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Research Article

Optimization of DNA Extraction and PCR Conditions for Genetic Diversity Study in *Artemisia Herba-Alba* from Algeria

Abstract

Background: *Artemisia herba alba* is an aromatic species very rich in secondary metabolites that can be used in traditional medicine.

The context and purpose of the study: This species suffers from the degradation of its natural habitat. The creation of collections containing diverse ecotypes is important for its preservation. This species contains essential oils which limit the extraction of DNA. The objective of this study is to optimize the DNA extraction and PCR conditions. The method will be used to evaluate the genetic diversity of *Artemisia herba alba* in the highlands of Western Algeria as a starting point.

Results and main findings: The use of different reagents and the modification of the incubation time allow obtaining white and clear DNA. Moreover, the modification of temperature and duration of hybridization results in clearer ISSR profiles. The use of the genetic markers for the analysis of genetic material ISSR, carried out on 12 individuals, indicates that *A. herba alba* is characterized by a broad genetic polymorphism.

Conclusions: This study permits the optimization of protocol of DNA extraction and amplification by PCR for *A. herba alba*. Large variability was found between the studied individuals for *A. herba alba*. The developed markers could be used for screening a large collection of individuals.

Brief summary: *Artemisia herba alba* is a popular medicine plant in North Africa. This plant shows a degradation of its natural habitat. It is necessary to constitute a collection in order to reintroduce it in the highlands of North Africa. For this purpose the genetic characterization is obviously important. Nevertheless, no information is available on this species. Moreover, its essential oils limit markedly the DNA extraction. This study aimed the optimization of DNA extraction and PCR conditions. The results showed, by using different reagents and both time and temperature of incubation, a clearer DNA and ISSR markers profiles. This study also revealed a large variability within *A. herba alba* which could help investigation on a large collection of individuals and therefore select the most efficient ecotypes for re-introduction this species in highlands of Algeria.

Introduction

Artemesia herba Alba, known as "wormwood" is a perennial small shrub with pubescent leaves. This Asteraceae species grows in desert wadi beds in North Africa and South-West of Europe [1]. As other species of *Artemesia,* wormwood is an important source of biological compounds with biocidal and allelopathic activities [2]. This aromatic species is widely used in North African's traditional medicine as expectorant, analgesic, antispasmodic, stomachic, vermifuge, diarrheic and sedative [3].

In Algeria, *A. herba-alba* covers three million hectares of the steppes and its potential as a forage resource is important. *Artemisia herba-alba* is well adapted to arid climatic conditions and grows in clay and compacted soil with low permeability [4,5]. This species is a natural way to fight against erosion and desertification [6]. However, Tarik and Arslan [7], showed that the sagebrush steppes are replaced by indicator species of vegetation degradation or give way to bare soil that has resulted in scarcity or the disappearance of the species. Therefore, it appears necessary that to take actions aiming repopulation and protection of this spontaneous species. Nevertheless, these actions require a good knowledge of the genetic diversity of A. *herba-alba.* There are no information concerning the use of molecular markers for the study of *A herba alba* genetic variability. This work was interested in the study of intraspecific genetic diversity of *A. herba-alba* by using inter simple sequence repeat (ISSR–PCR) technique.

Material and Methods

Presentation of the study area

Our study sites known as El-Manseb (Altitude 1326m) are located in the highlands of the South East of the province of Tiaret (001° 48' 40.8" E, 35° 01' 19.2" N) in the North West of Algeria. The soil in this area is shallow and rocky or stony part, with generally calcareous-lime nature. Annual rainfall reaches 330mm; the range of temperatures varies between 2.5°C (cold season) and 35°C (warm season). The dry season took places during 6 months from the May to October.

Plant material

Twelve individuals collected in the area of study were used for

the DNA extraction which was realized in 2013 at the laboratory of Plant Biotechnology, Faculty of Natural and Life Sciences, University Ibn-Khaldun, Tiaret (Algeria). The DNA extraction was performed on fresh leaves of *A. herba alba* collected, transferred to the laboratory and stored at -20°C.

DNA extraction protocol

The fresh leaf tissue (70mg) of each individual of *A. herba alba* were ground in presence of liquid nitrogen using a vibro-crusher (RETSCH - MM 4000), after the addition of 1mL of CTAB and 2µL of ß-mercaptoethanol. The crushed solution was incubated at 65°C for one hour (Termomixer COMPACT EPPENDORF). The whole was then centrifuged at 11000rpm for 10min at 4°C; the supernatant was recovered and transferred to a new tube and a volume (v/v) mixture of chloroform: isoamyl alcohol (24:1) was added to another centrifugation at 13000rpm for 10min at 4°C. This step was repeated twice. In the tube where the last supernatant is recovered, a (2/3) volume of cold isopropanol was added and mixed gently by inverting the tubes and then incubated at -20°C overnight. After centrifugation at 700rpm for 20min at 4°C, the supernatant was removed and the pellet is recovered then washed with wash buffer (75% ethanol, 15mM ammonium acetate), centrifuged at 900rpm for 10min at 4°C (the washing is repeated three times) and air dried. The DNA was then washed with 500µl of 70% ethanol and then suspended in 50µL of TE buffer PH 8. The DNA pellet was treated with 7µL of RNase by 100µL of DNA and incubated for 30min at 37°C. Finally, the DNA obtained was stored at 4°C for a short time and at (-20°C) for a long time.

PCR reaction

Six primers were tested. For this study, the used primer was that with clear bands (X14 Primer TRAAR $(GCC)_4$). PCR reactions were performed in a total volume of 50µL containing 10µL buffer PCR10X (50mM KCl, 10mM Tris-HCl, 1.5mM $MgCl_2$, pH9.0), 2µL of DNA extract (50ng/mu.L), 3µL of $MgCl_2$ (25mM), 2µL dNTPs (2mM), 5µL oligonucleotide primer (20µM), 1µL of Taq DNA polymerase (0.5U/µL) and 27 µL H_2O. The different temperature cycles for the PCR reactions were performed according to the method of Zietkiewiez et al. [8] on a thermocycler (Eppendorf-TECHNETC 5000). The polymerization program womported the following steps: (i) initial denaturation of the DNA, (ii) activation Taq polymerase at 95°C for 2min followed (iii) 30 cycles of amplification. Each cycle consists of denaturation at 94°C for 30s, annealing at 55°C for 30s elongation of 2min at 72°C. After 30cycles, a final extension at 72°C was applied for 5min.

Separation and visualization of DNA

The PCR products were analyzed by electrophoresis using a 3µL of DNA added to 2µL of the ultrapure water and 1µL of Bromophenol blue in 1% agarose (w/v TBE) gel for 1h 80volts. DNA bands were detected by ethidium bromide (0.1%) for 90 min, visualized under UV radiation and photographed with a device for taking pictures UV (DOC-PRINT VX2). The quantification of the size of the amplified DNA bands is made by the software Image J Gen with reference to the control λDNA/Hind III.

Data analysis

Similarity of qualitative data was generated using the Nei and Li/Dice similarity index [9] (Nei and Li, 1979), and were analyzed using UPGMA (unweighted pair group method using arithmetic averages). The calculated coefficients of similarity were used to product a dendrogram.

Results and Discussion

We have used the method of DNA extraction developed for *Pennisetum glaucum* [10] on species *A. herba alba* gave a DNA brown. This method is characterized by good performance but the DNA obtained is yellow, non-cleavable by enzyme restriction and not amplifiable by PCR [11]. However, our protocol which was optimized for *A. herba alba* gives a clean white DNA. This was achieved through several adjustments. The incubation was longer (one hour) and at higher temperature (65°C) of the crushed leaves compared to the basic protocol at 60°C for 30min. The use of isopropanol instead of ethanol provides good DNA precipitation at -20°C overnight. Washing performed with a buffer composed by 75% ethanol and 15mM ammonium acetate instead of 75% ethanol, 3M sodium acetate and TE [12]. The centrifugation at 4°C promoted good phase separation during the various stages of extraction [13].

Optimizing conditions for the PCR amplification of ISSR allowed obtaining clear and reproducible profiles. The differences with method of Zidani [9], are very marked: (i) the concentration of DNA used was less important, (ii) the number of PCR cycle (30 cycles) and the temperature of hybridization 55°C for 30s instead of 60°C for 1min allowed to more clearer profiles (Figure 1).

The revealed bands fall very different ways among the studied individuals. A very large genetic variability was evidenced by 37 amplified bands of different sizes (Figure 1).

Amplification product size ranged from 709 to 17353bp; thirteen bands among them presented sizes greater than 10000bp, and twenty-nine bands varied between 1000 and 10000bp. One band showed a size less than 1000bp. Among analyzed individuals, 29 bands (78.4%) of the ISSR bands were polymorphic.

The dendrogram obtained using UPGMA cluster analysis Figure 2 showed that individuals diverge in seven different groups. Four of them presented a single individual. Two other groups include two individuals each having the same number of bands. Finally one group stands out and is composed by four individuals. The distribution of 12 individuals in different groups indicates that this population is characterized by a high intraspecific genetic variability which is probably due to spontaneous mutations affecting this species. Nevertheless, this study constitutes a first step to investigate diversity on larger collection of this species.

Conclusion

Our study permits the optimization of DNA extraction and PCR conditions for *A. herba alba*. The modification of the reagent for DNA precipitation as well as the temperature of incubation allowed obtaining white DNA. The modification of the temperature of hybridization and its duration permits to obtain clearer profiles.

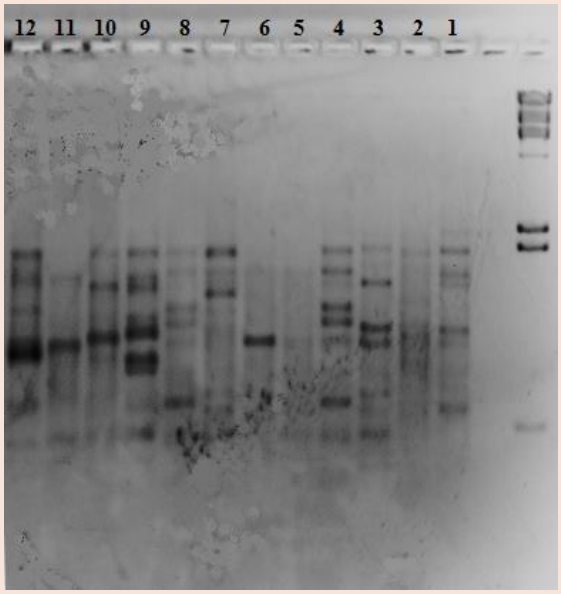

Figure 1: DNA profiles generated by ISSR primer X14 TRAAR (GCC) 4 in 1% agarose gel of twelve individuals of Artemisia herba alba Asso of Tiaret region (May 2013).

M: 20kb DNA ladder; R= A+G.

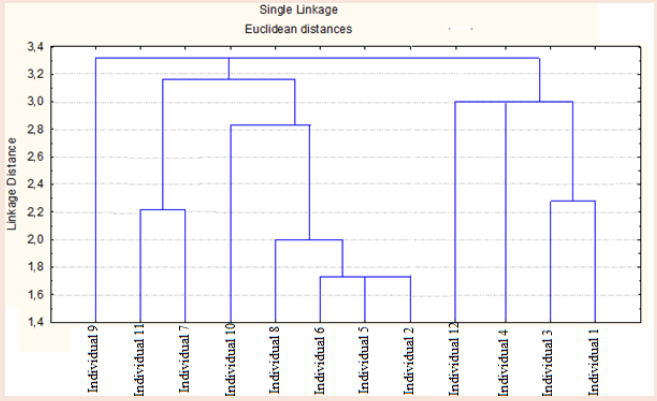

Figure 2: UPGMA cluster analysis of twelve Artemisia herba alba Asso individuals harvested at Tiaret (Algeria) with ISSR-PCR markers was established using Nei and Li/Dice coefficient.

The DNA extracted from twelve individuals harvested at the region of Tiaret (Algeria) was used for ISSR-PCR amplification to determine the genetic polymorphism of *A. herba alba*. In this study we have demonstrated the reliability of ISSR markers to detect DNA polymorphisms within the collection of *A. herba alba* in Tiaret. The ISSR markers showed multiband patterns in each individual. This primer amplified a total of 37 reliable bands from the DNA of 12 genotypes tested.

The DNA extraction protocol developed in this study can be used for other species with high production of secondary metabolites and essential oils.

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