

MICROPROPAGATION AND ASSESSMENT OF GENETIC FIDELITY OF REGENERATE BY RAPD MARKERS OF *SOLANUM NIGRUM*

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ABSTRACT

In vitro propagation approach has been adopted for micropropagation and conservation of *Solanum nigrum* (black nightshade) to guarantee its sustainable phyto-industry and research availability. Within 1 month, *in vitro* shoot differentiated was accomplished by culturing shoot tip, nodal segment, and leaf on MS medium enriched with various BA (Benzyl Adenine) concentrations. A significant difference was observed with shoot tips compared with nodal explants and leaf at the shoot induction percentage and mean number of shoot per explant. The highest percentage of shoot induction (100%) and average number of micro shoot per shoot tip (9.200 shoot) were recorded on MS medium fortified with 1 mg/l BA. The findings showed that a satisfactory rooting percentage (100%) was recorded when 1 mg/l IBA (Indole Butyric Acid) added to half MS medium with an average number of roots (2.600) root/shoot, a mean root length is 8.600cm. rooted plantlets could be successfully acclimatized in the shade house with 100% survival rate. The genetic stability of the micropropagated plants investigated by the RAPD markers indicated monomorphic and true to type when compare with the original plant.

Key words: medicinal plant, plant growth regulators, molecular markers

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الإكثار الدقيق والتقييم الوراثي لنباتات *Solanum nigrum* المتولدة باستعمال مؤشرات RAPD الجزيئية

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المستخلص

في هذه الدراسة، أُعتمد منهج الإكثار خارج الجسم الحي لأغراض الإكثار الدقيق وحفظ وصون نبات *Solanum nigrum* (black nightshade) لضمان إستدامة منتجاته الصناعية وتوافر بحوثه التطبيقية. في غضون شهر واحد، تمايزت الفروع الخضرية من الأجزاء النباتية الثلاثة (القلم النامية، العقد والأوراق) المزروعة على وسط MS الغذائي المجهز بتركيز مختلف من BA. لوحظت فروقات معنوية للقلم النامية مقارنة بالعقد والأوراق الخاصة بالنسبة المئوية لتكوين الفروع ومعدل عدد الفروع لكل جزء نباتي، إذ سُجّلت أعلى نسبة لتكون الفروع من القلم النامية (100%) ولمعدل عدد الفروع المتكونة لكل قلم نامية (9.200) في وسط MS المجهز بـ 1mg/l BA. وفي تجربة التجذير، أظهرت النتائج الى ان اعلى نسبة تجذير (100%) مع أعلى معدل لعدد الجذور لكل فرع (2.600) وأعلى معدل لطول الجذور (8.600) سم تم تسجيلها عند إضافة IBA بتركيز 1mg/l الى وسط MS الصلب بنصف قوة أملاحه. تأقلمت النباتات الصغيرة المجذرة والمزروعة بالظلة الخشبية بنسبة نجاح (100%). أظهرت نتائج الترحيل الكهربائي لنواتج التضاعف العشوائي المتعدد لمؤشرات RAPD (Randomly Amplified Polymorphic DNA) تطابقاً وراثياً بين النباتات النسيجية النامية من ألقم النامية والنبات الأم.

الكلمات المفتاحية: النباتات الطبية، منظمات النمو النباتية، المؤشرات الجزيئية.

INTRODUCTION

Solanum nigrum L. (black nightshade) is a species of herbal plant in the Solanaceae family (17;32). It is one of the most popular of the African Leafy Vegetables (ALVs) which are a group of wild-growing, common vegetables, a fairly prevalent annual or bi-annual herb. As an African Leafy Vegetables offer much-needed nutrient improvement in the form of proteins, vitamins, minerals and essential amino acids, the incorporation of these vegetables in dominantly starchy diet is incredibly beneficial to the consumers (33). In some countries, boiled leaves and ripe fruits of this species have been used as food (31), and it is widely used in different countries as a traditional medicine to treat different diseases because of its hepatoprotective, antioxidant and anti-inflammatory impacts (12; 34). For rapid multiplication, *in vitro* propagation via plant tissue culture is an essential alternative to conventional techniques, and in order to maintain clonal fidelity, micropropagation through shoot culture often used (16). This case makes organ cultures a preferred choice especially when the target is establishing an *in vitro* method of propagation from shoot tip, nodal explants and leaf (27). However, PGRs, the explant type, and the seedlings' *in vitro* regeneration pattern may modify the genome of plants resulting in somaclonal variation (7; 26). So, it's mandatory to validate the genetic uniformity of micro-propagated plants to evaluate their genetic quality which considers an important aspect for elite genotypes conservation and their commercial implications (36; 42). Random Amplified Polymorphic DNA (RAPD) has been used in many plant species to assess the genetic fidelity of regenerated plantlets (5, 6, 11, 13, 24, 40). In current study, achieved the accomplishments of target via a healthy set of seed explants and their culture *in vitro*, shade house acclimatization, and genetic fidelity using RAPD markers for regenerated black nightshade.

MATERIALS AND METHODS

Initiation stage

This study was implemented from February 2019 to March 2020 in the laboratory of plant tissue culture, department of plant genetic resources, seed testing and certification

director, Ministry of Agriculture, Baghdad. The seeds provided by Iraqi Gene Bank were cultured using two pathways:

1. The seeds were cultured in plastic pots and kept in a shade house to follow their growth reaching to the flowering and fruit set.
2. The seeds were cultured on MS medium (20) free of plant growth regulators. Seed surface sterilization was achieved after use for five minutes of a 5% solution of commercial sodium hypochlorite. After that, a triple rinsing with distilled sterile water for 3 times under the cabinet of laminar airflow was applied (29). The sterilized black nightshade seed cultured individually in test tubes containing free hormone MS media. 2 weeks later, all the seeds germinated and the grown seedlings were used as a source of explants.

Multiplication stage

Three types of explants (shoot tip, nodal explants and leaf) were used for multiplication stage. The explants were cultured on a medium which supplemented with different concentrations of BA (0.0, 0.5, 1.0, 1.5, and 2.0) mg/l. The medium was distributed and sterilized with an autoclave at 121 ° C and 1.04 kg cm⁻² for a period of 15 minutes (30). The cultures were incubated at 25±2°C, light exposure 16 hours per day and 1000 lux light intensity from cold white light (9, 37). The response percentage (%) for explants and the mean number of shoot/explants were recorded after one month of culture.

Rooting and acclimatization stage

The shoots that produced from the best explants and best BA concentration were planted on half strength MS medium enriched with various concentration of IBA (0.0, 0.5, 1.0, and 1.5) mg/l. The rooting percentage (%), roots length (cm) and mean number of root per shoot were recorded after one month of culture. The acclimatization of rooted plantlets that produced from the best treatment were achieved. Plantlets were removed from the test tube, washed the roots under running tap water to remove agar from, then soaked with Benlate herbicide, after that, plantlets were planted in sterilized small paper pots contained 2:1(peat moss: soil) and followed till flowering and fruit set.

DNA isolation and genetic stability analysis by RAPD marker: To evaluate the genetic

fidelity, genomic DNA was extracted from 500 mg of leaves, of both, *in vitro* acclimatized and mother plants through CTAB method. Agarose gel electrophoresis (0.8%) was used to analyze the purity of isolated plant DNA, while we used 6 types of RAPD marker to study the genetic homogeneity. A required volume of 50 µl PCR reaction which containing: DNA (100 ng/µl), 10 p mole primers and 2X master mixes was carried out. The PCR technique was applied in a thermal cycler (Eppendorf, Germany) under circumstances of 95°C denaturation step for 5 min, proceeded by 35 cycles of 95 °C denaturation for 30 s and primer annealing 45 s at 52 °C, and extension for 2 min at 72 °C with a final extension for 10 min at 72 °C, then the temperature was reduced to 4 °C with a final extension for 10 min. we isolated the amplified PCR samples by electrophoresis of agarose gel (1%) using a (50 V) TAE (1X) buffer for 2 h and 30 minutes. By using the gel documentation system, the gel was imaged and the bands of amplified PCR sizes was calculated via 1 kb DNA marker.

Statistical analysis

Table 1. Effect of BA on multiple shoots induction from shoot tips, nodes and leaves of *Solanum nigrum*

BA Concentration s (mg/l)	Shoot Tip	Explants (%of Response)			Mean	Explants (Mean no. of shoots/explant)			
		Node	Leaf	Mean		Shoot tip	Node	Leaf	Mean
0.000	0.400	0.000	0.000	0.133	1.000	0.000	0.000	0.330	
0.500	0.400	0.200	0.000	0.200	1.600	0.400	0.000	0.670	
1.00	1.000	0.600	0.200	0.600	9.200	2.200	0.400	3.930	
1.500	0.600	0.800	0.600	0.667	4.400	4.800	3.200	4.130	
2.000	0.200	0.200	0.000	0.133	1.800	0.600	0.000	0.800	
Mean	0.520	0.360	0.160		3.600	1.600	0.720		
LSD (0.05):					LSD (0.05):				
A (explants): 0.2062					A (explants): 1.203				
B (BA concentrations):0.2661					B (BA concentrations):1.554				
A x B: 0.4610					A x B: 2.691				

The average number of shoots (the optimist) (9.200) per explant with *in vitro* flowering (Figure 1, A, B) was produced by shoot tips cultured on an MS medium fortified with BA (1mg/l) which had a significant differences from nodes and leaves. Kavitha *et al.* (16) reported that the direct multiple shoots was differentiated when shoot tips and nodal explants placed on MS medium supplemented with Benzyl Adenine (1-5) mg/l, also Osama (22) used BA at (1mg/l) for the direct propagation of *Vitis Vinifera L.* using single

In each experiment, each treatment had 10 replicates containing 1 explant in every test tube. All data were statistically analyzed using Completely Randomized Design (CRD). Significant differences between treatments were determined using least significant differences (L.S.D) at the 0.05 level.

RESULTS AND DISCUSSION

In this study, the objective has been to evaluate a protocol for black nightshade propagation *in vitro* by using different explants and various concentrations of BA. Shoots were observed from shoot tips of the media that lacking plant growth regulators or BA-containing media. On the second week of culture, the explants response became obvious, and after 1 month, it was found the highest percentage (100%) of shoot induction in MS medium supporting by BA (1 mg/l) from shoot tips placed on. However, we observed a significant differences at the shoot induction stage where shoot tips and nodes gave (52%) (36%) in respectively, then leaves (16%) (Table1).

nodes. while Venugopal *et al.* (39) obtained *in vitro* flowering and direct organogenesis in *S. nigrum* from nodal explants and leaf on MS medium fortified with BA and IAA (Indole Acetic Acid). These results indicated that endogenous plant hormone rates existing in such explants are not adequate to sustain their development in the free medium. On the other hand, complemented with BA, MS medium produced numerous shoots from shoot tips, leaf, or nodal explants, this result has been verified by Pirek (23) when he reported that

BA is the super cytokinin in promoting the highest frequency of adventitious shoot in most plant species. In *S. nigrum*, Kavitha *et al.*, (16) found that shoot tip is an excellent source to promote organogenesis than leaf and nodes, also in several plants, for example, date palm (1), strawberry (41), *Abrus precatorius* (28) and chestnuts (4), the shoot tips were considered to be the preferred micropropagation explant for shoot proliferation due to the higher cytokinin-auxin ratio present in (10). For rooting, the resulted shoots were separately transferred to

solid half strength MS media fortified with various concentrations of IBA (0.5-1.5) mg/l, the analysis indicated that the micro shoots responses undergone the experiment of rooting differed with the added IBA concentrations. In addition, the data gathered showed that an increase IBA level in the media, increased the percentage of rooting to achieve an optimum of 100% rooting at 1mg/l IBA point (Table 2, Figure 1: C). On an MS medium fortified with 1 mg/l IBA, the highest mean of root number (2.600 root) and root length (8.600 cm) were recorded.

Table 2. Effect of IBA concentrations on the induction of rooting in *Solanum nigrum*

IBA Concentrations	Shootlet (% of Rooting)	Mean No. of Roots/shootlet	Mean Root Length (cm)
0.000	0.000	0.000	0.000
0.500	0.400	0.600	1.800
1.000	1.000	2.600	8.600
1.500	0.200	0.400	1.400
LSD (0.05)	0.5187	1.075	3.268

These findings were similar to the results obtained by AL-Dabagh and Salih (2); Chirumamilla *et al.* (7); Vemula *et al.* (38); Jogam *et al.* (15) and Hussain *et al.* (14) who emphasized that IBA's high *in vitro* frequency going to its fine structure, durability, and fast tissue translocation. The optimal IBA concentration increases root initiation, number of roots per shootlet and the mean root length due to the induction of carbohydrate and nitrogen portion mobilization and utilization then metabolites accumulation which causes new proteins synthesis, enlargement of cells and cell division (35). While Al-Jubori, et.al(3) referred that using IBA at 2 mg/l was more effective for rooting of paulownia explants. The highest

rooted *in vitro* plantlets were transferred to sterilized peat moss and soil (2:1) filled paper in a growth room and produced healthy leaves after two weeks of transplantation, then they moved to the shade house where they start growing normally (Figure 1:D). Rooted shoots have the highest survival (100%) due to the efficient root system, this could result in stronger soil absorption of water and mineral nutrients and eventually sustain a higher percentage of field survival (18). These results agree with the most findings of *in vitro* propagation of *S. nigrum* which showed that after acclimatization, the high survival rates of plants referred to the weedy aspect of the black nightshade as it helps its survival under sever environmental conditions (8; 17; 25; 33).

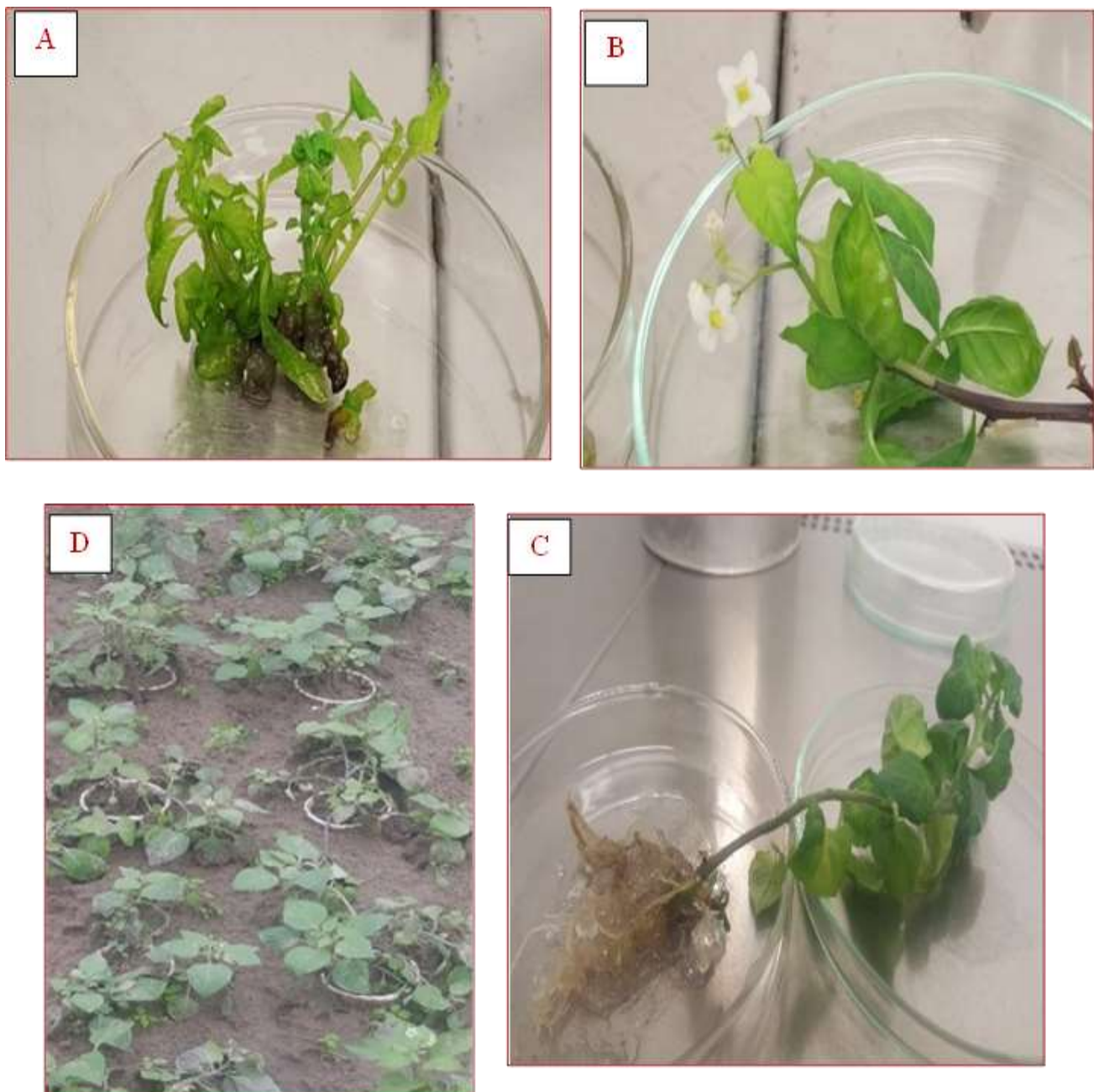


Figure 1. Micropropagation of *Solanum nigrum*

A *In vitro* multiplication

B *In vitro* flowering of *S. nigrum* in solid MS supplemented with 1 mg/l BA

C *In vitro* rooting

D Acclimatization in the shade house

In vitro culture is more complex and demanding than the natural environment, which increases the likelihood of genetic variation. As a result, and before certifying the success of micropropagation strategy, it is required to assess the genetic homogeneity of the regenerated plantlets. In this study, six RAPD primers (Table 3) generated 12 scorable bands in same molecular weight which ranging from 300 to 800 base pair. There were no

polymorphic bands between the mother plant and the regenerated plantlets (Figure 2), indicating the genetic consistency and stability of black nightshade plantlets. Due to their ease of use and competitive prices, RAPD markers have been successfully used to determine the genetic stability of *in vitro* regenerants in a variety of medicinal plants such as *Artemisia vulgaris* (15), *Rhynchosyilis retusa* (21) and *Lycium schweinfurthii* (19).

Table 3 . code and sequence of six RAPD primers used to detect the genetic stability of *S. nigrum* mother plant and plantlets produced from *in vitro* culture

Item	RAPD primer code	Primer sequences 5' to 3'	Number of bands (mother plant A)	Number of bands (acclimatized regenerated plantlets B)
1	OPT-18	GATGCCAGAC	1	1
2	C-88	ATCATCGTGGGG	3	3
3	B-41	GACAGCGTCCTA	1	1
4	CP-11	CCGCATCTAC	1	1
5	OPS-238	TGGTGG CGTT	4	4
6	UPC-90	GGGGGTTAGG	2	2

Conclusion

The proposed *in vitro* system could demonstrate the importance of the shoot tip in sustaining morphogenetic competence while maintaining regeneration frequency. Before it is licensed for commercial use, the established approach will give an alternate technique for large-scale multiplication and effective establishment of genetically stable plants.

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