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Evaluation of Genetic Diversity in Jordanian *Solanum nigrum* Plants and Genetic Stability of *invitro* Grown Plant using (AFLP) Technique

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Abstract

Solanum nigrum is an herbal plant that is reported for its healing powers against many ailments. In Jordan *S. nigrum* grows wild as a toxic weed in neglected fields and without any consideration of its medicinal value. This research aimed to collect and examine the genetic relationships of *S. nigrum* from different sites in Jordan. The *in vitro-grown S. nigrum* was established from the collected wild populations. Results of wild populations of *S. nigrum.* were arranged in two clusters (A & B) at the top range of hierarchy (genetic similarity of 0.13). Cluster A separated Bergesh Reserve population into its own group, while cluster B contained the remaining populations which were divided into two sub-clusters at a genetic similarity of 0.65, B1 contained Deer Alla and Jerash (2014 + 2015) populations at genetic similarity of 0.69 and B2 contained Almojeb Valley and Salt populations at genetic similarity of 0.71. However, the highest genetic similarity (0.87) was obtained between Jerash 2014 and Jerash 2015 from the same location. This dissimilarity might be due to the probability of cross-pollination AFLP (Amplified Fragment Length Polymorphism) molecular analysis gave no genetic variations between *in vitro* grown *S. nigrum* before and after micropropagation compared with wild- type mother plants collected from the Jerash site in 2014. A solid conclusion needs more research on the genetic diversity of Jordanian wild-grown *S. nigrum*. This can be achieved by extending the collection scale to include all sites where this valuable plant grows.

Keywords: AFLP, Callus, Cell suspension culture, Genetic diversity, Genetic stability, *In vitro*, microshoots, *Solanum nigrum.*

1. Introduction

Plant biodiversity is vital for all living as they comprise the main source of food besides being the prominent source of medicine (Ochoa-Villarreal et al., 2016; Shibli et al., 2018; Tahtamouni et al., 2021). Nowadays, folk medicine is getting a universal attention, especially in developing countries, as it represents an affordable approach for their medications (Aware et al., 2022; El-Saadony et al., 2023).Secondary metabolites extracted from plants have a long history as row resources of remedies for folk and modern systems of medicine (Pedrosa et al., 2023; Al-Qura'n, 2011, Wang et al., 2022).

Jordan is reputable for its huge plant biodiversity due to the nature of its multifarious geography and ecology (Oran, 2014). Unfortunately, Jordanian plant biodiversity has been exposed to extensive drops as a result of uncontrolled collection, urbanization, and climate change (Al-Eisawi et al., 2000; Oran 2014, Shibli et al., 2016). Many threatened Jordanian plant species have remarkable curative values, but are sadly still neglected and left without any collection, characterization, or documentation.

Solanum nigrum is one of the Solanaceae family members and is known as the black nightshade (Mandal et al., 2023). *S. nigrum* is considered as a weed and can be found in neglected areas and waste lands (Al- Kiyyam et al., 2019). According to Jordan's red list (Taifour, 2016), *S. nigrum* grows wild in different parts of Jordan in the north, such as Jerash and Ajloun, until reaching the south parts such as Aqaba and Petra. Till now, there is no adequate data about its population in Jordan.

Like most members of the Solanaceae family, *S. nigrum* was reported to contain many medically important compounds like glycoalkaloids (solasodine and solanine), glycoproteins, polysaccharides, polyphenolic compounds alkaloids and flavonoids (Churiyah et al., 2020.). Consequently, this plant was prescribed against bacteria, fungi, cancer and cytotoxic activities, and prescribed to heal cardiac ailments, skin cancer, and kidney diseases (Mahajan and Shaikh 2023).

We investigate the genetic relationships and diversity in this study for *S. nigrum* samples collected from five locations throughout Jordan using (AFLP) technique. Furthermore, we conduct an assessment of the genetic

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stability of the samples taken from selected treatments of tissue culture experiments (micro shoots, callus, and cell suspension), and compare them with the mother wild plant that was used for in vitro propagation part (taken from the Jerash site in 2014). The overall goal of this research is to provide comprehensive conservation strategies for *S. nigrum* by assessing the genetic diversity of *S. nigrum* populations in five locations and then validating the genome stability for the tissue culture plant samples (a conservation method) and comparing them with wild mother plants.

2. Materials and Methods

2.1. Genetic diversity and stability of S. nigrum

Seeds from wild grown plant samples were collected randomly from different five locations throughout Jordan (Table 1), planted in greenhouse- School of Agriculture at Jordan University, and then we investigated the genetic diversity or these samples using (AFLP) technique. The samples were taken from germinated seeds that were taken from each location. About one hundred seeds were collected from different plants in each location and were used for germination in the greenhouse. About ten samples of these germinated plants were taken from each location and were assessed using AFLP.

Also, mature seeds were collected from a mother plant grown at Jerash - Jordan (N: 32.27372, S: 35.89464) and *in vitro* propagated plants were established from mother plants that are grown in Jerash- Jordan location according to Al-Kiyyam et al. (2019). The microshoots were developed in MS media (Murashige and Skoog, 1962) plus 1.2 mg.L^{-1}Thidiazuron (TDZ), while the callus and cell suspension were established on MS media plus2.0 mg.$L^{-1}$of 2,4-Dichlorophenoxyacetic acid (2,4- D) and1.5 mg.L^{-1}TDZ. Then, the samples from the three types of plant material were tested for their genetic stability by comparing their results to data taken from their mother plant collected from Jerash using (AFLP) technique. All chemicals in this part were purchased from sigma Aldrich company.

In vitro propagation system was only applied to the Jerash population. The in vitro propagation protocol was mentioned in detail previously in (Al-Kiyyam et al., 2019). The establishment of the in vitro material was from seeds collected from the Jerash population. After seed germination; micro shoots were established from in vitro germinated seeds and further callus and cell suspension cultures were produced from microshoots according to (Al-Kiyyam et al., 2019); and each culture was maintained in the determined media for four weeks.

Table (1): Coordination of the locations of collected *S. nigrum* in Jordan

No.	Govornorate	Population	Coordinate		
			Altitude (m)	Latitude (N)	Longitude (E)
1	Jerash	Jerash	505	32.27372	35.89464
2	Irbid	Bergesh Reserve	865	32.41532	35.75775
3	Madaba	Almojeb Valley	400	31.44506	35.81405
4	AL- Balqaa'	Salt	962	32.05115	35.71655
5	AL-Balqaa'	Deer Alla	-210	32.12677	35.60668

2.2. The extractionof DNA

The extraction of DNA was done using Cetyltrimethyl ammonium bromide (CTAB) from collected plants according to Saghai Maroof et al.(1994) protocol with slight modifications. Leaves from each collected sample were merged and mashed in LN (Liquid Nitrogen). About 200 mg of young plant tissues was added to 2 ml tube has600 µL of CTAB (2% CTAB, 3 M sodium chloride,20 mM EDTA at pH: 8.0, 100 mM Tris at pH: 8.0 with 0.2% Beta-Mercaptoethanol (BME)). The tested plant samples were put under 65 °C for half of hour and were mixed every 10 minutes. Next, 800 µL Chloroform isoamylalcohol was applied at the concentration 24:1. After that, samples were put in centrifuge at the maximum speed (10 min at 13800 rpm). About 600µLof the aqueous phase was removed, the liquid phase was transferred into new tubes, and1µL RNase was put in fora quarter hour at 37°C. The precipitation method was applied to nucleic acids using 700 µL of pre-chilled isopropanol, and then the centrifugation was done with 13000 rpm for up to ten minutes. Ethanol of 70% concentration was used for DNA washing and samples were then air dried for 15 minutes. The DNA was suspended again in 75 µl 0.1X TE and left at -20°C. Stock solution concentration is 10 ng.$\mu L^{-1}$and 3 ng were added to each sample. The quality DNA was tested by 1% agarose using gel electrophoresis (Intron, Bio-tek, Korea). Genomic DNA was then inspected using a spectrophotometer (BIO-RAD, Smart spec Tm plus USA).

2.3. AFLP analysis:

The digestion of DNA was done using 30 ng of DNA, adaptor ligation, and amplification. About ten plants from germinated seeds were used for each location to conduct the AFLP analysis. The genetic diversity was applied only to five locations and not to the in vitro grown plants. In-vitro grown plants were only assessed for their genetic stability in comparison to their mother plants (Jerash location) using AFLP technique.

To determine the genetic variation between the samples, 8 primers were used (Table 2). AFLP procedure was applied according to Vos et al. (1995) with few alterations. AFLP protocol for digestion of DNA was done with*Eco*RI and *Mse*I enzymes, while the *Eco* RI and *Mse* I adaptors were used for ligation, pre-amplification with E-A and M-C primers, and selective AFLP amplification using labeled E-AAC IRDye 700 and E-ACT IRDye 800 primers and unlabeled M-CTA, M-CTT, M-CTG and M-CAG primers (Table 2).

Table 2. Adapters and primers used for AFLP analysis

Primer/Adapter	The Sequence (3'-5')
Eco RI adapter (top strand)	CTCGTAGACTGCGTACC
(bottom strand)	AATTGGTACGCAGTC
Mse I adapter (top strand)	GACGATGAGTCCTGAG
(bottom strand)	TACTCAGGACTCAT
E-A	GACTGCGTACCAATTCA
M-C	GATGAGTCCTGAGTAAC
E-AAC	GACTGCGTACCAATTCAAC
E-AGC	GACTGCGTACCAATTCACT
M-CTA	GATGAGTCCTGAGTAACTA
M-CTT	GATGAGTCCTGAGTAACTT
M-CTG	GATGAGTCCTGAGTAACTG
M-CAG	GATGAGTCCTGAGTAACAG

Selective amplification was conducted using the program of touchdown at 65 ºC with13 cycles. Other 23 cycles were performed at 94 ºC for 30 seconds. LI-COR Bioscience 4300 DNA Analyzer was used to perform AFLP markers

The genetic diversity was assessed using AFLP bands which were scored at 1 for presence, and 0 means for absence. Data were analyzed using (NTSys- PC) program (version 2.02) according to (Dice, 1945; Rohlf, 2000). After that, the cluster was analyzed using (UPGMA) the unweighted pair group method with arithmetic average.

3. Results and Discussions

3.1. Genetic diversity analysis

Genetic variation among *S. nigrum* plants collected from the five locations (Jerash 2014 and 2015, Almojeb Valley, Deer Alla, Barges Reserve and Salt) was analyzed using AFLP technique (Fig. 1), and generated polymorphic bands and their number from eight primer combinations were recorded (Table 3).The polymorphic bands are marked with arrows as shown in Fig. 1. Genetic diversity was evaluated previously for *Solanum elaeagnifolium* using AFLP and SSR which showed high genetic diversity in collected *Solanum elaeagnifolium* samples (Qasem et al., 2019). Qasem et al., (2019) found that a sixth of AFLP bands used were polymorphic with (PIC) of 38.0% in *Solanum elaeagnifolium.* Besides that, the genetic relationship was analyzed for *S. nigrum* populations in Kenya by SSR method (Mafuta et al., 2023). Furthermore, another method of (SNP) Markers was used also in *S. nigrum* to evaluate population structure and genetic diversity in China (Li et al., 2023).

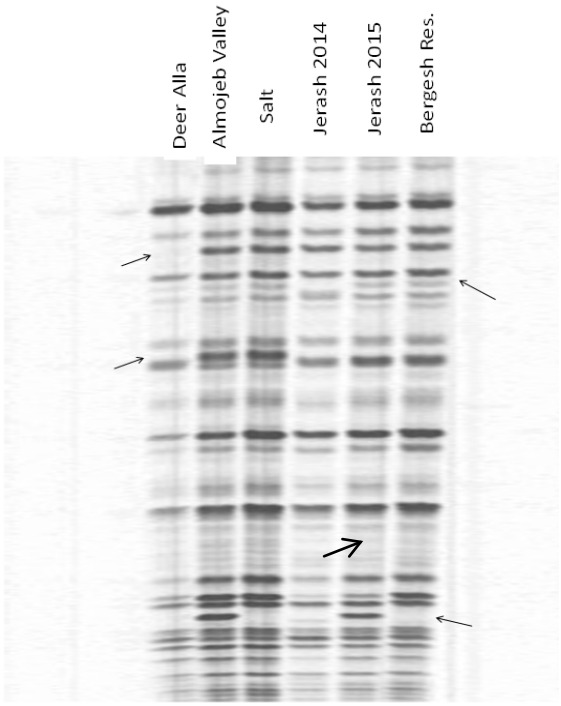

Figure 1 Amplified DNA patterns using AFLP primers for *S. nigrum* gathered from different sites. The primer combinations are (M-CTG with E-AGC), and arrows indicate the polymorphic bands.

Table 3. Number of generated polymorphic bands from eight primer combinations in *Solanum nigrum*.

Primer combinations	Selective nucleotides		Number polymorphic of bands
	*Mse*l	*Eco*RI	
1	CTA	AAC	5
2	CTA	AGC	4
3	CTT	AAC	7
4	CTT	AGC	5
5	CTG	AAC	5
6	CTG	AGC	7
7	CAG	AAC	3
8	CAG	AGC	1
Total			37

A genetic similarity matrix was constructed to investigate genetic diversity among the populations. Results showed that the genetic distances (1 minus Dice coefficient) between the studied populations were distributed as close as 0.13 to those genetically different as 0.63.In contrast, the overall mean of genetic dissimilarity was 0.585 (Table 4). The distance between the DNA of different species was measured and a good diversity was found between while the relationship between them was close (Jacoby, 2003).*S. nigrum* from Kenya and Germany was characterized using AFLP and the distance between the markers were correlated morphological characters (Matasyoh et al., 2015).

Table 4: Dice (1945) coefficient of genetic similarity matrix among *S. nigrum* wild populations using eight AFLP primers.

	Almojeb Valley	Salt	Jerash 2014	Jerash 2015	Deer Alla	Bergesh Reserve
Almojeb Valley	1					
Salt	0.7108511	1				
Jerash 2014	0.7058824	0.7179487	1			
Jerash 2015	0.6868421	0.6511628	0.8789474	1		
Deer Alla	0.6500000	0.6818182	0.6945946	0.6780488	1	
Bergesh Reserve	0.4000000	0.5263158	0.3703704	0.4516129	0.4375000	1
Means	0.6307152	0.6443113	0.6479708	0.5648305	0.4375	
Over all means	0.585					

From the dendrogram (Fig. 2) of the six *S. nigrum* plants were two clusters in two main groups (cluster A and cluster B) (Fig. 1) at the maximum level of hierarchy (genetic similarity of 0.13) (Fig. 1 and 2). Cluster A separated Bergesh Reserve population into its own group, while cluster B contained the remaining populations which were divided into 2 clusters with a 0.65 similarity index; B1 contained Deer Alla and Jerash (2014 + 2015) populations at genetic similarity of 0.69 and B2 contained Almojeb Valley and Salt populations at genetic similarity of 0.71. However, the similarity of (0.87) was the highest obtained between Jerash 2014 and Jerash 2015 which were collected from the same location, but in different years, and this dissimilarity might be due to the probability of cross-pollination occurrence (citation). Genetic dissimilarity (0.585) between the six collected samples increases the possibility of cross-pollination occurrence as a causal agent behind these genetic dissimilarities, taking into account that *S. nigrum* is a self-pollinated plant with little chance of cross-pollination occurrence. Similarly, Kapesa et al. (2021) found that the molecular analysis of *Solanum nigrum* collected from Congo was separated into two distinct groups. Additionally, Mafuta et al., (2022) found that the genetic diversity of *Solanum nigrum* from Western Kenya gave a dendrogram that was grouped into three clusters. These clusters gave high variety which was attributed to cross ability between accessions (Mafuta et al., 2022).

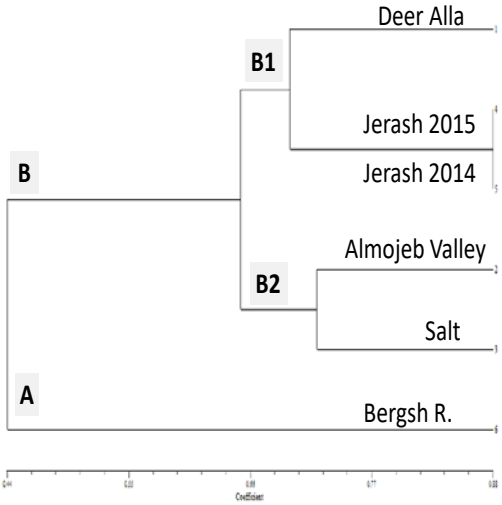

Figure 2. Unweighted pairwise group method with UPGMA (arithmetic mean)- clustering 6 wild *S. nigrum* plants, according to AFLP markers.

3.2. Effect of in vitro propagation on genetic stability of S. nigrum L. explants (microshoots, callus, and cell suspension)

The results obtained after AFLP molecular analysis indicated that there were no genetic variations between *S. nigrum* (microshoots) before and after micropropagation in comparison to the mother plant (Fig.3 and 4). These results were expected due to the fact that microshoots are differentiated tissues that are known (like any other differentiated tissue) for their ability to remain true type even after being undergone tissue culturing systems (Tikendra et al., 2019; Zhang et al., 2023; Biswas et al., 2023). Luo *et al.* (2023) confirmed the genetic stability in their study on *Manglietiastrum sinicum* when RAPD or ISSR markers were used after *in vitro* propagation. Our results are similar to Parzymies *et al.* (2023) study on the effects of micropropagation on the genetic stability of *Salix myrtilloides*, as they found no differences between microshoots and mother plants.

Although plant genetic stability was put under validation in cases where undifferentiated explants like cell suspension or callus were used as plant material due to some tissue culture protocols and in vitro preservation (Tahtamouni et al., 2017; Qahtan et al., 2022), our results revealed that genetic makeup was unaltered in callus and cell suspension cultures (Fig. 3 and 4). However, genetic alterations in tissue-cultured plant materials reported in some studies were rare and referred to personal error rather than the tissue culture technique itself and described to be epigenetic as the original DNA was not altered (Smulders and Klerk, 2011;Biswas et al., 2023).The tissue culture of plants could be affected by somaclonal variation and other factors like the type of plant hormones used, the time of in vitro propagation, direct- versus indirect regeneration, etc (Biswas et al., 2023; Bulbarela et al., 2023). However, most reports found a low percentage of soma clonal variation occurrences in tissue-cultured plants (Taji et al., 2002; Duta-Cornescu et al., 2023). Finally, this is a primary study on tissue-cultured plants because the major aim was genetic diversity as a tool of conservation and the other aim was to show if there is any genetic variation after tissue culture. Soma clonal variation analysis needs deeper studies and may be used as recommendations for further studies of the genetic stability of the tissue cultured samples.

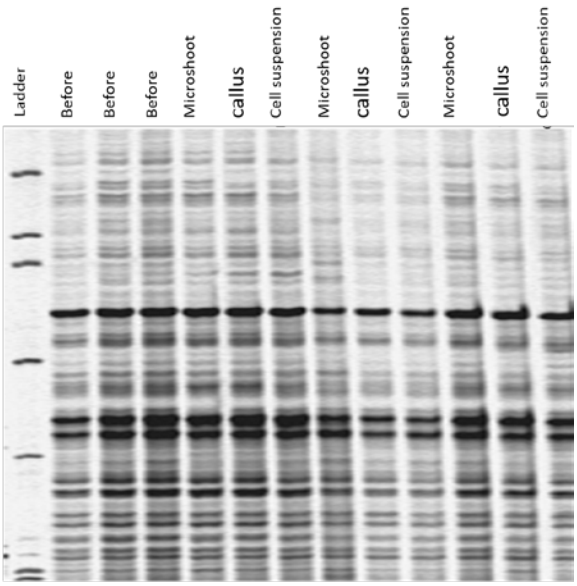

Figure3. DNA banding patterns using AFLP for *S. nigrum.* Pre-amplified DNA products were selectively amplified using four *Eco*RI+*MseI* primer combinations (M-CTA IR Dye 700 with E-AAC and E-AGC). The panel labeling includes three replicates for each label (before: refers to the plant before tissue culture, micro shoot, callus, and cell suspension).

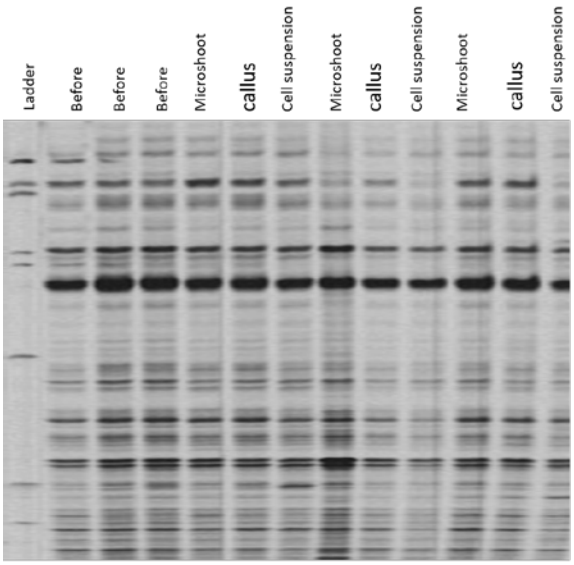

Figure 4. Bands of DNA via AFLP technique for *S. nigrum*, using four *Eco*RI+*MseI* primer combinations (M-CTT IR Dye 800 with E-AAC and E-AGC). The panel labeling includes three replicates for each label (before: refers to the plant before tissue culture, micro shoot, callus, and cell suspension).

4. Conclusion

The results obtained after AFLP molecular analysis indicated that six *S.nigrum* wild populations separated and prated in two clusters (A & B) at the highest level of hierarchy (genetic similarity of 0.13). Cluster A separated into Bergesh Reserve population into its own group, while Cluster B contained the remaining populations which were divided into 2 clusters with 0.65 similarities, B1 contained Deer Alla and Jerash (2014 + 2015) populations at the genetic similarity of 0.69 and B2 contained Almojeb Valley and Salt populations at the genetic similarity of 0.71.

Also, AFLP molecular analysis indicated that there were no genetic variations between *S. nigrum* (microshoots) before and after micropropagation in comparison to the mother plant (taken from the Jerash sitesite in 2014), which proved the ability of *S. nigrum* to be micropropagated without any change in genetic makeup.Conducting more research is necessary to make a solid conclusion about the genetic diversity of *S. nigrum* which grows wild in Jordan.This can be achieved by extending the collection scale to include all sites where this valuable plant grows.

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