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Study of Genetic Diversity of Berseem Accessions (Trifolium Alexandrinum L. SPP) With RAPD Markers

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***Abstract* – This study aimed to screening genetic diversity and ancestral relationship between Tunisian berseem accessions. The analysis of the genetic diversity of 29 accessions of berseem kept and recorded in the fodder index of Tunisian National Institute of Agronomy has been conducted via RAPD markers. Several prompts have been tested, but 5 only were selected for their reproductive potential and their high polymorphism rates. A total of 388 reproducible bands were obtained during PCR-based amplifications. The bands have a size ranging between 200 and 2000 pb; though, most of the bands are in the range of 200 to 600 pb. The correlations between the accessions have been evaluated by means of analyses according to the UPGMA method. The extent of dissimilarity between the accessions varies from 16 to 100%, which represents a high polymorphism level. The dendrogram, worked out via the Treecon software, reveals seven sets of accessions at the 60% threshold. *T. berytheum*, ancestor of berseem, clearly emerged from the other accessions. It is of interest for intensive crop-growing systems and may be used as parent in selection programs. *T. meironense* is genetically very close to the *T. alexandrinum var serotinum*. Three sets are generated by *T. alexandrinum* and *T. berytheum* crosses. The present study showed a high genetic diversity of studied berseem accession. Showed results can be used as a pathway for preservation and productivity improvement under different agricultural systems.**

Keywords – Trifolium Alexandrinum, Berseem, Genetic Diversity, T. Berytheum.

I. INTRODUCTION

Zohary and Heller, 1984; Lange and Schifino-Wittmann, 2000; Le Floc'h *et al*., 2010 reported that Tunisia is characterized by a great genetic diversity of the fodder and pastoral legumes of the genus *Trifolium*, having 32 species among a total of 237. According to Morris & Greene, 2001The berseem (*Trifolium alexandrinum* L.) is one of the most important crops in the Mediterranean agricultural system. The geographical area of the berseem is the Mediterranean Basin, the ranging from the Middle East to Irak and Central Asia, like Pakistan and India (Knight, 1985; Sardana and Narwal, 2000; Badr *et al*., 2008).

The intensive use of a limited number of improved varieties of berseem reduces the genetic base of this species and contributes to genetic vulnerability of its biodiversity. The *ex-situ* conservation and the evaluation of genetic resources of berseem has begun since the 1980s in the Tunisian National Institute of Agronomy (INAT). The morphological identification of the genetic variability in this collection through morphological traits poses a major issue due to possible inter-crossings amongst species close to berseem and belonging to the subsection Alexandrina (Putiyevsky *et al*., 1975). The RAPD (Random Amplified Polymorphic DNA) had been used by Bullita (1995) to characterize varieties and populations of *Trifolium*. It demonstrated an efficiency to reveal the genetic polymorphisms between species and populations within the species. This method can be used for the improvement of berseem and for the management of its genetic resources.

In this work we analyze and try to determine 29 entries of the genetic diversity of Egyptian clover available in the collection of the INAT by the RAPD method using primers which already gave successful results for the other species of clover (Bullita, 1995).

II. MATERIAL & METHODS

In this study, twenty nine entries of berseem are studied including a commercial cultivar Miscawi, two local cultivars, two naturalized cultivars and the rest are natural populations obtained by exchange with diverse research institutions, recorded in the fodder index of INAT. The Origin, the lot number, the name of the genotype and the code are summarized in table 1.

Table 1. Biologic material, number and origin

Species	Number	Origin
T. alexandrinum L.	**02**	TUNISIA-(Local)
T. alexandrinum L.	**01**	USA
T. alexandrinum L.	**02**	EGYPT
T. alexandrinum L.	**24**	INAT

These entries of berseem have been cultivated in 2007/08 in the experimental station of the Agricultural College of Mograne; situated at a height of 156 m, a longitude of 10°04' and a latitude of 36°26', in a homogeneous clay-limestone soil. The sowing has been done in the same date, and the morphological and agronomic parameters have also been determined (height of plants, number of stems by plant, date of bloom, color of flowers, number of cutting, yield dry matter for each cut,…).

Extraction and Quantification of the DNA

To extract and purify the total DNA from berseem leaves, we adopted the protocol described by Ben Naceur (1998) which is a combination of three methods of extractions (Murray and Thomson, 1980; Saghai-Maroof *et al*., 1984; Webb and Knapp, 1990) bringing CTAB.

A representative sample of 1g of leaves from every entry has been taken from 10 plants of berseem and crushed in the mortar, in the presence of liquid nitrogen, until obtaining of a very fine powder. Each mix has been transferred to a 10 ml tube, adding 7.2 ml of extraction

tampon (3% CTAB, 20 mM EDTA; 1,44 mM Tris HCl, 1% β-mercaptoethanol, at pH 8). All the tubes are incubated at 65°C for 60min with a periodic agitation every 5 minutes. After a 5 minute-break, 4ml of chloroform: isoamylalcool (24:1 v /v) were added. The mixture was manually shaken during 15 mins to maintain the emulsion and finished with 30 minutes centrifugation in 4500 rpm in a temperature of 4°C.

After centrifugation, the aqueous phase was collected and nucleic acid was precipitated with and equal volume of isopropanol, then, the second centrifugation was realized in the same conditions during 20 minutes. DNA collected, was washed with 2-3 ml of ethanol (70%).

In the term of this wash (centrifugation), Supernatant was eliminated; tubes are air-dried for one hour. DNA was afterwards, dissolves in 400 µl of re-suspension (Tris HCl 10 mM with pH 8, EDTA 1 mM with pH 8). Finally, we added RNase (10 µg/ml) in each tube and incubated at 37°C during 60 minutes or more to eliminate RNA in the solution. Samples are used, afterwards, or stored at -20°C.

The DNA was quantified by agarose gel electrophoresis (0.8%) and colored with the bromide of ethidium (BET), in comparison with the fluorescence of another DNA of known concentration. We opted for this method for its simplicity, despite, the fact that the RAPD technique doesn't depend on the amount of DNA.

RAPD Method Application

The application of RAPD on 29 entries of berseem is realized by testing using 20 universal kits belonging to Operon Technology, USA (OpA, OpC, OpD, OpE, OpG, OpH) (Bullita, 1995). Oligonucleotids of 10 bases with more than 50% C-G. All the kits were tested on the studied entries. Then, five reproducible and polymorphic primers are used for the study of the molecular polymorphism (Table 2). Reaction mixtures contained in PCR tube are placed into a thermocycler (Biometra Uno II), then programmed to make a pre-denaturating, at 94 °C during 3 minutes, followed by 35 cycles each containing a step of denaturating, at 94 °C for 1 minute, a step of hybridization at 36 °C for 1 minute, and a stage of elongation at 72 °C for 1 minute. The last step of the amplification reaction consists in a post elongation during 3 minutes at 72°C.

Table 2. List of primers and nucleotide sequence

Primers	Nucleotide sequences (5'-3')
OPC06	GAACGGACTC
OPG13	CTCTCCGCCA
OPC07	GTCCCGACGA
OPA06	GGTCCCTGAC
OPG03	GAGCCCTCCA

DNA Electrophoresis

Agar gel electrophoresis (2%) was used to determine the presence of polymorphism. 10µL of each sample added to 1µL of blue/orange 6X Loading Dye were introduced in each hole. Marker used is 5 µL of DNA Ladder (100pb: Promega) and 1µL of blue. The migration was conducted in 80 V during approximately 3 hours. Observation under UV allowed the distinction between several bands, which were either heavier or lighter. The presence of these bands varies between samples.

Statistical and Numeric Analysis of Results

Results of RAPD amplification can be shown on the gel's pictures. Only bands clearly visible were considered. Profiles were transformed into a binary matrix (presence: 1; absence 0); matrix' lines showed studied accessions and columns showed generated markers, considering uniform lengths as the same fragment. Matrix data was analyzed by "treecon for windows version 1.3b program according to the method UPGMA (Unweighted Pair-Group Method using arithmetic Average) (Nei, 1987).

III. Results and Discussion

Polymorphisms of RAPD Generated Bands

The number of produced bands with each primer varies from 2 to 15. A total of 338 reproducible bands were obtained using the 5 chosen primers. Band sizes are included between 200 and 2000 pb. The majority of bands are situated between 200 and 600 pb. The average of the reproducible bands is 3.47 bands per primer and per genotype.

The phylogenetic constructions based on UPGMA method showed dissimilarity coefficients, varying from 16.7% (for AK107 and AK167) to 100% (for AK121) with an average of 69.1% (Table III). This interval confirms a clear genetic distinction between the studied plants and corroborate with the dispatching into several different groups. Accessions were divided into seven groups according to the dissimilarity threshold of 60% which we call: A, B, C, D, E, F and G. (Plate 1).

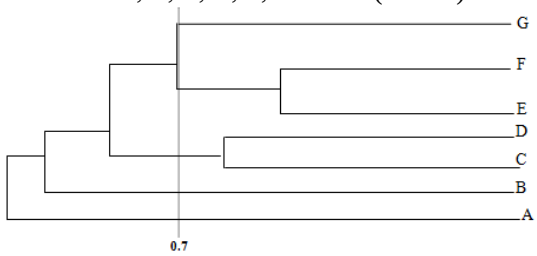

Fig. 1. Dendrogram of berseem accessions

The number of bands obtained and the molecular weight are similar to those obtained by Bullita (1995) in similar conditions. The RAPD characterization permitted the genotypic distinction between 29 berseem accessions from the INAT collection. This characterization can be verified with the morphological, biological and agronomic characters obtained. These observations accordingly match with the thesis of Putiyevsky *et al.*, (1975).

AK121 is the only one accession which gives a single cut and for which the regrowth is absent. It has a strong orthotropic stem apically branching. The average number of ramifications at the base is 5. Leaflets are longer than those in other accessions with the main longer of 5.2 cm. These observations assure that this entry belongs to the botanical group 'Fahli' (Putiyevsky *et al.*, 1975) and the species *T. alexandrinum* var. *berytheum* (Oppenheimer, 1959). It's also named *T. alexandrinum* L. Var., *alexandrinum* Boiss (Zohary et Heller, 1984) or Beirut

clover. It represents the primitive form of berseem and constitutes the main genetic resource from which Man domesticated berseem through a natural selection in Syria and Palestine (Badr *et al*., 2008). This accession can be used for intensive crop system because of its high yield in a single cut.

The accession AK120 can be harvested two to three times a year. It has basal and apical ramification with a high yield in the third cut in April. This accession belongs to the botanical group Saidi (Badr *et al.*, 2008). AK 106 has wide cotyledonary leaves and can be harvested more than six times per year. The link between AK106 and AK120 can be the result of same DNA sequences for used primers. AK14 can be harvested only twice, AK169 three times while AK142 gives more than four cuts.

The accessions that can be harvested two and four times (Groups B, C and D) are the hybrids issued from the cross between *T. alexandrinum* L. and *T. berytheum* Boiss. This corroborates with the results obtained by Oppenheimer (1959) and Bakheit, (1996). The genes responsible for the number of cuts pass from *T. alexandrinum* L. to *T. berytheum* Boiss while the genes responsible for the precocity pass from *T. berytheum* Boiss. to *T. alexandrinum* L (Bakheit, 1996).

The groups E, F and G give several cuts in irrigated systems due to the most important agronomic character which is the structure in crown. Erected stems are grown from buds situated between 1 and 2 cms of the ground at the level of cotyledonary leaves and the first trifoliate leaves (Graves *et al.*, 1989). Stems are scrolled. These accessions belong to botanical group Miscawi and *T. alexandrinum* L. Var. *serotinum* Zoh. & Lern. (Oppenheimer, 1959; Putiyevsky *et al*., 1975; Badr *et al.*, 2008). They also prove that berseem is monophyletic species (Badr *et al*., 2008)..

The local cultivar El Alem AK176 from group E is better adjusted to the Tunisian conditions than the commercial cultivar Miskawi (AK 175) from group F because it presents a higher dry matter and a larger flexibility. These cultivars can be harvested seven to eight times a year if the sowing is made at the beginning of September.

The accession AK164 presents small leaves and inflorescences which are smaller and more cylindrical than *T. alexandrinum* L. Var. *serotinum* Zoh. & Lern. and *T. berytheum* Boiss. It belongs to *T. meironense* Zoh. & Lerner. (Putiyevsky *et al*., 1975). The low dissimilatrity (27.3%) between AK 164 and AK175 shows the genetic similarity between these two species. This ensures the thesis of Putiyevsky *et al*., (1975) et Badr *et al*., (2008).

AK22 & AK 147 from G1 group are characterized by a high growth if sown in winter or early spring. Ramification is typically basal and they have secondary and tertiary ramifications which generate many apical inflorescences per plant and consequently a high seed production potential. This result is in accordance with that obtained by Morris (2002) who made a success in the production of sufficient seeds of berseem targeted to the regeneration of seeds in the gene bank.

G2 group includes the accessions (AK05, AK67, AK167, AK111 and AK136) with the highest dry matter production. AK05 is native of Egypt and naturalized in the USA; it gives further several cuts to its crossing with the botanical group Miscawi (Rethwisch *et al.*, 2002; Ranjbar, 2007).

IV. Conclusion

The present study showed a high genetic diversity of studied berseem accession. Showed results can be used as a pathway for preservation and productivity improvement under different agricultural systems.

V. References

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