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# RESEARCH ON THE USE OF MOLECULAR MARKERS TO IDENTIFY THE *NPTII* GENE INVOLVED IN GENETIC MUTATION PROCESSES

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***Abstract***

*Because, for medicinal and aromatic plants there is the ease of being genetically modified both naturally through mutations and through external intervention. In this context, the effectiveness of the present protocol to produce good quality DNA suitable for the detection of genetically modified crops was evaluated. Genomic DNA isolated from different parts of coriander, fennel and sage plants through was done. For this, through the PCR technique, three molecular markers were used to amplify the neomycin phosphotransferase gene (nptII), which is used as a marker gene in mutation identification processes. The existence of nptII (target of 173 bp) was investigated in the plant material studied in the 3 plant species (coriander, fennel and sage), vegetative parts (leaves) but also the seeds considered in this work. Successful PCR amplification of the nptII gene with a randomly amplified polymorphic DNA primer and complete digestion of the isolated DNA with the restriction enzyme HindIII validated the quality of the isolated DNA.*

***Key words:*** *Salvia officinalis, genomic DNA, mutations, Coriandrum sativum, Foeniculum vulgare, genes.*

## INTRODUCTION

The efficient selection of plants modified by natural mutations as well as transgenic plants is essential in plant biotechnology and plant exploitation. For this reason, the development of efficient, specific marker systems plays a crucial role in identifying genes involved in these complex processes. The evaluation of an improved spectinomycin resistance construct to provide strong selection in transformation processes has been highlighted (Eustice et al., 1983; Jefferson et al., 1987). The new construct was compared, in several plant species, with the standard selectable spectinomycin marker gene currently used for the transformation of soybean and other medicinal, aromatic and crop plants and with the neomycin phosphotransferase (*nptII*) gene that confers resistance to kanamycin. La *Arabidopsis thaliana*, potato and citrus, the transformation efficiency provided by the improved spectinomycin marker was approximately equal to that of kanamycin and considerably better than that observed for the standard spectinomycin marker. (Miki et al. 2004, Dekeyser et al.1989). For soybeans, the improved spectinomycin resistance marker performed as well as the standard spectinomycin gene. These results demonstrate that the improved spectinomycin construct provides strong resistance comparable to kanamycin in several species, offering an alternative tool for the selection of transgenic and mutated plants (Nitovska et al., 2015; Guerche et al., 1987). Antibiotic and herbicide resistance genes are the most effective and widely used selectable markers (Miki et al., 2004). The *hygromycin phosphotransferase-II* (*hptII*) gene is the most used antibiotic selectable marker in genetic transformation and mutation detection in various plants. However, the antibiotic hygromycin that selects transgenic or mutated plants expressing the *hptII gene* is expensive in the setting of developing countries. The other gene most widely used for selection of transgenic and mutated plants is *neomycin phosphotransferase*-II (*npt II*) (Martinell et al., 2013).

The *neomycin phosphotransferase-II* gene has been used as a selection marker in many commercialized transgenic plants as well as those that have undergone natural or chemical mutations and may face fewer regulatory hurdles for future commercialization of transgenic crops. *Neomycin phosphotransferase-II* has been approved by the U.S. Food and Drug Administration (FDA) as a food additive for tomatoes, cotton, and canola

(U.S. Food and Drug Administration, FDA in 1994). The *nptII* gene encodes the enzyme aminoglycoside 3′-phosphotransferase and is inactivated by phosphorylation by several aminoglycoside antibiotics such as neomycin, kanamycin, paromomycin, ribostamycin, butyrosin and geneticin (G418). However, native rice callus has been observed to exhibit varying degrees of natural innate resistance to kanamycin (Caplan et al., 1992). Selection on G418 is more effective than kanamycin for transgenic or mutated plants expressing the *nptII* gene, probably because G418 is more toxic than kanamycin to wheat, rice and soybean cells (Twyman et al., 2022). Chan et al., 1992, used G418 for Agrobacterium-mediated transformation of various plants, however, following selection on 40 mg $l^{-1}$ of G418, a total of only four transgenic plants were produced.

Also, two aminoglycosides, G418 and paromomycin, were used for sweet potatoes to develop a reliable selection system for both mutated and transgenic plants (*Ipomoea batatas* (L.) Lam.) (Jones, 1980; Shin YM et al., 2007). For soybean plants, genetic transformation is limited by the lack of multiple efficient, specific marker systems. The most commonly used soybean transformation relies heavily on selection with hygromycin phosphotransferase II (*hptII*). Transgenic events were detected using *nptII* with enhanced G418 selection without generating contamination. Six independent, fertile transgenic plants were identified using *nptII* and G418, a frequency similar to that obtained with hygromycin selection. Soybean embryogenic cultures co-transformed with *hptII* and *nptII markers* demonstrated resistance to both hygromycin B and G418, while plant regeneration and fertility were not adversely affected. *nptII* has been useful as a second selectable marker for multiple genetic transformations in basic and applied soybean research. (Li R. et al., 2011). Also, for medicinal and aromatic plants, there is the easiness of being genetically modified both naturally through mutations and through external intervention. (Wang et al., 2011). The applications of PCR technology are very diverse, starting from those aimed at identifying, characterizing or controlled modification of genes using as a template either directly purified DNA molecules from various sources, or cDNA molecules derived from reverse transcription of the mRNA of interest. The applications have also developed in practice, especially in the horticultural field, for diagnosing harmful diseases by detecting the pathogens that cause them. (Ion et al., 2019; Church et al., 2012; Dilhari et al., 2017; Don et al., 1991; Dellaporta et al., 1983).

## MATERIALS AND METHODS

The plant material consisted of plant and seed samples from the species common sage (*Salvia officinalis* L.), coriander (*Coriandrum sativum*) and fennel (*Foeniculum vulgare),* originating from the Buzău Research Station, and the fennel samples from the Bucharest Botanical Garden.

Molecular research began with the classic genomic DNA isolation protocol following the CTAB method of Doyle and Doyle (1987) for the species, common sage (*Salvia officinalis* L.), coriander (*Coriandrum sativum*) and fennel (*Foeniculum vulgare).*

DNA concentration was determined spectrophotometrically at an absorbance of 260 nm (A260) using NanoDrop1000 (Thermo Scientific). The purity of DNA from protein and polysaccharide contamination (Reinoso et al., 2016) was assessed by reading the absorbance ratio at A260/A280 and A260/A230, respectively. The quality of the DNA extract was also assessed by electrophoretic separation for all DNA samples from all 3 species studied and also on particular parts of the plants evaluated, respectively the aerial part and seeds. For this, a 2% agarose gel was prepared, stained with ethidium bromide (1 µg/ml). Standard protocol isolation, genomic DNA according to the CTAB method of Doyle and Doyle (1987) involves the following work steps: grinding in liquid nitrogen approximately 0.5 g of young leaves/sample until a fine powder was obtained. The powder thus obtained was transferred to Eppendorf tubes, with 700 µl of extraction buffer (maintained at 6°C); after which the tubes were inverted several times. The samples were then placed on a water bath at 65°C for 25 minutes, inverting the tubes to ensure a constant temperature. After the water bath (Bain Marie), the tubes were allowed to cool to room temperature. Then 700 µl of chloroform: isoamyl alcohol (24:1) was added,

homogenization was performed by consecutively inverting the tubes 20-25 times, until a homogeneous emulsion was obtained. Centrifugation was done at 12,000 rpm for 15 minutes. The aqueous phase (above) was transferred to another sterile tube. If the aqueous phase is cloudy, it is recommended to repeat the chloroform: isoamyl alcohol extraction (steps 4-6). 0.5 volumes of NaCl were added to the solution recovered in the previous step and the contents of the tubes were homogenized. 2 volumes of 95% ethyl alcohol kept in the freezer at -20°C were added and the tubes were placed in the refrigerator at 4-6°C for 15-20 minutes, until the DNA filaments became visible in solution. Centrifugation was performed at 3,000 rpm for 3 minutes, after which the speed was increased to 12,000 rpm for another 5 minutes. The supernatant was carefully removed without detaching the DNA pellet from the walls or bottom of the Eppendorf tube. 700 µl of 76% ethyl alcohol was added and kept at 0-4°C. A new centrifugation at 10,000 rpm for 5 minutes followed, after which the supernatant was carefully removed. The opened tubes were placed in a speed-vac until the alcohol evaporated , approximately 20-30 minutes. The DNA pellet was redissolved in 50 µl of TE buffer solution. The genomic DNA thus extracted from leaf tissue collected from sage, fennel and coriander plants was used by PCR technique to amplify the 575 bp region of *the nptII fragment by using nptII* specific primer sequences:

P52- 5`- CCG GTG TCT G - 3`

P53 - 5`- TGC ATT CCG A - 3`

P54 - 5`- TGC ATT CCG A - 3`

Each PCR reaction was performed in 25 µL (total volume) of reaction mixture, comprising 100 ng of template DNA, 0.2 µL (1 unit) of Taq DNA polymerase (MBI Fermentas, UK), 2.5 µL of 10X PCR buffer (MBI Fermentas, UK), 2.5 µL of 2 mM dNTP mix (MBI Fermentas, UK), 0.5 µL of forward primer and reverse primer (10pM/µL) each, and the volume was completed using molecular grade double-distilled water. The thermal cycling conditions used comprised 3 min at 94°C for initial denaturation, followed by 35 cycles of 94°C for 30 s, 58-62°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min, finally stopping the reaction at 4°C. The PCR protocol was performed using the Mastercycler (EP Gradient Eppendorf). DNA from a transgenic plant was included in the experiments as a control. *The nptII* amplified DNA fragments were separated by electrophoresis on a 1.2% agarose gel, detected by ethidium bromide staining, visualized, and documented on the Molecular Imager Gel Doc XR system (Bio-Rad Laboratories, CA, USA).

## RESULTS AND DISCUSSIONS

The DNA yield obtained with the CTAB method of Doyle and Doyle (1987) was 450.2 ng DNA/ µl, of dried leaf tissue and 480.4 ng DNA/ µl for samples obtained from seeds for the Coriander species, and the absorption ratios of 260/280 and 260/230 nm were on average 1.78, 1.45 for plant samples and 1.98 and 1.45 for seed samples, respectively, revealing slight contaminations in the case of coriander. It is observed that the samples obtained from seeds have a higher amount of DNA than those obtained from leaves (Ion et al. 2018). For the *Fennel species,* the amount of DNA was lower, 344.2 ng DNA/ µl, of dry leaf tissue and 485.1 ng DNA/ µl for samples obtained from seeds, and the absorption ratios of 260/280 and 260/230 nm were on average 1.88, 1.85 for plant samples and 1.51 and 1.50 for seed samples, respectively, revealing slight contaminations.

Table 1. Results regarding the quantity of genomic DNA isolated from coriander, sage and fennel species using the standard protocol CTAB method of Doyle and Doyle (1987)

| Plant genotypes | Parameters | | | |
|---|---|---|---|---|
| | Quantity ng/µl dsDNA | R260/280 (DNA/protein >1.7) | R260 /230 (Leftovers alcohol <1.5) | 320 (Polysaccharides -contaminations <0.1) |
| Coriander-plant | 450.2 | 1, 78 | 1.45 | 0.100 |
| Coriander-seed | 480.4 | 1.98 | 1.45 | 0.021 |
| Fennel - plant | 344.2 | 1.88 | 1.51 | 0.053 |
| Fennel - seed | 485.1 | 1.85 | 1.50 | 0.049 |
| Salvia officinalis - plant | 389.5 | 1.92 | 0.89 | 0.076 |
| Salvia officinalis-seed | 552.2 | 1.89 | 1.35 | 0.013 |

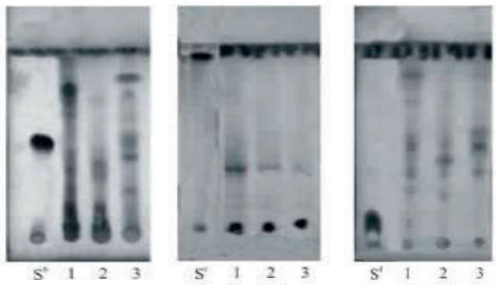
For *the Salvia officinalis species*, the amount of DNA was quite uneven with a large difference between the two types of samples (plant and signs), of 389.5 ng/ µl DNA/ µl, of dry leaf tissue and 552.2 ng DNA/ µl for the samples obtained from the signs and the absorption ratios of 260/280 and 260/230 nm were on average 1.92, 1.89 for plant samples and 0.89 and 1.35 for seed samples, respectively, achieving a difference of 162.7 ng DNA/ µl.

For all three species, it was observed that seeds are the parts of the plants richest in genetic material.

Since medicinal and aromatic plants are prone to genetic modification both naturally through mutations and through external intervention, in this context, the effectiveness of the present protocol to produce good quality DNA suitable for the detection of genetically modified or mutated crops was evaluated. PCR analyses performed on genomic DNA isolated from sage, fennel and coriander plants confirmed the presence of the *nptII* gene in the genome of fennel plants and in the presence of the P53 marker (Figure 2).

Table 2. Results regarding the structure of primers used in DNA amplification

| Primer | Primer name | Primer sequence | Molecular weight |
|---|---|---|---|
| P 52 | PC 12 | 5`- CCG GTG TCT G - 3` | 3124.1 g/mol |
| P53 | Pf 2.3 | 5`- TGC ATT CCG A - 3` | 2994.2 g/mol |
| P 54 | Pso 3.6 | 5`- CTG TAC CCC G - 3` | 3222.4 g/mol |




A - Primer P52 b- Primer P53 c- Primer P54

Figure 1. Migration of DNA fragments in coriander, fennel and sage species for Primers P52, P53, P54

Figure 2. Results of PCR product analysis

The primer sequences were

P52- 5`- CCG GTG TCT G - 3`

P53 - 5`- TGC ATT CCG A - 3`

P54 - 5`- TGC ATT CCG A - 3`

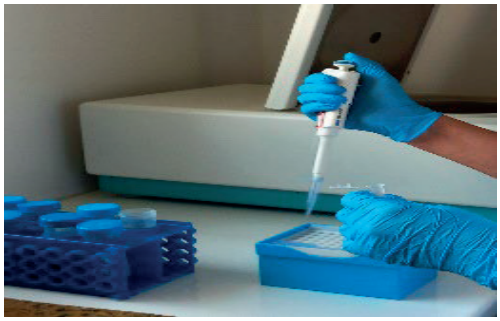


Figure 3. DNA isolation from coriander, fennel and sage

The existence of *nptII* (size 173 bp) was investigated in the plant material taken into study in the 3 plant species (coriander, fennel and sage) vegetative parts (leaves) but also the seeds considered in this work. For this, specific primers were used, following which of them are able to highlight the presence of the gene. Primers P52, P53 and P54 amplified fragments of 575 bp, 478 bp and 175 bp, proving the presence of the *nptII* gene only primer P 53 in the sampled genomes. Taken together, the PCR experiments and the genomic DNA of the presumed plants that underwent mutations confirmed the existence of the gene *nptII* in the genome of the fennel genotypes.

Figure 4 Results regarding the identification of the *nptII gene* in the three species sage, fennel and coriander

The results obtained from electrophoresis demonstrate the presence of the *nptII gene* in the fennel species with a visible band in the agarose gel. The results validate the molecular markers used and the reasoning refers to the source of the plant material procured from the botanical garden and not from a controlled culture.

Intact DNA resulted in bands very close to the edges (Figure 4, upper band), indicating high purity of the extracted DNA, without RNA contamination, especially since the recommended and most accurate way to determine RNA contamination is to run a sample on an agarose gel where another RNA band, if present, will be visible in the gel. Therefore, the higher absorbance ratios A260/A280 in our case can be attributed to slight changes in the pH of the extracted samples (Williams J.G., 1990). A polysaccharide contamination was also assessed (Table 1) by estimating the absorbance ratio A260/A230 as a secondary measure of nucleic acid purity. This ratio is important to assess the level of salt residues in the purified DNA. It is recommended to be greater than 1.5 and preferably close to 1.8. (Tamari et al., 2013; Valentini et al., 1996).

## CONCLUSIONS

The effectiveness of the protocol proves its suitability for other applications for food safety assessment by detecting naturally mutated or genetically modified (GM) crops and constitutes a valuable practice in biodiversity conservation. Isolation of quality DNA from sage, fennel and coriander plants is an essential step for satisfactory results in molecular studies, especially those involving plant genetics. For all three species, it has been observed that the seeds are the plant parts richest in genetic material.

The DNA isolation method used in this work has proven to be successful and applicable for DNA extraction with high yield and purity for the 3 different plant species *Salvia officinalis, Foeniculum vulgare and Coriandrum sativum.* The DNA extracted through the standardized protocol gave detectable results and reproducible bands for the detection of the *npt* II gene (173 bp target) for PCR amplification with the ultimate goal of identifying naturally mutated or genetically modified crops. The A260/A280 purity ratio is an important measure for estimating the levels of polyphenol contamination of the extracted DNA. The primer that succeeds in detecting the *nptII gene* is the primer P53- 5`- TGC ATT CCG A - 3`. The target fragments can be amplified by electrophoresis in 1.2% agarose gel. The present work has achieved its goal of developing a protocol for identifying plants that may undergo mutations in technological processes or organisms that have been genetically modified, especially since aromatic and medicinal plants can constitute the raw material for nutritional supplements. Continuing research in this field is important for the development of new applications and the improvement of existing techniques capable of exploring and better understanding the complexity of biological molecules.

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