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Micropropagation of Black Nightshade (*Solanum nigrum* L.): A promising medicinal plant in Libya

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ABSTRACT

Black nightshade *(Solanum nigrum* L.) is a promising medicinal plant that grows wild as a weed in Libya. Tissue culture approach was utilized for Micropropagation of this plant to insure its stable availability for phytoindustry and research. *In vitro* shoot multiplication was examined by culturing nodal explants on MS media supplemented with either BAP, kinetin or TDZ at 0, 0.5, 1.0 or 1.5 mg/L plus 0.1 mg/L NAA. For rooting, microshoots were cultured on MS media enriched with either, IBA, NAA or IAA at 0, 0.5, 1.0 or 1.5 mg/L and were added to the media to enhance microshoots rooting. The results revealed that a satisfactory shoot multiplication was obtained when TDZ was added to MS media, and the maximum number of the new shoots per explant 15 shoot/ explant was recorded in nodal segments treated with 1.5 mg/L TDZ with an average height of 2.06 cm. Best rooting 6.7 roots/ explant was obtained in microshoots cultured on Growth regulators –free media, While auxin- treated microshoots showed best rooting by using IBA at 1.5 mg/L as it yielded 80% rooting percentage with 4.9 root/ explant and average root length of 1.95 cm. Moreover, all potted plantlets were successfully acclimatized, and 100% survival rate was recorded in all plants one month after being transplanted to greenhouse conditions.

Keywords: Black nightshade**,** *Solanum nigrum* , Micropropagation**.**

Introduction

Solanum nigrum L. commonly known as Black Nightshade is an important medicinal plant of the family Solanaceae. It is a herbal plant widely distributed throughout the world, extending from tropical regions to temperate regions (Särkinen, *et al.*, 2018). The species of this family have great economic importance, because many plants are sources of chemical compounds of importance in modern medicine and pharmacology, as well as important sources of human food.

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Many research studies have reported that S. *nigrum* showed anti-cancer activity for hepatocellular carcinoma cells (Wang *et al*., 2015), human ovarian carcinoma cells (Wang *et al*., 2015), human colorectal carcinoma cells (Tai *et al*., 2012), and human endometrial carcinoma cells (Tai *et al*., 2013). The leaves can provide appreciable amounts of protein and amino acids, minerals including calcium, iron, and phosphorus, vitamins A and C, fats and fibers, and appreciable amounts of methionine, which is an amino acid limited in other vegetables (Ikeda *et al*., 2000).

Black nightshade plants are extensively harvested from the wild, leading to the possibility of its becoming an endangered species in the near future. However, to meet the demand of the pharmaceutical industry and to provide a continuous supply throughout the year, there is need to explore modern biotecnological approaches such as micropropagation.

Micropropagation is the process of multiplication from field –grown plants tissues or from in vitro seedlings, it is carried out in aseptic and favorable conditions on sterile growth media, using various plant tissue culture techniques (Pierik, 1987). Micropropagation is considered to be easier and rapid for herbaceous plants than woody species. In conventional cultivation many plant seeds do not germinate, flower and produce seed under certain climatic conditions or have long periods of growth and multiplication. Micropropagation insures a good regular supply of medicinal plants, using minimum space and time (Prakash and Van Staden, 2007). The success of the micropropagation depends on several factors like genotype, medium, plant growth regulators and type of explants (Kim *et al*., 2003). But still all possible types of explants and hormonal combinations have not been exploited to develop more various and efficient protocols. *Results of Several research papers dealt with micropropagation of black nightshade showed the possibility of micropropagatiing this plant successfully.* Ugandhar *et al*., (2010) reported that, highest rate and number of multiple shoots of *S. nigrum* were obtained from leaf and nodal explants cultured on MS medium supplemented with BA and IAA*, while* Kannan *et al*., (2005) reported *In vitro* regeneration of *S. nigrum* using different plant growth regulators and showed that BAP at 0.5 mg/l, and at 2, 4-D 1.0 mg/l and IBA gave the highest rate of the well-growing shoots. Bhat *et al*., (2010), obtained a high rate of shoot multiplication directly from leaf explant of *S. nigrum* on MS medium supplemented with BAP and KIN without any callusing stage. Hassanein and Soltan (2005), reported that, cultured shoots from shoot cuttings of germinated seeds of *S. nigrum* cultured on d B5, MS and SH, and observed that the best culture conditions for shoot formation was the culture of stem internode segments on B5 medium supplemented with 0.5 mg/l BAP.

The aim of this study was to micropropgate *black* nightshade, that grows wild in Libya, to facilitate the production of a large number of plants to be readily available for phytoindustry and research at any time.

Materials and Methods

This research work was conducted in the plant Tissue Culture Laboratory, Department of Horticulture and Crop Science at University of Jordan, (Amman-Jordan).

In vitro establishment of plant material

Mature seeds of *S. nigrum* were collected in September (2014) from a single wild type mother plant growing wildly in Tripoli – Libya (32°54′8″ N, 13°11′9″E). Then, seeds were surface sterilized by washing thoroughly under running tap water for 15 min with few drops of mild detergent, antibacterial soap (devomycin 5 ml/L water), and fungicide solution 1%. Then, seeds were dipped in 3.5% sodium hypochlorite for 5 min Followed by three rinses for 10 min each with sterile distilled water under laminar air-flow cabinet. Next, seeds were transferred into ethanol 70% (v/v) for 30 seconds and then rinsed with sterile distilled water for three times 10 min each under laminar air-flow. MS (Murashige and Skoog, 1962) salts present as MS premix (Duchefa Biochemiea Murashige and Skoog including vitamins; Duchefa-Postbus 809,2003 RV Haarlem, The Netherlands), at concentration of 4.4 g/L , 30 g/L sucrose and 8.0 g/L bacto agar (Agar A CAS #9002-18-0). Sterilized seeds were cultured into 100 ml bottles containing water media WM which consists of water and agar, then maintained in the growth room under a daily regime of 24±1 °C under a 16/8 (light/dark) photoperiod of 45–50 μmol $m^{-2}s^{-1}$ irradiance provided by cool white fluorescent tubes, until seed germination.

***In vitro* shoot multiplication:**

Nodal segments (1.0 cm) were excised from germinated seedlings and cultured into Erlenmeyer flasks containing MS media supplemented with different cytokinins: Kinetin, TDZ (Thidiazuron) or BAP (Benzyle aminopurine) at different concentrations 0.0 control, 0.5, 1.0 or 1.5 mg/L combined with 0.1 mg/L NAA. Each treatment consisted of five replicates (flasks), with four explant / replicate. Cultures were incubated at growth room and kept under a regime of 16- hr. light, 8- hr. dark and 24 ± 1°C. Data was collected after 4 weeks for: number of shoots/ explant, shoot height, callus Formation and rooting percentage.

In vitro rooting

Rooting was experimented by subculturing microshoots (1.0 cm) into test tubes containing 10 ml MS medium Containing different concentrations 0.0 (control), 0.5, 1.0 or 1.5 mg /L of IBA (indole butyric acid), NAA (naphthalene acetic acid) or IAA (indole acetic acid), with 20 replicates (test tube) per treatment and one explant / replicate. Cultures then were kept under similar conditions described earlier. Data was recorded after 4 weeks for rooting percentage, number of roots/ explant, root height and callus Formation.

Acclimatization:

For acclimatization, the test tubes with plantlets of well-developed roots were opened and maintained under growth room conditions for five days. Next, agar was removed by washing the roots under running tap water, then the plantlets were cultured into sterilized plastic cups with

sterilized growth medium peat: perlite (1:1, v/v) and were covered with plastic bags. The plantlets were irrigated with distilled water every 2 days for 10 days before being transferred to normal greenhouse conditions (33 ±1°C) and the survival percentage was recorded after 4 weeks.

Experimental design and statistical analysis:

All treatments were arranged in a completely randomized design (CRD) and data was statistically analyzed using statistical package for the social sciences (SPSS). Standard error was calculated for all the experiments. Analysis of variance (ANOVA) was used to analyze the obtained results for shoot height, root length in addition to number of shoots and roots per explant, and means were separated with probability level of 0.05 according to the Tukeys HSD.

Results and discussion

***In vitro* shoot multiplication:**

The obtained results revealed that shoot multiplication of explants varied with cytokinins type and concentration in the media. For example, shoot multiplication was unsatisfactory in response to BAP at all concentrations (Table 1).

Table 1. Effect of BAP on number of shoots, shoot height, callus formation and rooting formation of *in vitro* grown *S. nigrum.*

concentration (mg/L)	Number of shoots/ explant	Shoot height (cm)	Callus (%)	Rooting (%)
Control	1.00± 0.00a*	6.85±0.38 a	0.00±0.00	100±0.00
0.5	1.15±0.41a	1.95±0.16b	100±0.00	100±0.00
1.0	1.20±0.36a	1.77±0.77b	100±0.00	50±8.20
1.5	1.20±0.41a	1.60±0.12b	100±0.00	15±11.40

*Values represent means ± standard error. For microshoot height and number of shoots/explant, means within column for each growth regulator having different letters are significantly different according to Tukey HSD at P ≤ 0.05. Y: explant has formed more than 15 recognized microshoots.

These results were in complete contrast with Kavitha *et al.* (2012), as they reported the beneficial effect of BAP for the enhancement of shoot multiplication in *S. nigrum* However, the satisfactory shoot multiplication (20.4 shoot/ explant) reported by Kavitha *et al.* (2012) was obtained as they used BAP at higher rates 1.0-5.0 mg/L than those used in the current study. The results of the current study also, showed increasing BAP concentration in the media had a negative effect on shoot elongation, as the maximum shoot height 6.85 cm was recorded in the control treatment growth regulators –free media compared to 1.60 cm recorded in explants treated with 1.5 mg/L BAP (Table 1). Moreover, callus was developed at the bases of the stems segments in all BAP treatments, while no callus was obtained in the control (Table1) which agrees with the results obtained by Pandhure *et al.* (2010) during *in vitro*

multiplication of *S. nigrum*. On the other hand, increasing BAP level in the media has adversely affected rooting percentage, and the minimum rooting rate 15% was recorded in explants cultured in 1.5 mg/L BAP supplemented media (Table 1).

In kinetin experiment, trend of shoot multiplication results were similar to those obtained in BAP experiment, as shoot multiplication was very poor at all kinetin levels (Table 2). However, these results were opposite to those reported by Schuelter *et al.* (2009) and Kumar *et al.* (2011), as kinetin was recommended for best shoot multiplication during Micropropagation of Solanum *sessiliflorum and Solanum trilobatum.* Meanwhile, unlike the results obtained in BAP experiment, shoot height increased significantly with increasing kinetin level in the media as it increased from 1.40 cm at 0.5 mg/L kinetin to 2.55 cm at level of (1.5g/L) (Table 2). Also, full rooting percentages 100% were obtained at all kinetin levels which completely contrasted the results obtained in BAP experiment (Table 2).

Table 2. Effect of Kinetin on number of shoots, shoot height, callus formation and rooting formation of *in vitro* grown *S. nigrum.*

concentration (mg/L)	Number of shoots/ explant	Shoot height (cm)	Callus (%)	Rooting (%)
Control	1.00± 0.00a*	6.85±0.38 a	0.00±0.00	100±0.00
0.5	1.15±0.08a	1.40±0.11c	100±0.00	100±0.00
1.0	1.20±0.09a	1.92±0.11bc	100±0.00	100±0.00
1.5	1.25±0. 1a	2.55±0.10b	100±0.00	100±0.00

*Values represent means ± standard error. For microshoot height and number of shoots/explant, means within column for each growth regulator having different letters are significantly different according to Tukey HSD at P ≤ 0.05. Y: explant has formed more than 15 recognized microshoots.

The differences in responses of black nightshade nodal segments to the application of BAP or kinetin in the media could be due to the fact that, type and level of a given growth regulator in association to specific genotypes might lead to physiological and morphological responses that are completely different from those obtained when treated with another growth regulator belonging to the same group (Magioli *et al.*, 1998).

Meanwhile, a very satisfactory shoot multiplication was achieved when TDZ was used. The number of the new shoots increased significantly with increasing TDZ level in the media, and more than 15 new microshoots were recognized at TDZ level of 1.5 mg/L (Table 3). Enhancement of new shoot multiplication in response to TDZ was also reported in Solanacea plants (Khan *et al.*, 2006). For example, Magioli *et al.* (1998) reported that TDZ was best for successful microshoot multiplication in

eggplant. Meanwhile, maximum callus formation were obtained at all TDZ levels (Table 3). This agrees with Guo, *et al.* (2011) as they reported that, callus was found to develop in woody plants microshoots when high rates of TDZ more than 0.001 mg/L were added to the media during shoot multiplication phase. Meanwhile, rooting was completely inhibited as TDZ was applied to the media (Table 3). Inhibition of root development in response to TDZ application to the culture media while as also reported in Pelargonium explants (Mutui *et al.*, 2005).

Table 3. Effect of different TDZ Concentrations on number of shoots, shoot height, callus formation and rooting formation of *in vitro* grown *S. nigrum.*

concentration (mg/L)	Number of shoots/ explant	Shoot height (cm)	Callus (%)	Rooting (%)
Control	1.00± 0.00c*	6.85±0.38 a	0.00±0.00	100±0.00
0.5	1.30±0.10c	1.80±0.12b	100±0.00	0.0±0.00
1.0	5.35±1.45b	2.07±0.03b	100±0.00	0.0±0.00
1.5	15.00Y ±0. 1a	2.06±0.03b	100±0.00	0.0±0.00

*Values represent means ± standard error. For microshoot height and number of shoots/explant, means within column for each growth regulator having different letters are significantly different according to Tukey HSD at P ≤ 0.05. Y: explant has formed more than 15 recognized microshoots.

In vitro rooting:

The results revealed that, best rooting of microshoots were obtained in explants cultured in control treatment (Table 4, 5, 6). This could be due to the fact that, microshoots descended from seeds as a starting material (which it was the case in this study) always tend to form roots. Also, the results showed that responses of the microshoots undergone rooting experiment varied with the type and concentration of the added auxin.

Table 4. Effect of IBA on rooting percentage, number of roots/explant, root length and callus formation of *in vitro* grown *S. nigrum*.

concentration (mg/L)	Rooting (%)	Number of roots/explant	Root length (cm)	Callus (%)
Control	90.0± 10.0*	6.70±1.08 a	3.90±0.48a	60±0.00
0.5	20.0±13.33	1.40±0.20b	0.60±0.40b	100±0.00
1.0	40.0±16.33	1.70±0.80b	1.30±0.54b	100±0.00
1.5	80.0±13.33	4.90±1.64ab	1.95±0.46b	100±0.00

*Values represent means ± standard error. For microshoot height and number of shoots/explant, means within column for each growth regulator having different letters are significantly different according to Tukey HSD at P ≤ 0.05. Y: explant has formed more than 15 recognized microshoots.

Table 5. Effect of NAA on rooting percentage, number of roots/explant, root length and callus formation of *in vitro* grown *S. nigrum*.

concentration (mg/L)	Rooting (%)	Number of roots/explant	Root length (cm)	Callus (%)
Control	90.0 ± 10.0*	6.70±1.08a	3.90±0.48a	60±16.32
0.5	40.0±16.32	2.10±1.01b	1.80±0.35b	100±0.00
1.0	40.0±16.32	1.70±0.39b	1.03±0.40b	100±0.00
1.5	0.00±0.00	0.00±0.00b	0.00±0.00b	100±0.00

*Values represent means ± standard error. For microshoot height and number of shoots/explant, means within column for each growth regulator having different letters are significantly different according to Tukey HSD at P ≤ 0.05. Y: explant has formed more than 15 recognized microshoots.

Table 6. Effect of IAA on rooting percentage, number of roots/explant, root length and callus formation of *in vitro* grown *S. nigrum*.

concentration (mg/L)	Rooting (%)	Number of roots/explant	Root length (cm)	Callus (%)
Control	90.0 ± 10.0*	6.70±1.08a	3.90±0.48a	60±16.33
0.5	30.0±16.60	0.80±0.44b	0.80±0.44b	100±0.00
1.0	60.0±13.33	3.40±1.14ab	1.50±0.48b	100±0.00
1.5	80.00±13.33	3.60±0.96ab	2.10±0.41b	100±0.00

*Values represent means ± standard error. For microshoot height and number of shoots/explant, means within column for each growth regulator having different letters are significantly different according to Tukey HSD at P ≤ 0.05. Y: explant has formed more than 15 recognized microshoots.

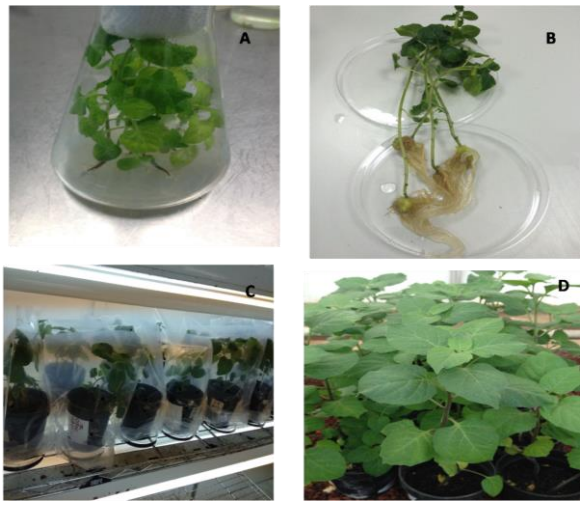

Figure 1. Stages of *in vitro* multiplication and acclimatization of *S. nigrum*. A: *in vitro* shoot multiplication, B: *in vitro* rooting, C: acclimatization in growth room, D: acclimatization in the greenhouse.

Moreover, the best rooting results in the growth regulators treated microshoots were obtained in those cultured on IBA supplemented media, which in accordance with the results obtained by Pandhure *et al.* (2010) and Padmapriya *et al.* (2011) protocols for *in vitro* rooting of black nightshade. Additionally, IBA was reported as best growth regulators for *in vitro* rooting in many other plant species such as, *Salvia fruticosa* Mill. (Arikat *et al.*, 2004), *Fagopyrum esculentum* (Klcova and Marcela, 2008) and *Phyllanthus amarus* Schum. (Sen *et al.*, 2009). Moreover, the obtained results showed that rooting percentages increased with increasing IBA level in the media to reach a maximum of (80%) rooting obtained at 1.5 mg/L IBA level (Table 4). Similar trends were also obtained in the results of root number and height, as the maximum number of roots/ explant 4.9 and root height 1.95 cm were recorded at IBA level of 1.5 mg/L (Table 4). These results were in full agreement with those reported in black nightshade which was *in vitro* grown by Padmapriya *et al.,* (2011), but disagreed with the results obtained by Pandhure *et al.* (2010) as they found that number and length of new roots formed in stem segments of black nightshade decreased as IBA level in the rooting media exceeded 0.5 mg/L. Moreover, all microshoots under IBA treatments had developed callus at their bases compared to (60%) callus development rate obtained in the control treatment (Table 4). Development of callus was also reported by (Younis, 2012) when IBA was used for *in vitro* rooting of *Acheillea fragrantissima.*

In NAA experiments, increasing NAA level in the media had negatively affected rooting of *S. nigrum*, as only 40% rooting was recorded in explants treated with either 0.5 or 1.0 mg/L NAA, while rooting was completely inhibited at level of 1.5 mg/L. Similar responses were also obtained for number of root/ explant and root length, as they tended to decline as NAA level increased in the media (Table 5). However, these results completely contrasted those obtained by Kavitha *et al.,* (2012) as they reported that increasing NAA level in the media up to 1mg/L had improved significantly rooting percentage, root number and length in the nodal stem segments of *s. nigrum*. The unsatisfactory rooting in response to NAA in the current study could be attributed to use of microshoots as explants material in the rooting experiment instead of nodal segments that might did encourage rooting as it was the case in Kavitha *et al.,* (2012) study. In IAA experiment, data showed that rooting percentage, root number and root height were positively affected by increasing IAA level in the media, but the maximum values were obtained in the control (Table 6). The maximum number of roots per microshoot (3.6 root/explant) and root height 2.10 cm in IAA treated microshoots were recorded at level of 1.5 mg/L (Table 6). These results agreed with other studies that reported IAA for best rooting in some plants belonging to Solanacea family such as *Solanum melongena* L. (Magioli *et al.*, 1998) and *Solanum sisymbriifolium* (Kim *et al.*, 2005; Oda *et al.,* 2006).

Acclimatization:

S. nigrum potted plantlets were successfully acclimatized and recorded 100% survival rate

(Figure 1). Also, the plantlets appeared healthy and more vigorous under greenhouse conditions (Figure 1). This agrees with most the *in vitro* propagation studies on this plant, which referred the high survival rates of the plantlet after acclimatization to the weedy nature of black nightshade as it supports its survival at harsh environmental conditions (Pandhure *et al.*, 2010; Padmapriya *et al. ,* 2011; Rathore and Gupta, 2013; Choudhary *et al.*, 2014).

Conclusions

Black nightshade *S. nigrum*, a promising medicinal plant that grows wild in Libya was successfully undergone *in vitro* micropropagation and acclimatization. However, more research is still needed to investigate the effect of *in vitro* propagation on the quality and quantity of its active ingredients.

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الإكثار الدقيق لنبات عنب الذئب (*Solanum nigrum* L.): نبات طبي واعد ينمو في البرية في ليبيا

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1. مركز البحوث الزراعية - طرابلس - ليبيا
2. قسم البستنة - كلية الزراعة - الجامعة الأردنية -عمان - الأردن
3. قسم العلوم التطبيقية - كلية الأميرة عالية - جامعة البلقاء التطبيقية - عمان - الأردن
4. مركز حمدي مانجو للبحث العلمي (HMCSR) - الجامعة الأردنية - عمان - الأردن

المستخلص

عنب الذئب *Solanum nigrum* L. نبات طبي واعد ينمو برية كحشيش في ليبيا. تم استخدام زراعة الأنسجة في التكاثر الدقيق لهذا النبات لضمان توفره للصناعة النباتية والبحوث. تم مضاعفة إعداده في المختبر عن طريق تعريض العقد الساقية إلى BAP أو الكينيتين أو TDZ عند مستويات 0 ، 0.5 ، 1.0 أو 1.5 مجم / لتر بالإضافة إلى 0.1 مجم / لتر NAA. وفي الوقت نفسه ، تم إضافة معدلات مختلفة 0 ، 0.5 ، 1.0 أو 1.5 ملغم / لتر من IBA، NAA و NAA تم إضافته إلى الوسط الغذائي لتعزيز تجذير المتأصلات. أوضحت النتائج أنه تم الحصول على تضاعف مُرضي للأفرع عند إضافة TDZ إلى الوسط الغذائي ، وتم تسجيل اكبر عدد من التفرعات الجديدة لكل مستأصل 15 تفرع /مستأصل عند معاملتها 1.5 ملغم / لتر TDZ مع متوسط ارتفاع 2.06 سم. تم الحصول على أفضل تجذير 6.7 جذر / مستأصل في أوساط خالية من منظمات النمو ، في حين تم الحصول على أفضل تجذير للمستأصلات التي خضعت لمنظمات النمو ليظهر أفضل مستأصل عند معاملتها 1.5 ملغم / لتر IBA حيث انتتجت 80 ٪ نسبة التجذير مع 4.9 جذر /مستأصل ومتوسط طول الجذر 1.95 سم. علاوة على ذلك ، تم تأقلم جميع النباتات بنجاح ، وسجل معدل النباتات الحياة 100 ٪ بعد شهر واحد من زراعتها في الصوبة.

الكلمات الدالة: *Solanum nigrum*، عنب الذئب، الإكثار الدقيق.

للاتصال: ليلى يونس، مركز البحوث الزراعية والحيوانية، طرابلس، ليبياز.

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