 11 primer
1 – pressed mucilage, 2 – pressed mucilage grounded in liquid nitrogen, 3 – centrifuged mucilage and 4 – centrifuged mucilage grounded in liquid nitrogen
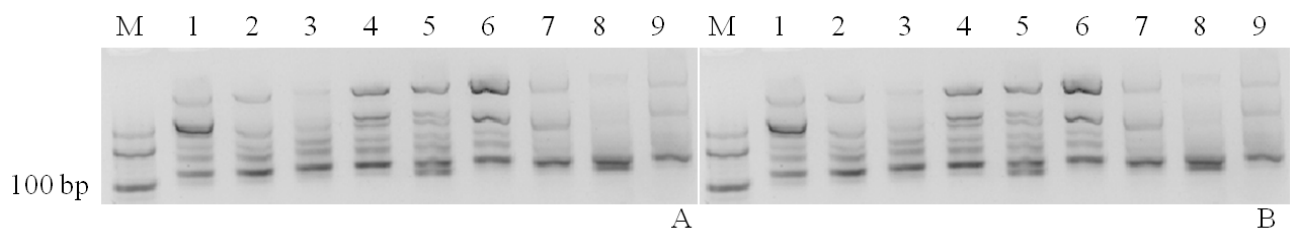

Figure 4 MiRNA-based DNA fingerprinting of the DNA isolated from non-lyophilized mucilage of nine flaxseed genotypes generated by the lus-miR168 (A) and lus-miR408 (B) markers. Electrophoresis on 3% agarose gel with size marker (M)
1 – Libra, 2 – Lola, 3 – Astella, 4 – Agriol, 5 – Natural, 6 – Agram, 7 – Flanders, 8 – Raciol, 9 – CDC Bethune
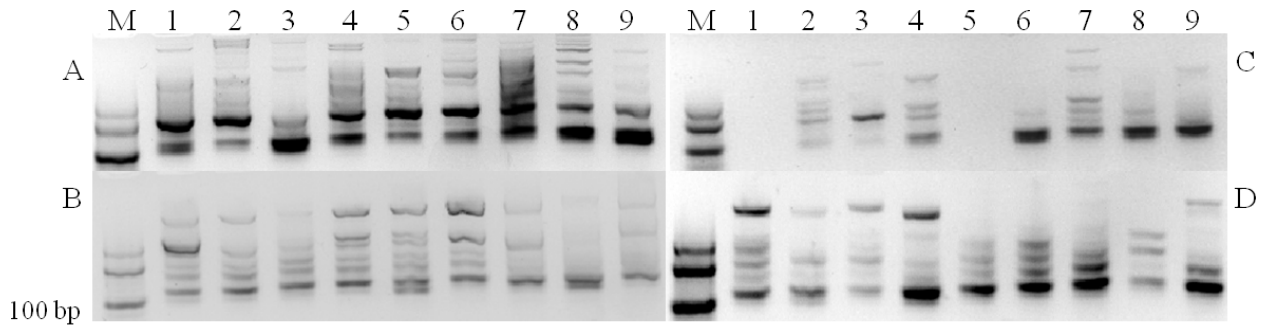
Figure 5 DNA amplification by touchdown PCR using the lus-miR408 marker in non-lyophilized (A, B) and lyophilized (C, D) mucilage samples before (A, C) and after (B, D) the DNA purification procedure. Electrophoresis on 3% agarose gel with size marker (M)
1 – Libra, 2 – Lola, 3 – Astella, 4 – Agriol, 5 – Natural, 6 – Agram, 7 – Flanders, 8 – Raciol, 9 – CDC Bethune

In addition to the use of random RAPD primers, the amplification efficiency of isolated DNA was tested using the sequence-specific markers designed based on the sequences of short endogenous molecules with a regulatory function, microRNAs (Figure 4). Two types of miRNA-based markers have been tested (lus-miR168 and lus-miR408) whose amplification effectivity was confirmed by our previous studies (Ražná et al., 2016; Ražná et al., 2020; Ražná et al., 2021). In addition to the amplification potential of the isolated DNA from the mucilaginous matter, we were also interested to see whether the quality of PCR products could be affected by the consistency of mucilaginous matter – fresh or lyophilized. Therefore, the extracted mucilage was lyophilized under the conditions described above, followed by the extraction of DNA as described previously. The spectrophotometric measurement of DNA purity showed that DNA is contaminated mainly by carbohydrates, which occurred in a much higher concentration when DNA is isolated from lyophilized mucilage compared to fresh mucilage matter. For this reason, it was necessary to perform the purification of DNA. Figure 5 compares PCR amplification efficiency between the non-lyophilized and lyophilized mucilage samples before and after the DNA purification procedure.

We observed that DNA purification is crucial for the amplification efficiency of the DNA isolated from the mucilaginous mass of flax seeds. The initial consistency of the mucilage before the isolation itself does not affect this result, although it should be noted that it acquires a consistency similar to that after the lyophilization process, even by the action of liquid nitrogen on the extracted mucilage.

4 Conclusions

Since flax seed mucilage is a plant product of significant value for human and animal nutrition and health, it is necessary to continue in the research of this viscous biological mass. The importance of this research is also confirmed by the fact that molecular markers of mucilage are genotype-dependent. Therefore, our future research aims to identify the molecular nature of this diversity. We established an optimized linseed mucilage extraction protocol, which meets the criteria of minimizing the mucilage degradation. It is essential to avoid pressing in gauze when isolating mucilage from seeds, which is likely to cause mechanical damage to the cell molecules, and to choose a lower temperature for mucilage extraction, as such mucilage contains fewer secondary metabolites.

For the isolation of DNA from mucilage, it is essential to use a lysing solution that allows incubation on ice, as the mucilage must be in solid form and not liquid to separate the mucilage mass from the DNA effectively. The efficiency of amplification of isolated DNA can be increased by DNA purification since polysaccharides are present after initial isolation.

We will continue in the research of the genotypic variability of the mucilage properties at the molecular level, specifically by identifying microRNAs involved in the regulation of the biosynthesis of mucilaginous substances and analyzing the expression of these molecules depending on the genotype.

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