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RESEARCH PAPER

Improving solanine production in *in vitro* cultures of *Solanum nigrum* L. using different chemical and physical factors

Mu,ad A. AL- Kiyyam[1], Rida A. Shibli[2], Reham W. Tahtamouni[3], Tamara S. AlQudah[4], Bashaer B Abu-Iramaileh[4]

التحكم ببعض الظروف الزراعية يعزز من إنتاج السولانين في مزارع السويقات النباتية والكالوس والمعلقات الخلوية لنبات المغد الأسود *Solanum nigrum* L.: نبات طبي بري

معاذ أ. القيام ورضا أ. شبلي ورهام و. تهتموني وتمارة س. القضاة وبشائر ب. أبو الرمايلة

Abstract. *Solanum nigrum* L. is a medicinal plant of solanaceae family with distinguished therapeutic properties. Traditionally, *S. nigrum*. had been used as an anti-tumorgenic, antioxidant, hepatoprotective, diuretic, and antipyretic agent. The most important alkaloid member in this plant is solanine. Therefore, this study was conducted to utilize tissue culture techniques for the enhancement of solanine production in the in vitro grown cultures of this promising neglected plant. For callus growth and development experimental part, the highest callus growth parameters (callus diameter (21.4 mm) and callus fresh weight (2202.4 mg) were obtained in callus grown on Murashige & Skoog MS media supplemented with 2,4-Dichlorophenoxyacetic acid (2.0 mg·L^{-1}) plus 1.5 mg·L^{-1} Thidiazuron. Similar trend was also obtained in cell suspension culture experiment, as maximum growth was recorded at similar hormone combination. Moreover, High-performance liquid chromatography analysis revealed that, solanine was affected by growth regulator type and concentration. The highest solanine levels were obtained when the explants were treated with 6-benzylaminopurine at level of 2.0 mg·L^{-1}, as solanine content reached up to (2.61, 1.53 mg.g^{-1}) for callus and cell suspension, respectively, while, microshoot contained the highest solanine (4.52 mg.g^{-1} DW) at 6-benzylaminopurine level of 1.6 mg.L^{-1}. Additionally, carbon source had positively affected solanine level, where 0.2 M sucrose resulted in production of the highest amounts (3.13, 2.03 and 1.20 mg.g^{-1} DW) of solanine in microshoots, callus and cell suspension, respectively. Also, exposing microshoots and callus to light intensity of (100 µmol.$m^{-2}s^{-1}$) yielded the highest solanine content (4.03 and 1.26 mg.g^{-1} DW, respectively),while the lowest solanine levels (1.50 and 0.48 mg.g^{-1} DW) were observed in plant material exposed to the lowest light intensity treatment (25 µmol.$m^{-2}s^{-1}$). Our results were promising for production of solanine especially in the microshoot (4.52 mg.g^{-1} DW) using 1.6 mg.L^{-1} of BA, as compared with previous studies which maximized production of solanine only up to 2.33 mg. g^{-1} DW using 0.5 mg.L^{-1} of cholesterol in *Solanum lyratum*.

Keywords: Callus; Carbon source; Cell suspension; Light, Solanin.

المستخلص: المغد الأسود *Solanum nigrum* L. نباتٌ طبيٌّ ينتمي للعائلة الباذنجانية ويتميز بخصائصه العلاجية المميّزة، فقد استخدم تقليدياً كعامل مضاد للأورام ومضاد للأكسدة ومضاد للسمية الكبدية، وكمدر للبول وخافض للحرارة. يعد السولانين القلويد الأكثر أهمية في هذا النبات. لذلك أجريت هذه الدراسة مستخدمة تقنيات الزراعة النسيجية لتعزيز إنتاج السولانين في هذا النبات الواعد والمستحق للمزيد من الاهتمام وباستعمال مزارع النمو المختبرية. لوحظ في القسم التجريبي المتعلق بنمو الكالوس وتطوره أنّ القيم الأعلى لنمو الكالوس المزروع (قطر الكالوس: 21.4 مم، ووزنه الرطب: 2202.4 مغ) قد تم الحصول عليها باستعمال وسط موراشج وسكوج MS مضافاً له حمض 2,4–Dichlorophenoxyacetic 2) مغ.ل1–) و1.5) Thidiazuron مغ.ل1–). كما تم التوصل إلى تأثير مماثل في تجربة زراعة المعلق الخلوي، حيث سُجلت أقصى قيمة للنمو باستعمال المزيج الهرموني ذاته. علاوة على ذلك، كشف التحليل اللوني السائل عالي الأداء أن السولانين قد تأثر بنوع وتركيز منظم النمو، فقد تم تسجيل أعلى مستويات للسولانين عندما عولجت العينات النباتية بـ(2.0 مغ.ل1–) من 6–benzylaminopurine، حيث وصل محتوى السولانين إلى (2.61، 1.53 مغ.غ1–) في كل من الكالوس والمعلق الخلوي، على التوالي، في حين احتوت السويقات النباتية على أعلى مستوى من السولانين (4.52 مغ.غ1– ماء مقطر) عند إضافة (1.6 مغ.ل1–) من 6–benzylaminopurine. كما كان لمصدر الكربون أثر إيجابي في مستوى السولانين، حيث أدى استعمال 0.2 مول من السكروز إلى إنتاج كميات أعلى من السولانين (3.13، 2.03، و1.20 مغ.غ1– ماء مقطر) في كل من السويقات النباتية والكالوس والمعلق الخلوي على التوالي. وكذلك فإن تعريض السويقات النباتية والكالوس لشدة الضوء البالغة (100 ميكرومول.متر2– ثانية 1–) قد حقق أعلى محتوى من السولانين (4.03 و1.26 مغ.غ1– ماء مقطر(على التوالي، بينما لوحظت أدنى مستويات للسولانين (1.50 و0.48 مغ.غ1– ماء مقطر) في المواد النباتية عند تعرضها لأدنى معالجة من شدة الضوء (25 ميكرومول.متر2– ثانية 1–).

الكلمات المفتاحية: الكالوس، مصدر الكربون، المعلق الخلوي، الضوء، السولانين.

Introduction

Plant cell and tissue culture technologies have been established from different explant types, such as plant leaves, stems, roots, and meristems under sterile conditions for the production of secondary metabolites besides micropropagation purposes. Recently, successful production of many elite chemical compounds from plant cell suspension cultures has been

Table 1. Effect of plant growth regulators type and level on callus fresh weight and diameter of *in vitro* grown *S. nigrum* in combination with (2.0 mg.L^{-1}) 2,4-D, except for (C & 0.0) concentrations.

	Plant growth regulator Concentration (mg.L^{-1})	
TDZ	**Callus Weight (mg)**	**Callus Weight (mg)**
0.0	551.7^{z} f	6.3 d
C^{x}	613.4 e	6.9 d
0.5	679.8 d	7.0 d
1.0	1729.2 b	16.1 b
1.5	**2202.4 a**	**21.4 a**
2.5	1229.5 c	10.9 c
		Kinetin
0.0	551.7 d	6.3 a
0.5	589.8 c	6.8 a
1.0	**623.3 a**	**7.0 a**
1.5(C^{x})	613.4 ab	6.9 a
2.5	601.9 b	6.9 a
		BAP
0.0	551.7 d	6.3 d
C^{x}	613.4 c	6.9 c
0.5	809.9 b	8.1 b
1.0	**1137.8 a**	**10.2 a**
1.5	615.9 c	7.0 c
2.5	605.5 c	7.1 c

^{x} C (Control treatment) represents callus establishment media consisted of MS solid media plus 2.0 mg.L^{-1} 2,4- D and 1.5 mg.L^{-1} kinetin. ^{z} Means within columns for each growth regulator having different letters are significantly different according to Tukey HSD at p≤0.05.

reported in various medicinal plants by exposing the cultured explants to different growth conditions *in vitro* such as, using different types of culturing media, growth regulators, sugars and manipulating some physical environmental factors, such as, temperature, moisture and light (Robbins et al., 1996; Shibli and Ajlouni, 2000; Chawla, 2002; Jasmin et al., 2011).

Solanum nigrum L. (black nightshade) (Fig. 1A) is an annual herbaceous plant of 30-90 cm height with a green, smooth and semi-climbing stem. This plant grows wild in wet woods, near river, waste land, old field, ditches roadside and cultivated land (Pronob and Islam, 2012). Despite of being considered as a noxious weed that grows in many agro-climatic regions (Sundari et al., 2010), *S. nigrum* has been recently classified as a medicinal plant with distinguished therapeutic properties (Rajani et al., 2012). Traditionally, *S. nigrum* had been used in oriental systems of medicine for various purposes as, an anti-tumorgenic, antioxidant, hepatoprotective, diuretic, and antipyretic agent (Lee et al., 2003 ; Raju et al., 2003).

Solanine is considered the most important alkaloid produced in the green fruits of *S. nigrum* and many studies have confirmed Solanine activity as a cardiac anti-accelerating agent (Cham; 1994). This is in addition to its use as an antimicrobial agent against many strains of bacteria and fungi (Roddick, 1996). Moreover, Solanine is recently recommended as a new therapy for treatment of many cancer cell lines (Cham, 2008; Ji et al. 2008; Sutkovic et al., 2011).

Solanine can be found in the wild plants at high concentrations; but if we want to study how secondary metabolites are produced in plant cells in details we must adopt the tissue culture approach where the interference of all other factors such as, environmental factors is excluded. Moreover, wild plants in general are not effective sources to study secondary metabolites, as most medicinal wild plants are subjected to heavy loss due over collection; climate fluctuations; grazing ;urbanization , etc. Tissue culture is a potent technique that is used to enhance massive plant *in vitro* propagation and huge plantlets production under controlled conditions in few times and all around the year (Vinod and Dipali , 2013). Furthermore; *in vitro* culture permits better understanding of the mechanism by which plant cells synthesize secondary metabolites and the factors the can maximize their production by optimizing the cultural conditions of plant cells (DiCosmo and Misawa, 1995; Saito and Mizukami, 2002; Jasmin et al. 2011). Additionally, *in vitro* propagation of medicinal plants with enriched medicinal compounds and cell culture methodologies for selective metabolite production was found to be highly useful for commercial production of medicinally important compounds, as tissue culture techniques guarantee sustainable supply of true to name plants with high purity of the medicinally important compounds by excluding environmental factors and any possibility of genetic drift due to outcrossing (Hussain et al., 2012).

Large-scale plant tissue culture is found to be an attractive alternative approach to traditional methods of plantation as it offers controlled supply of biochemi-

Rida A. Shibli[*2] (✉) Mu,ad A. AL-Kiyyam[1], Department of Plant Production and Protection, Faculty of Agriculture, Jerash University, Jordan. Rida A. Shibli[*2], Department of Horticulture and Agronomy, Faculty of Agriculture, University of Jordan, Jordan, email: r.shibli@ju.edu.jo. Reham W. Tahtamouni[3], Department of Applied Sciences, Princess Alia University College, Al-Balqa Applied University, Jordan. Tamara S. AlQudah[4], Hamdi Mango Center for Scientific Research (HMCSR), University of Jordan, Jordan. Bashaer B Abu-Iramaileh[4], Hamdi Mango Center for Scientific Research (HMCSR), University of Jordan, Jordan.

Table 2. Effect of different combinations of BAP and 2,4-D on growth rate of cell suspension cultures of *S. nigrum* within different periods.

	Days	Growth regulator				
	0.0	**C[x]**	**BAP 0.1 mg.L^{-1}+ 2,4-D 0.1 mg.L^{-1}**	**BAP 0.1 mg.L^{-1} + 2,4-D 0.25 mg.L^{-1}**	**BAP 0.25 mg.L^{-1} + 2,4-D 0.1 mg.L^{-1}**	**BAP 0.25 mg.L^{-1} + 2,4-D 0.25 mg.L^{-1}**
7	11.5d[z]	**39.9 a**	19.5 c	19.7 c	18.7 c	27.5 b
15	37.7d	**88.5 a**	44.9 d	59.2 c	56.1 c	68.7 b

[x] C (Control treatment) represents callus multiplication media consisted of liquid MS media plus 2.0 mg.L-1 2,4 D + 1.5 mg.L-1 TDZ. [z]Means having different letters are significantly different according to Tukey HSD at P≤0.05. Cell suspension culture growth expressed as % packed cell volume (PCV)

cal's independent of plant availability (Sajc et al., 2000). Therefore, this study was conducted to investigate the possibility of improving solanine production in *S. nigrum*in *in vitro* grown cultures by manipulating some tissue culture growth conditions (chemical and physical factors).

Materials and Methods

Establishment and multiplication of plant material

Microshoots

Mature seeds of *S. nigrum* were collected from the mother plants grown in wild in Jerash- Jordan (N: 32.27372, S: 35.89464).Then the seeds were surface sterilized before being subcultured into full strength MS (Murashige and Skoog, 1962) solid media at concentrations of 4.4 g.L^{-1} and supplemented with 1.0 ml.L^{-1} MS vitamin mixture (Sigma Aldrich Murashige and Skoog Vitamin Powder 1000X) pluse 0.1 M sucrose. Seeds were cultured and kept up in the growth room under a daily temperature of 24±1 °C under a 16/8 (light/dark) photoperiod of 45–50 µmol m-2s^{-1} irradiance, until germination. For shoot multiplication, a prelimenary experiment was conducted to determine which growth regulatores would result in best shoot multiplication (data not shown), and based on the obtained results nodal segments (2 cm) from the germinated seedlings were subcultured into MS media supplemented with (1.2 mg·L^{-1}) Thidiazuron; 1-Phenyl-3-(1,2,3-thiadiazol-5-yl (TDZ) plus 0.1 mg·L^{-1}alpha-Naphthalene acetic acid free acid (NAA) (media that was found to be best for shoot proliferation). The cultures were kept under growth room conditions described earlier.

Callus

Callus was successfully established from excised *in vitro* grown leaf discs subcultured into callus establishment media (MS solid media supplemented with 2.0 mg·L^{-1} of 2,4-dichlorophenoxy acetic acid (2,4-D) and 1.5 mg·L^{-1} of kinetin) and kept under complete dark condition for 8weeks. After callus establishment, calli with diameter of (5.0 mm) and weight of (500 mg) were subcultured into a hormone free MS medium (HF-MS) for one week to remove the carry-over effects of plant growth regulators (PGRs.). Later, the calli were sub-cultured into fresh MS media supplemented with (2.0 mg·L^{-1}) 2, 4-D in combination with different levels (0.0, 0.5, 1.0, 1.5 or 2.5 mg·L^{-1}) of kinetin, 6-Benzylaminopurine (BAP) or TDZ. Control treatment was consisted of solid MS media plus (2.0 mg·L^{-1}) 2, 4-D in combination with 1.5 mg·L^{-1} kinetin (callus establishment media). Data were obtained after 8 weeks for callus diameter and weight.

Cell suspension cultures

Approximately 1.0 g of friable callus, which was still in its active growth phase (i.e., after the 15th day of subculture) was placed in 250 ml jar containing 50 ml liquid MS medium (Murashige and Skoog, 1962). The culture media used in this experiment were as follows: hormone free MS solid media , MS media plus hormone combination which was found to be optimum for maximum callus growth in callus multiplication experiments (2.0 mg·L^{-1} 2,4-D + 1.5 mg·L^{-1} TDZ) (control) or MS media plus (0.1 or 0.25 mg·L^{-1}) BAP in combinations with (0.1 or 0.25 mg·L^{-1}) 2,4-D. Cultures were incubated in complete darkness at 25°C on a horizontal shaker at 100 rpm for 15 days. Growth of cells was determined by measuring packed cell volume (PCV) described by Allan (1996) under sterile conditions at 7and 15 days of which was expressed as ml cell pellet per culture.

For PCV measurements, which were repeated three times per treatment, the cell suspension in flasks was gently shaken and then 10 ml aliquots were transferred into 15ml graduated conical centrifuge tubes followed by centrifugation at 200 g for 5 min using a swing-out rotor (Allan, 1996) in order to determine the best hormonal combinations that can give the maximum cell suspension culture weight.

Effect of chemical factors and light intensities on the *in vitro* production of solanine

Effect of Cytokinins

Microshoots were grown in MS media supplemented with 0.1 M sucrose, 0.1 mg·L^{-1} NAA in addition to different concentrations of different cytokinin growth regulators: BAP, kinetin or 6-(gamma, gamma- Dimethylallyl amino) purine (2iP) at levels of 0.0, 0.4, 1.0, 1.6 or 2.0 mg·L^{-1}. The subcultured microshoots were kept under normal growth room conditions. For callus and cell sspenssion, a part of plant material was treated with the different cytokinin types and levels mentioned before and kept under dark, while the other part was cultured on a media consisted of MS media plus best hormone formula resulted in the callus multiplication experiments (2.0 mg·L^{-1} 2,4-D and 1.5 mg·L^{-1} TDZ) and maintained under complete dark conditions. After 8 weeks, plants material (microshoots, callus and filtered cell suspension) from each experiment were collected, dried in the oven (35 °C for 2 days). Next, the dried plants material were grounded in a mortar and pestle, and pooled before being analyzed for alkaloids quantification (extraction and analysis), and the results were compared with those obtained from wild mother plant from Jerash grown in green house at Faculty of Agriculture/ University of Jordan, Amman -Jordan.

Effect of carbohydrate sources

Microshoots of *S. nigrum* (1.0 cm long) were subcultured into a hormone free MS medium for one week to remove the carry-over effects of plant growth regulatores (PGRs). Next , the microshoots were transferred into hormone free MS media supplemented with elevated concentrations of sucrose, glucose and fructose (0.05, 0.1, 0.15 and 0.2 M). For the callus and cell suspension, plant material were subcultured into callus multiplication media described earlier and supplemented with the different sugar types as mentioned above and kept under dark. After 8 weeks, the plant materials were dried and grounded as described earlier before being analyzed for alkaloids quantification (extraction and analysis) and the results were compared with those obtained from wild mother plant.

Effect of different light intensities

Microshoots and callus samples were kept at different light intensities (25, 50, 75 or 100 µmol. $m^{-2}.s^{-1}$) irradiance for 8 weeks. The plant material was collected, dried and grounded as described earlier before being analyzed for alkaloids quantification (extraction and analysis), and the results were compared with those obtained from wild mother plant from Jerash grown in green house at Faculty of Agriculture, University of Jordan, Amman -Jordan.

Determination of alkaloids content using high performance liquid chromatography(HPLC):

Alkaloids extraction

Samples (3 g) from each powdered plant material taken from each experiment, in addition to samples taken from the wild plants collected from the five different places in Jordan (including the wild mother plant from Jerash) were extracted thrice with 200 mL of 5% aqueous acetic acid and its pH was adjusted to 11 with ammonium hydroxide before being vacuum filtered. The alkaline extract was partitioned with water-saturated butanol and evaporated to dryness, purified and the residue was weighed and dissolved in acetonitrile and analyzed.

Preparation of solanine stock solution and working standards

Solanine stock solution at concentration of 20 ppm was prepared by weighing 2.0 mg of solanine in 100ml volumetric flask, dissolved and completed up to volume by methanol HPLC grade .The prepared stock solution was stored at 4°C in dark. Working solutions were prepared by serially diluting stock solutions using the mobile phase at concentrations of 0.5, 1, 2.5, 5 or 10 ppm. Fresh working standards were prepared daily. About 2.0 mg (±0.01mg) of solanine reference standard was dissolved into a 100 m1 volumetric flask and about 100 ml of acetonitrile until complete dissolve. Four points were constructed for the calibration curves (Fig. 2) and HPLC chromatogram (Fig. 3) for solanine was obtained before starting chemical analysis.

Chromatographic conditions

Chromatographic separation was carried out on HPLC-Shimadzu system (Japan), equipped with (LC-10 AT) pump, a manual injector, a system control (SCL-10A),a UV/VIS detector (SPD-10A), and an analytical column – C18 (250 - 4.6 mm I.D. 5 µm, Merck, Germany). The software, Shimatzu CLASS-VP (V6.14 SP1) chromatography data system was used as the data acquisition system. An ultrasonic cleaner (JEIOTECH- US) , a VM-300 vortex (Germany industrial CORP), an electronic balance (Precisa 410AM-FR, Switzerland), a SUPRA 30K (HANIL science industrial co., Ltd., Germany) ultra-centrifuge, a microfiltration unit (Advantec, MFS, Inc) with a rotary pump (Rocher 300) were used in sample treatment.

The alkaloids were analyzed using HPLC apparatus consisting of Shimadzu LC-10A system equipped with a model LC-10AT pump, an SPD-10A variable wavelength detector, a CBM-10A interface module with class LC-10 HPLC software using a Merck C-18 column (250×4.6, i.d., 5 µm particle size).

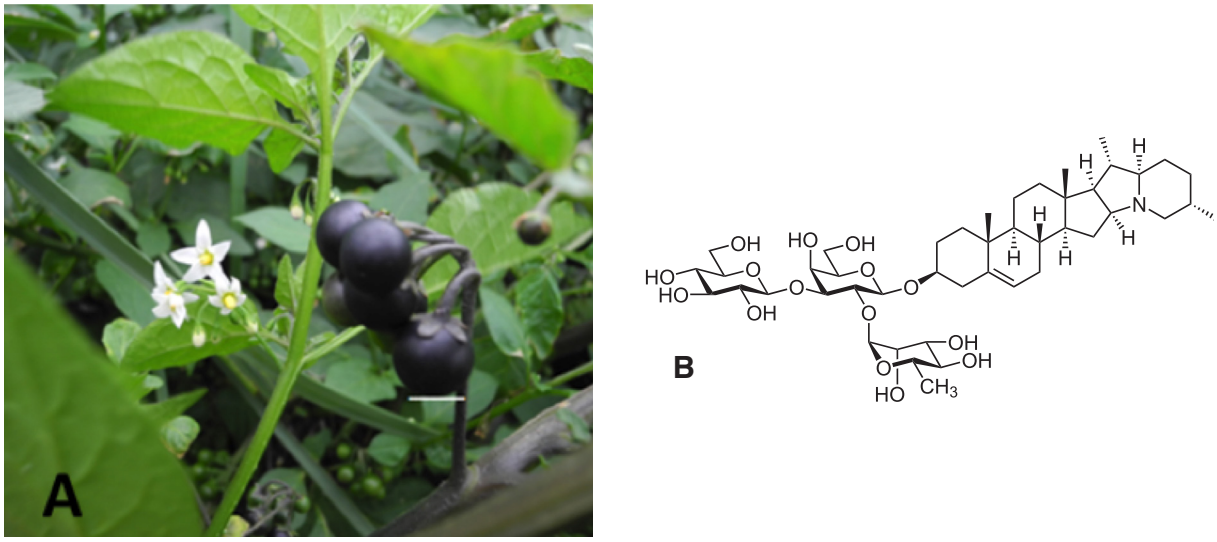

Figure 1. A: *Solanum nigrum* plant. (http://www.homeredies.com/solanum-nigrum-medicinal-uses-and-images). B: Chemical structure of solanine (Nino et al. 2009).

Solanine identification test

The basic method for solanine identification followed in this research was that set by Mohy-Ud-Din et al., (2010).

After conditioning the column and reaching equilibrium with the mobile phase, 20 µl of each of the solanine standard solution was injected at 0.5ml/min flow rate and tested at the range of detection wavelength 204 nm in order to determine solanine peak.

Method development

When the basic solanine identification method was applied, no separation between solanine peak and a matrix impurity peak was achieved. Therefore, it was necessary to develop this method, to improve the separation of solanine from the peak of impurity in plant extract matrix. The best separation of solanine was achieved on the following detection conditions: mobile phase: 25 acitonitrile (ACN), 75 potassium dihydrogen phosphate and 5ml Triethylamine at pH 7.5, the flow rate was 0.5 ml/min and UV detector wavelength set at 204 nm.

Experimental design and arrangement

All treatments in each experiment were arranged in a completely randomized design (CRD) and consisted of five replicates with 4 explants /replicate. Meanwhile, in alkaloids content determination experiment each treatment consisted of 3 replicates with 3 samples /replicate and sample dry weight of (3.0 g). The collected data were statistically analyzed using SPSS analysis system and analysis of variance (ANOVA) was used to analyze the obtained results, and mean was separated with probability level of 0.05 according to the Tukeys HSD.

Results and Discussion

Callus induction and multiplication:

Effect of TDZ

In callus multiplication experiments, the combination of 2,4,D (2.0 mg·L^{-1}) plus TDZ resulted in increasing callus growth in terms of fresh weight and diameter at all TDZ levels compared to control (C) and 0.0 mg·L^{-1} (Table 1). Similar results were reported by Sajid and Faheem (2009) who investigated the effect of Thidiazuron (TDZ) on *in vitro* micropropagation of Solanum tuberosum and they reported that, TDZ promoted callus induction and multiplication due to its biological activities in inducing the synthesis and accumulation of endogenous cytokinins which would direct cell division and callus clumps formation. The highest callus diameter (21.4 mm) and callus fresh weight (2202.4 mg) (Table 1) were recorded in callus grown in hormone combination of 2,4-D (2.0 mg·L^{-1}) plus 1.5 mg·L^{-1} TDZ. Improving callus growth using different combinations of 2, 4-D and TDZ of was also reported to be efficient in *Stephania tetrandra*, as it was found that, best callus growth and multiplication resulted on MS media plus 1.0 mg·L^{-1} 2, 4- D and 0.5 mg·L^{-1}TDZ (Chao et al., 2011). However, increasing TDZ level to 2.5 mg·L^{-1} resulted in decreasing callus fresh weight and diameter (Table 1), which might be attributed to the high TDZ concentration that might had inhibitory influence on callus growth and development.

Effect of kinetin

Callus fresh weight and diameter increased significantly with increasing kinetin level in the media up to 1.5 mg·L^{-1} (Table 1). The maximum values for both tested

parameters were obtained in the combination of 1.0 mg·L^{-1} kinetin and 2.0 mg·L^{-1} 2, 4-D as it yielded the highest callus fresh weight (623.3 mg) and diameter (7.0 mm) as shown in Table (1). Generally, kinetin performance was poor in terms of callus growth compared to the results obtained in TDZ treated explants. This contrasted the results reported about callus induction and multiplication of *Securinega suffruticosa* and *Viola uliginosa*, as they were maximized when their explants were grown on media supplemented with 2,4-D plus Kinetin (Raj et al., 2015; Slazak et al., 2015; respectively). This might refer to the fact that, plant responses to the different types and combinations of the growth regulators are species dependent.

Effect of BAP

Adding BAP in combination with 2,4-D improved callus growth significantly up to BAP level of 1.0 mg·L^{-1} compared to the results recorded in control (C and 0.0 mg·L^{-1}) (Table 1). The maximum callus weight (1137.8 mg) and diameter (10.2 mm) were obtained in BAP level of 1.0 mg·L^{-1} (Table 1). These results agreed with those reported by Sridhar and Naidu (2011), Sheeba and Palanivel (2013) and Sheeba et al., (2013) through their investigation on callus induction procedure of *S. nigrum*, *Solanum surattense* and *Physalis minima*, respectively.

The obtained results from callus multiplication experiment can lead to a conclusion that, TDZ (1.5 mg·L^{-1}) and 2,4, D (2.0 mg·L^{-1}) was the best combination that gave the maximum callus fresh weight and diameter of *S. nigrum*.

Cell suspension culture

The obtained results indicated that, adding 2.0 mg·L^{-1} 2,4-D plus 1.5 mg·L^{-1} TDZ (callus multiplication hormonal combination (C)); to the culture medium had yielded the maximum cell growth rate compared to the other treatments (Table 2). Meanwhile, for BAP and 2,4-D combination treatments, the highest cell growth rate (68.7%) was recorded in BAP (0.25 mg·L^{-1}) and 2,4-D (0.25 mg·L^{-1}) treatment (Table 2), this agrees with Song et al., (2002) results, as they reported that the media containing high BAP (0.25 mg·L^{-1}) and 2,4-D (0.25 mg·L^{-1}) produced greater rates of cell division in sugar Beet (*Beta vulgaris*) than either medium with lower BAP (0.1 mg·L^{-1}) and 2,4-D (0.1 mg·L^{-1}) levels or the control (hormone free MS liquid media). This agrees with Kshirsagar et al. (2015) findings in their study on *Swertia lawii* Burkill; as the maximum growth of cells suspension cultures (fresh weight = 2.8 g and dry weight 0.6 g) was observed after 15 days of culture in MS liquid medium supplemented with 2, 4-D (2.0 mg l–1) and BAP (2.0 mg l–1). Similarly, maximum growth of cells in suspension were observed between 10–15 days were reported in *Passiflora alata* using similar combination of growth regulators (2, 4-D and BAP) (Pacheco et al., 2012).

Table 3. Effect of cytokinine type and concentration on solanine % (mg.g^{-1}) dry weight (DW) in microshoots, callus and cell suspension of in vitro and wild (in green house) grown *S. nigrum*.

Concentration (mg.L^{-1})	BAP	Kinetin	2iP
Microshoot			
W*	4.92 a^{z}	4.92 a	4.92 a
C^{y}	**1.82 d**	1.82 d	1.82 e
0.4	2.20 c	2.00 d	2.16 d
1.00	3.93 b	2.60 c	3.00 c
1.6	**4.52ab**	3.83 b	**3.87 b**
2.0	3.77 b	**4.00 b**	3.43 bc
Callus			
W	4.92 a	4.92 a	4.92 a
C^{y}	0.73 d	0.73 d	0.73 e
0.4	0.95 d	1.97 c	1.10 d
1.00	1.62 cd	2.13 c	1.53 c
1.6	2.01 c	2.21 c	1.62 c
2.0	**2.61 b**	**2.82 b**	**2.55 b**
Cell suspension			
W	4.92 a	4.92 a	4.92 a
C^{y}	0.43 c	0.43 d	0.43 c
0.4	0.63 c	0.50 d	0.42 c
1.00	1.00 bc	0.50 d	0.76 b
1.6	1.11 bc	0.98 c	**1.01 b**
2.0	1.53 b	1.33 b	0.86 b

W: represents mother plant collected from Jerash. C^{Y}: control treatment for microshoots consisted of hormone free solid MS media. CY: control in callus experiment consisted of callus multiplication media (MS solid media + 2.0 mg.L^{-1} 2, 4-D + 1.5 mg.L^{-1} TDZ). CY: control for cell suspension cultures experiment consisted of MS liquid media plus 2.0 mg.L^{-1} 2,4-D + 1.5 mg.L^{-1} TDZ. ^{z}Means within columns having different letters for each growth regulator type are significantly different according to Tukey HSD at P≤0.05.*

Effect of chemical factors and light intensities on the *in vitro* production of solanine

Calibration curve

A calibration curve of alkaloids (Solanine) reference standard solution was prepared (Fig. 2) in order to calculate solanine content in the microshoots, callus and cell suspension of *S. nigrum* and comparing the results to solanine content in the wild type plant as mentioned above. Figure (2) explains how solanine contents was determined and calculated in the samples (a quantification method) using different quality control points includes different concentrations of the solanine standard to build up the calibration curve of solanine. Alkaloids

compounds were eluted at 6.26 min for solanine, and (Fig. 3) represents the peak of solanine, areas and linear calibration curve (r^2= 0.9999) as they were measured in the range of 12.5-12800 µg.ml^{-1} (ppm or mg·L^{-1}). Solanine was determined based on Figure 2 (calibration curve) using the following equation provided by the calibration curve: y = ax + b

Where: y = area, a = constant, x = solanine concentration and b = constant

Effect of plant growth regulator on solanine content

Effect of BAP

The obtained data in BAP experiment revealed that, solanine percentage in microshoots had increased remarkably in response to BAP level in the media to reach a maximum level of (4.52 mg.g-1 DW) at BAP concentration of 1.6 mg·L^{-1} compared to (1.82 mg.g-1 DW) recorded in the control treatment (C) (Table 3). This means that BAP level of (1.6 mg·L^{-1}) was able to enhance solanine production in the microshoots to reach a level which was very close to solanine content obtained in Jerash mother plant grown in the greenhouse. Meanwhile, solanine tended to decrease (3.77 mg.g^{-1} DW) at higher BAP level (2.0 mg·L^{-1}).These results fully agreed with Yogananth et al., (2009) and Bhat et al., (2010), where BAP enhanced the solanine accumulation in *S. nigrum* shoots. Also, Jayabalan et al., (2014) studied improve solasodine (another important alkaloid in Solanacea family) accumulation *in vitro* Solanum trilobatum using different concentration of BAP, and they concluded that 2.0 mg·L^{-1} of BAP was the best levels that gave the maximum solasodine percentage. Additionally, Al-Hawamdeh et al., (2013) reported the effect of BAP on the content of silymarin compounds in *Silybum marianum*, as they found that Silybin, silydanin, and silymarin content increased as BAP concentration increased compared with the control.

In callus experiment, solanine was recorded to increase with increasing BAP level to reach a maximum concentration (2.61 mg·L^{-1} DW) at 2.0 mg·L^{-1} of BAP (Table 3). Moreover, solanine was positively influenced by BAP level in the samples taken from the cell suspension, but in lower percentages than those obtained in the microshoots and callus cultures (Table 3), as the maximum level of solanine recorded in the cell suspension was only (1.53 mg.g^{-1} DW compared to 3.77 and 2.61 mg.g^{-1} DW) extracted from the microshoots and callus; respectively at similar BAP level (Table 3). This could be attributed to high water content in the cells cultured in the liquid MS media, as high water content in the cell was always reported to have a negative impact on the production and accumulation of secondary metabolites including alkaloids (Morales et al., 1993; Tahtamouni et al., 2016).

Table 4. Effect of charbohydrate type and concentration on solanine % (mg.g^{-1} DW) in microshoots, callus and cell suspension of in vitro grown *S.nigrum*, in addition to wild (in green house) grown *S. nigrum*.

Concentration (mg.L^{-1})	Sucrose	Glucose	Fructose
Microshoot			
W*	4.92 a^z	4.92 a	4.92 a
0.05	0.90 e	0.43d	1.68 c
0.1 (C^y)	1.82 d	2.67 c	1.85 c
0.15	2.43 c	3.00 b	2.13 bc
0.2	3.13 b	2.00 c	2.40 b
Callus			
W	4.92 a	4.92 a	4.92 a
0.05	0.21 e	0.58 c	0.58 c
0.1 (C^y)	0.73 d	0.85 b	0.61 c
0.15	1.50 c	1.03 b	0.93 b
0.2	2.03 b	0.48 c	1.05 b
Cell suspension			
W	4.92 a	4.92 a	4.92 a
0.05	0.10 d	0.33 c	0.13 d
0.1 (C^y)	0.43 c	0.38 c	0.26 c
0.15	1.16 b	0.56 b	0.50 b
0.2	1.20 b	0.40 c	0.66 b

W: represents wild mother plant from Jerash. Cy: for microshoots control treatment is consisted of hormone free MS media + 0.1 M of each carbohydrate types. CY: control in callus experiment consisted of callus multiplication media (MS solid media+2.0 mg.L^{-1} 2,4- D+ 1.5 mg.L^{-1} TDZ) + 0.1 M of each sugar types. CY : control in cell suspension cultures experiment consisted of MS liquid media plus (2.0 mg.L^{-1} 2,4-D + 1.5 mg.L^{-1} TDZ)) + 0.1 M of each sugar types. zMeans within columns having different letters for each sugar type are significantly different according to Tukey HSD at P≤0.05.*

Effect of kinetin

In kinetin experiment, the results showed that, increasing kinetin concentration positively affected solanine percentage (mg.g^{-1} DW) in shoots, callus, and cell suspension at all levels. (Table 3). Similar to the results trend obtained in BAP experiment, the highest solanine level were recorded in the microshoots (4.00 mg.g^{-1} DW) and callus culture (2.82 mg.g^{-1} DW) while the lowest values were recorded in the cell suspension cultures at all kinetin levels (Table 3).

The positive impact of kinetin on alkaloids production was also reported by Al-Hawamdeh et al., (2013) as kinetin enhanced the content of silymarin compounds in *Silybum marianum* shoots at 1.6 mg·L^{-1} kinetin. Moreover, effects of kinetin on biosynthesis of vindoline and other indole alkaloids in *Catharanthus roseus* callus cultures was investigated by Jian et al., (2001) and

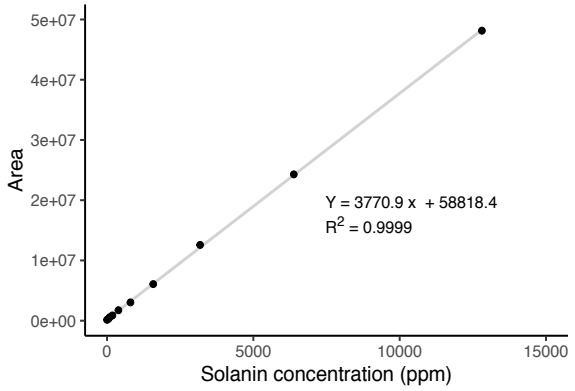

Figure 2. Standard calibration curve of solanine. Y: area, R2: retention time.

they reported that, indole alkaloids increased as BAP increased, which was attributed to enhanced peroxidase activity, reflected the pattern of alkaloid biosynthesis under the same culture conditions.

Effect of 2iP

The obtained data in 2iP experiment concluded that, solanine percentage in the microshoots had increased significantly in response to 2iP level in the media to reach a maximum level of (3.87 mg.g^{-1} DW) at 2iP concentration of 1.6 mg·L^{-1} compared to (1.82 mg.g^{-1} DW) recorded in the control treatment (Table 3). Meanwhile, solanine in microshoots tended to decrease (3.43 mg.g^{-1} DW) at higher 2iP level (2.0 mg·L^{-1}). In callus experiment, solanine was observed to increase with increasing 2iP level to reach a maximum concentration (2.55 mg.g^{-1} DW) at 2.0 mg.L^{-1} of 2iP (Table 3). In cell suspension experiment, solanine content increased as 2iP concentration increased; 1.6 mg·L^{-1} of 2iP gave the highest solanine content (1.01 mg.g-1 DW) in cell suspension after that solanine content decreased to (0.86 mg.g^{-1} DW).

Al-Hawamdeh et al., (2013) studied the effect of 2iP on the content of silymarin compounds in *Silybum marianum* shoots and they reported that Silybin, silydanin, and silymarin content increased as 2iP concentration increased compared with the control, and the maximum percentages for both silybin (0.76%) and silydanin (0.24 %) were obtained at 1.0 mg·L^{-1} 2iP and 0.1 mg·L^{-1} NAA, while higher concentrations of 2iP (more than 1.0 mg·L^{-1}) was reported to reduce the production of all silymarin compounds. Karolak et al., (2015) explained that cytokinin significantly suppresses the transport of macronutrients such as nitrate, ammonium, sulfate and phosphate, while nitrate regulates the expression of genes involved in the phenylpropanoid and flavonoid pathways.

On the other hand, the concentrations of solanine in the control (C) treatments were lower compared to solanine concentrations found in the plant materials treated with the growth regulators (2iP, BAP, kinetin) (Table 3). This could refer to the fact that, C media was designed for each explants type to be optimum for cell division and growth which would direct all plant cell resources towards cell division and primary metabolites (proteins, carbohydrates ...etc) synthesis rather than production of secondary metabolites, which was very obvious in the results obtained in callus and cell suspension cultures where cell division is the predominant task for the cells (Table 3). Meanwhile, the obtained data revealed that, the highest values for solanine in this experiment were those extracted from mother plant (W) collected from Jerash.

Table 5. Effect of different light intensities on solanine % (mg.g-1) in microshoots and callus of *in vitro* grown *S. nigrum*, in addition to wild (in green house) grown *S. nigrum*.

Light Intensity (µmol/m $^{-2}$ s^{-1})	Solanine (mg.g^{-1})
Microshoot	
W*	4.92 a^{z}
25	1.50 b
50 (C^{y})	1.82 b
75	3.53 a
100	4.03 a
Callus	
W*	4.92 a
25	0.48 d
50 (C^{y})	0.73 cd
75	0.93 bc
100	1.26 b

**W: represents mother plant from Jerash grown under ordinary light intensity (50 (µmol.m $^{-2}$ s^{-1}) . ^{y}C: control for microshoots, was microshoots grown in MS solid media + 0.1 M sucrose under normal growth room light intensity. For callus control (C^{y}) represents callus grown in callus multiplication media + 0.1 M sucrose under normal growth room light intensity. ^{z} Means within columns having different letters are significantly different according to Tukey HSD at P≤0.05.*

Effect of carbohydrates sources on solanine content

Sucrose

Different levels (0.05, 0.1, 0.15 and 0.2 M) of sucrose were investigated for their impact on the solanine content of *S. nigrum* microshoots, callus and cell suspension. As sucrose level increased in the media, higher solanine in the microshoots than control (C) and the maximum solanine content (3.13 mg.g^{-1} DW) at sucrose level 0.2 M (Table 4). Similarly, solanine content in the callus cultures increased significantly with increasing sucrose level in the media and 2.0 M sucrose treatment resulted in production of the highest solanine content (2.03 mg.g^{-1} DW) (Table 4). However, solanine content

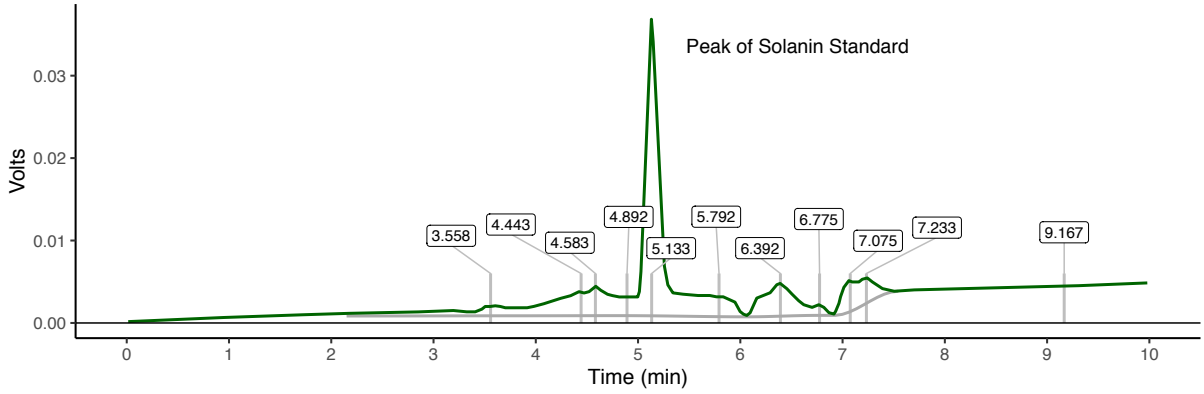

Figure 3. HPLC chromatogram for the solanine content from *in vitro* grown plantlets of *S. nigrum.*

in cell suspension culture was less than those obtained in microshhots and callus experiments, as the maximum solanine content was only (1.20 mg.g^{-1} DW) obtained at the highest sucrose level (0.2 M) (Table 4). This agrees with another research, where sucrose (60% = 0.2 M) was found to improve alkaloid content in callus culture of *Catharanthus roseus* (Ashutosh et al., 2012). Also, in cell suspension cultures of *Gymnema sylvestre*, different sugars types were tested, and sucrose was found to be the perfect carbohydrate source for biomass accumulation (11.56 g.L^{-1} DW) and gymnemic acid production (9.95 mg.g -1 DW) (Nagella et al., 2011).

High sucrose concentrations was reported as a technique for inducing osmotic stress in plants which forces plant cell to produce more electrolytes and secondary metabolites inside the cell as a defense mechanism to increase osmolarity and to decrease water loss from the cells (Shibli et al., 2006), which might explain the increase in solanine level resulted in our experiments.

Glucose

Adding elevated levels of glucose had improved solanine content in all of the experimented explants types, and 0.15 M glucose produced the highest solanine content in microshoots, callus and cell suspension culture, respectively (3.0, 1.03 and 0.56 mg.g^{-1} DW) (Table 4).

Al-Hawamdeh et al., (2013) investigated the effect of glucose on the content of silymarin compounds in *Silybum marianum* shoots and they reported that, these compounds increased as glucose concentration increased to reach the maximum at 0.1 M of glucose, while exceeding this concentration led to the reduction of secondary metabolites production.

Similarly, Wang and Weathers, (2007) investigated the effect of equal concentrations (0.1M) of carbohydrates types such as sucrose, glucose, or fructose on artemisinin production from the *in vitro* grown of *Artemisia annua,* and they concluded that a dramatic increment in the production of artemisinin in the medium treated with glucose compared with other sugar types.

Fructose

Solanine content of *in vitro* grown *S. nigrum* was significantly and positively affected in microshoots, callus and cells suspension culture (Table 4). Maximum solanine content (2.40, 1.05 and 0.66 mg.g-1 DW) were obtained in micoshoots, callus and cell suspension; respectively, treated with 0.2 M of fructose as shown in (Table 4). This agrees with Al-Hawamdeh et al., (2013) who reported that, Silybin, silydanin, and silymarin content increased as fructose concentration increased up to 0.15 M of fructose. In general, the result obtained from our study indicated that solanine content responded positively to increasing the concentration of sucrose, glucose and fructose. However, based on current study sucrose was for the solanine production in all the tested plant materials. For all sugar types; wild collected plants from Jerash; produced the highest solanine contents.

Effect of light intensity on solanine content

Solanine content in the microshoots and callus increased significantly with increasing light intensity compared to the results obtained in C plant materials which cultured on the ordinary light intensity of 50 (µmol/m^{-2} s^{-1}) (Table 5). Beside that wild plant gave the highest content of solanin at ordinary light intensity of 50 (µmol/m^{-2} s^{-1}) . Exposing both plant materials types to light intensity of (100 µmol/m^{-2} s^{-1}) yielded the highest solanine content in shoot and callus (4.03 and 1.26 mg.g^{-1} DW, respectively) (Table 5). Meanwhile, the lowest solanine level was observed in explants exposed to the lowest light intensity treatment (25 µmol/m^{-2} s^{-1}) for both microshoots (1.50 mg.g^{-1} DW) and callus (0.48 mg.g^{-1} DW)(Table 5). This might be justified by the fact that, solanine biosynthesis in tissues is highly dependent on the presence of active chloroplast (Moreira et al., 2010). Additionally, high light intensities might resulted in building a strong stress on the cultures that might cause cell dehydration

and yet increasing cell osmotic potential as a defense response, which shifted cell mission from division and production of primary metabolites into production and accumulation of secondary metabolites (Lenore et al., 1985)

Many studies investigated the influence of light on alkaloids production, for example, Lenore et al. (1985) concluded that the light increased the alkaloids of *Heimius alicifolia* , and they explained that there was a positive correlation between chlorophyll content and lupine alkaloid formation, and suggested that the lysine branch of the biosynthesis of the *Heimia alkaloids* might also be influenced by light. Similar findings were reported by Karimi et al., (2013) as they reported that, the maximum production of flavonoids and phenolic compounds were achieved at high light intensity (630 $\mu mol\ m^{-2}s^{-1}$) as increasing light intensity increases primary photosynthate, which leads to an increase in phenolic concentration in the plant (Warren et al., 2003). Moreover, effects of light on the biosynthesis of vindoline and other indole alkaloids in *Catharanthus roseus* callus cultures was studied by Jian et al., (2001) and they reported that light enhance all alkaloid biosynthesis in the callus, especially vindoline and serpentine about 3–4 folds higher than that in the dark.

Conclusion

Based on the outcomes of this study, it was found that manipulating some tissue culture growth conditions (chemical and physical factors) enhanced the production of solanine in microshoots, callus and cell suspension cultures of *Solanum nigrum* L. The highest solanine level (4.52 $mg.g^{-1}$) DW was obtained when the plant material were treated with BAP at level of 1.6 $mg{\cdot}L^{-1}$; this results were remarkable if compared to other previous studies on the production of solanine *in vitro* using other techniques. For example; in *Solanum lyratum* the addition of cholesterol at a concentration of 0.5 mg/l increased the synthesis of solanine (2.33 mg/g DW) compared to those extracted from the control (1.32 mg/g DW) (Lee et al., 2007) which were less than solanine level obtained in our study. Also, our data revealed that carbon source had positively affected solanine level, especially in plant material grown in media supplemented with 0.2 M sucrose. Moreover, exposing microshoots and callus to light intensity of (100 $\mu mol/m^{-2}s^{-1}$) yielded the highest solanine content compared to the other light intensities treatments. However, other culture growth conditions need to be under research for better enhancement of solanine production in *Solanum nigrum* L. *in vitro*.

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