Solanum nigrum is a model system in plant tissue and protoplast cultures

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Abstract

Solanum nigrum is a model system especially for newcomer to the subject of plant tissue culture. Shoot culture has been easily established from shoot cutting of germinated seeds on Gamborg (B5), or Murashige and Skoog (MS) medium without phytohormones. Direct regeneration was possible using basal media B5, B5C (B5 supplemented with 5 % coconut endosperm milk), Schenk and Hildebrandt (SH), and MS, leaf, stem, shoot tip as explants, cytokinins benzylaminopurine (BAP) or kinetin (KIN) at concentrations from 0.25 to 2 mg dm^{-3}, and different light treatments (dark, dim and normal light). The best culture condition for shoot formation was the culture of stem internode segments on B5 medium supplemented with 0.5 mg dm^{-3} BAP at 16-h photoperiod (irradiance of 100 μmol m^{-2} s^{-1}). Also, root formation was possible under different culture conditions. The best culture condition was the culture of microshoot segments on half strength MS medium supplemented with 1 mg dm^{-3} isobutyric acid. Induction of callus formation from young and mature tissues on MS medium supplemented with 0.5 mg dm^{-3} BAP, 0.1 mg dm^{-3} 2,4-dichlorophenoxyacetic acid and 1 mg dm^{-3} naphthalene acetic acid, and subsequent plant regeneration on B5C medium supplemented with 0.5 mg dm^{-3} BAP was easy. Regeneration of protoplasts isolated from shoot tips and fully expanded leaves was also simple. Finally, the transfer of rooted plantlets to the soil was successful.

Additional key words: shoot culture, explant, callus, regeneration, cytokinins, auxins.

Introduction

The ability to regenerate whole plant from selected or genetically altered cell or tissue is the key to the broad biotechnological potential of plant cell and tissue culture. It has been accomplished by reversing the conditions suitable for cell division and callus formation to other conditions stimulated organogenesis. Classical and somatic hybridization techniques indicate that both callus growth and regeneration capacity are inherited as a dominant traits. Therefore, highly regenerative species, such as *Solanum nigrum*, *Nicotiana* species, *Petunia hybrida* and others can be used as a parent in many successful somatic hybridization experiments.

S. nigrum is resistant to the herbicide atrazine (Gasquez and Barralis 1979). This trait is inherited maternally in *S. nigrum* and coded for by plastidal genome. Transfer of atrazine resistance trait into economic plants via protoplast fusion could be of great importance. In addition, substitution of *S. nigrum* plastids by other of *S. tuberosum* via protoplast fusion obtained cybrid plants containing *S. nigrum* genome and *S. tuberosum* plastome (Hassanein *et al.* 1993). This cybrid in comparison to the original plant could be used to study the incompatibility between nucleus and the transferred plastids as well as the effect of atrazine on resistant and sensitive plant lines (Hassanein 1998, Hassanein *et al.* 1998, 1999a,b).

The model plant in tissue culture must have specific characters such as high multiplication rates (*in vitro*), high regeneration capacity in short time under different culture conditions, amenable for different culture techniques, low frequency of somatic mutation if it does not intended, and it is preferably to harbor naturally a marker gene such as herbicide or antibiotic resistance. The term model plant was used before to many plant species, *e.g.*, potato as a model plant in somatic hybridization programs (Melchers *et al.* 1978, Binding *et al.* 1982) and *Arabidopsis thaliana* as a general model system in somatic cell genetics (Negrutiu *et al.* 1975).

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Abbreviations: BAP - benzylaminopurine; KIN - kinetin; IAA - indole-3-acetic acid; IBA - indolebutyric acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; NAA - naphthalene acetic acid.

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Induction of shoot formation from leaf explants (Zenkteler 1972, Bhatt *et al.* 1979) and regeneration from shoot tip protoplasts (Nehls 1978) of *S. nigrum* were reported. Also, many studies have been fulfilled by Binding and his coworkers (Binding *et al.* 1982, 1986, 1988a,b, 1992, Hassanein 1993) using *S. nigrum* protoplasts as a partner in somatic hybridization experiments or in co-culture with other species to improve its regenerability. The aim of the present work was *1*) to find the suitable techniques to establish shoot culture with high multiplication rate, *2*) to determine the effect of explant type, different growth regulators, basal media, and light treatments on shoot and root formation, *3*) to induce callus formation and subsequent plant regeneration from tiny and normal size of explants obtained from young and mature tissues, and *4*) to regenerate plants from protoplasts isolated from young and old plant materials.

Materials and methods

Establishment of shoot culture: Seeds of *Solanum nigrum* were disinfected by dipping in 5 % *Clorox* solution for 5 min followed by 5 min dip in 75 % ethanol. Seeds were germinated on hormone free B5 medium (Gamborg *et al.* 1968). Shoot part of seedling was cut at epicotyl or hypocotyl and transferred to B5 or (Murashige and Skoog (1962, MS) medium without phytohormones to establish shoot culture. After four weeks, the growing shoots were cut into segments (1 - 1.5 cm long) containing node or shoot apex and transferred to new media.

Also, shoot tip of seedlings were used to establish shoot culture in mass quantity in a short time. Immediately after seed germination, shoot tips still in contact with the cotyledons were cut and placed on B5 basal medium containing 0.5 mg dm^{-3} benzylaminopurine (BAP). After four weeks, the initiated shoots were cut and transferred to new B5 hormone-free-medium. Shoots may be subcultured at short period (2 weeks) if high multiplication rate was intended. In general, subculturing was at 4 - 5 weeks. All shoot cultures were maintained in 16-h photoperoid (irradiance of 100 μmol m^{-2} s^{-1}) using white fluorescent lamp at temperature of 25 ± 1 °C and without humidity control. Under these conditions, the effect of different media (MS and B5 media with and without phytohormones) on shoot growth were tested. It was accomplished by determine the mass of shoots grown on the tested media for three weeks.

Direct regeneration: All the experiments were carried out using leaf or stem segments from the established shoot culture. To test for the effect of different cytokinins on adventitious shoot formation, stem internodal sections (0.5 - 1 cm) were cultured on B5 basal medium, to which 0.25, 0.5, 1, 1.5, or 2 mg dm^{-3} of BAP or kinetin (KIN) was added. The most effective cytokinin concentration (0.5 mg dm^{-3} BAP) was used to study the effect of different basal media [B5, B5C, SH (Schenk and Hildebrandt 1972), MS, MS/2 (half strength MS containing 1.5 % sucrose)] on shoot formation. The best medium (B5 with 0.5 mg dm^{-3} BAP) were used to test for the effect of light and explant type on induction of shoot formation. Three types of explants were used stem internode (0.5 - 1 cm), leaf segments (0.3 × 0.3 cm) and shoot tips with cotyledons of the germinated seeds. The leaf segments were placed upside down and *vice versa*. Three different light treatments were used: complete darkness, dim light at 20 μmol $m^{-2}s^{-1}$ for 16-h and 16-h daily light at 100 μmol m^{-2} s^{-1}. Generally, in all the experiments a group of sixty explants was cultured in three Petri dishes (9 cm width) and considered as a replicate for each treatment. After three weeks, the percentage of explants showing shoot formation, fresh mass of each shoot and the number of initiated shoots/explant were determined.

Root formation: For adventitious root formation, microshoot or stem internodal sections was cultured on half strength MS basal medium supplemented with different concentrations of isobutyric acid (IBA; 0.5, 1, 3, 5 mg dm^{-3}). The best concentration of IBA (1 mg dm^{-3}) and the same concentration of indoleacetic acid (IAA) and napthalene acetic acid (NAA) were used to test auxin effected on root formation. Different media B5, SH, MS, MS/2 and MSC/2 (half strength MS containing 3 % sucrose) were used. After four weeks, the percentage of explants showing root formation, length and the number of initiated roots/explant were determined.

Induction of callus formation from tiny and large mature explants: Fully expanded leaves (6 weeks old) or corresponding internodes were sliced into small pieces (1 × 1 mm maximum) by razor blade and forceps in a drop of liquid media (V-KM) containing 0.6, 1.0 and 0.1 mg dm^{-3} of BAP, NAA and 2,4-dichlorophenoxyacetic acid (2,4-D), respectively (Binding and Nehls 1977). The resulted pieces were mixed with droplets of agarose solution in 5 cm Petri dishes. The agarose solution was prepared by melting it in liquid V-KM medium and used at a temperature of 40 °C (Lörz *et al.* 1983, Binding *et al.* 1988a,b, 1992). The size of agarose plate was about 1.3 mm high and 10 - 15 mm in diameter. This plate was divided into small parts (3 × 3 mm). Thereafter, 1.5 cm^3 of liquid V-KM medium was added. The dishes were

sealed and kept at 25 ± 1 °C under dim light condition for 2 - 3 weeks. The liquid medium was replaced 4 - 6 times with fresh medium at 3 d intervals. In order to establish the callus formation, the obtained microcalli in agarose sheets were transferred from the liquid medium onto MS agar medium (MTH) supplemented with the hormonal part of V-KM medium (Hassanein 1999). This medium was used to induce callus formation from normal size explants (0.5 - 1 cm long). These explants were taken from newly formed or fully expanded leaves or adjacent stem internode segments. The resulted calli were divided into small pieces (2 mm thick) and transferred to new medium at 3 weeks intervals for callus maintenance. Three replicates were used in the callus induction experiment each one represented the yield obtained from 1 g of internodal segments (60 stem segments) in case of large explants, 0.5 g of shoot tips or expanded leaves in case of protoplast culture and 0.3 g of tissue in case of tiny explants.

Callus formation and plant regeneration from protoplasts: Apical tips including about three young leaves or mature-fully-expanded leaves (6 weeks old) were collected, cut into small segments (1 - 3 mm). and incubated in the enzyme solution (3 % *Rohament CP*, cellulase and pectinase, *Röhm 80029*, *Röhm GmbH Chemische Fabrik*, Damstadt, Germany) for 14 h. The incubation was under dim light condition at 25 °C. The protoplasts were passed through steel sieve (pore 40 μm) to remove undigested cell clumps and centrifuged at 100 *g* for 5 min to remove enzyme mixture. Then, the protoplasts were washed and plated as described by Binding *et al.* (1988a,b). Medium V-KM (Binding and Nehls 1977) containing 0.56 mg dm^{-3} BAP, 1 mg dm^{-3} NAA and 0.1 mg dm^{-3} 2,4-D and the organic nutrient of the 8P medium of Kao and Michayluk (1975) was used for protoplast culture. After two weeks the protoplasts were transferred to B5 agar medium (0.8 %) containing 0.5 mg dm^{-3} BAP and 5 % coconut endosperm juice (B5C) for shoot regeneration or B5 containing the hormonal part of V-KM (BTM medium) for callus establishment. The established calli were transferred for induction of shoots or roots using the best culture condition for shoot and root formation. After four weeks on shoot induction medium the number of calli showing plant regeneration was determined.

Results and discussion

Shoot culture establishment: Shoot culture have been established easily from shoot tips or shoot segments of germinated seeds. In 6 weeks including one subculture, 16 shoots (each about 3 cm long) were obtained from one shoot tip. At the same time, 4 shoots could only be obtained when a shoot of seedling was directly used. Although the proliferation rate of the last method was slower than the first, it presumably provides more genetic stability and is easily achievable at any culture conditions. After establishment of shoot culture, high continuous multiplication was fulfilled by subculture the shoots at short period (2 weeks). The material obtained was large enough to mass protoplast isolation for protoplast culture or protoplast fusion (Binding *et al.* 1988a,b, Hassanein 1993). B5 basal medium without phytohormones was the best medium for establishment of shoot culture. Regenerants expressed normal growth with tendency to root formation and increased the shoot length (Fig. 1). The increase in the fresh mass of the shoots cultured on BAP-containing medium (Table 1) was resulted from the shoot enlargement and the formation of the adventitious shoots at the base of the cultured microshoots.

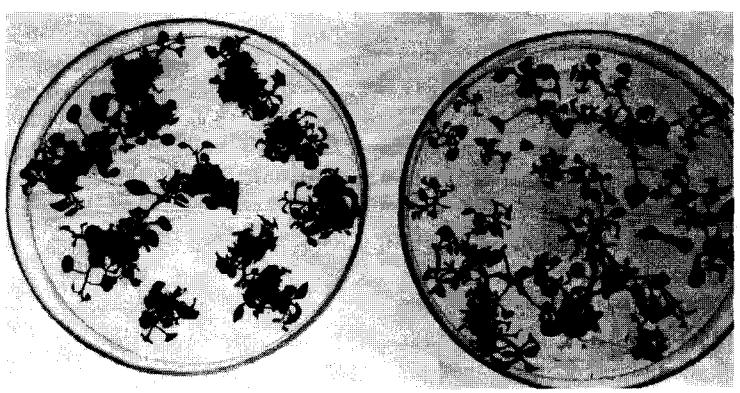

Fig. 1. Effect of BAP on establishment of shoot culture of *S. nigrum* on B5 medium. Microshoots were subcultured for three weeks on B5 medium containing 0.5 mg dm^{-3} BAP (*on the right*) or without phytohormones (*on the left*).

Table 1. Effect of different types of media on shoot culture of *S. nigrum*. Microshoots were cultured on B5 and MS media with and without BAP, each value was recorded after three weeks. Total number of explants was 40 (* - means significantly different (*t* - test) from shoots on B5 medium supplemented with 0.5 mg dm^{-3} BAP at $P < 0.05$).

Medium	BAP [mg dm^{-3}]	Explants give shoots [%]	Fresh mass [g $shoot^{-1}$]
B5	0.5	100	0.203
B5	---	0	0.117*
MS	0.5	100	0.096*
MS	---	0	0.080*

Direct shoot formation: After 5 d under shoot formation conditions the lower side of the explants, which immersed in the shoot induction medium, showed slight enlargements (Fig. 2). From these enlargements shoot primordia were arose.

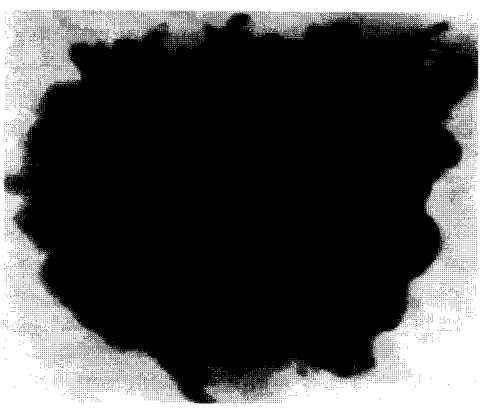

Fig. 2. The enlargement and the appearance of adventitious buds at the base of the explant cultured for one week on B5 medium supplemented with 0.5 mg BAP.

There are many cytokinins frequently used for initiation of shoot formation: BAP, KIN, N^6-(2-iso-pentenyl)-adenine (2ip), zeatin (ZEA) and SD8339. The low usage of the last cytokinins may be due to the high cost and limited availability (Hu and Wang 1984). Different concentrations of BAP (Table 2) and KIN (Table 3) were tested, and both the cytokinins induced shoot formation in 8 d. This is in agreement with the previous studies which reported that the plant regeneration from *S. nigrum* leaves (Hassanein 1999) or from protoplasts is high and fast in comparison to many other plant species (Nehls 1978, Binding 1985, Binding and Mordhorst 1984, Binding *et al.* 1982, 1988a,b, Hassanein 1993). Stem explants cultured on B5 medium supplemented with different concentrations of KIN produced fewer shoots than explants cultured on B5 medium supplemented with the comparable concentrations of BAP.

Table 2. Effect of different concentrations of BAP on shoot organogenesis of *S. nigrum* from stem internode segments on B5 medium. Each value was recorded after three weeks on shoot induction medium (* - means significantly different from shoots on B_5 medium supplemented with 0.5 mg dm^{-3} BAP at $P < 0.05$).

Medium	BAP [mg dm^{-3}]	Number of adventitious buds [$explant^{-1}$]
B5	0.25	8*
B5	0.5	21
B5	1.0	14*
B5	1.5	10*
B5	2.0	7*

Table 3. Effect of different concentrations of kinetin on shoot organogenesis of *S. nigrum*. from stem internode segments on B5 medium. Each value was recorded after three weeks on shoot induction medium (* - means significantly different from shoots on B5 medium supplemented with 0.5 mg dm^{-3} KIN at $P < 0.05$).

Medium	KIN [mg dm^{-3}]	Number of adventitious buds [$explant^{-1}$]
B5	0.25	7
B5	0.5	8
B5	1.0	6*
B5	1.5	4*
B5	2.0	4*

The five tested basal media (MS, MS/2, B5, B5C and SH) contained 0.5 mg dm^{-3} BAP induced shoot formation but B5 was the most effective one followed in order by MS and B5C media (Table 4). Decreasing the level of micro- and macro-nutrients (MS/2) decreased the shoot formation. This in agreement with Nin *et al.* (1996) who found that increased the micro-nutrients of the basal

Table 4. Shoot organogenesis of *S. nigrum* from stem internode segments cultured on different types of media supplemented with 0.5 mg dm^{-3} BAP. Each value was recorded after three weeks on shoot induction medium (* - means significantly different from internodal stem segments on B5 medium at $P < 0.05$).

Medium	Number of adventitious buds [$explant^{-1}$]	Shoot formation [%]
B5	14	100
SH	3*	81
B5C	8*	93
MS/2	6	90
MS	9*	100

medium increased the number of the formed shoots. On the other hand, half-strength MS medium supplemented with appropriate hormone concentration induced maximum regeneration in other plant species (Torregrosa and Bouquet 1996).

Three types of explants (shoot tips of germinated seeds, leaf and stem internode segments) were used (Table 5, Fig. 3). Shoot tip and stem internode segments formed shoots at the same time (8 d) but the number of shoots was influenced. The appearance of adventitious shoots from leaf segments was retarded; buds appeared after 12 d. After 3 weeks on B5 medium supplemented with 0.5 mg dm^{-3} BAP, 20, 13, and 6 shoots per explant were found when shoot tips, stem internode segments, and leaf segments, respectively, were used as explants. While the position of explant upon agar surface seems important in other works due to the polarity of auxin transport and supply of nutrition (Sinnott 1960), this and other explant factors (see Durzan 1984) were not critical in *S. nigrum*. Therefore, the orientations of leaf explants and the shoot tips with cotyledons on shoot induction medium (B5 medium supplemented with 0.5 mg dm^{-3} BAP) did not influence the number of shoots/explant. Shoot formation from *S. nigrum* leaves was in agreement with that previously reported (Zenkteler 1972, Bhatt *et al.* 1979).

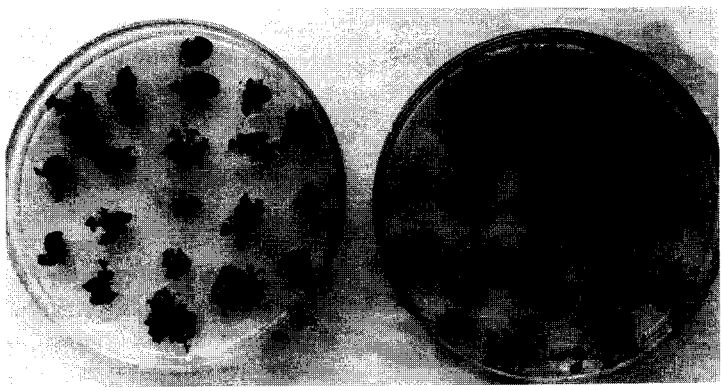

Fig. 3. The effect of the different types of explants on shoot formation. Leaf (*on the left*) and stem internode (*on the right*) segments cultured on B5 medium supplemented with 0.5 mg dm^{-3} BAP.

The percentage of stem explants which induced to form shoots and the number of shoots per explant varied widely under the influence of light treatments. The highest number of shoots was produced when the stem internode segments were cultured at 16-h photoperoid (irradiance of 100 μmol m^{-2} s^{-1}) and temperature at 25 ± 1 °C, where about 100 % of stem internode segments formed 16 shoots. Hu and Wang (1984) also reported that sufficient irradiance supported better shoot formation and production of plantlets which could be successfully transferred to soil. The regeneration potential decreased strongly under low irradiance of 20 μmol m^{-2} s^{-1} for 16-h photoperiod (9 shoots per explant) or complete darkness (5 shoots per explant). In the last case base of the explants formed friable-unregenerated calli. While the buds could be detected in 7 d under irradiance of 100 μmol m^{-2} s^{-1}, they could be detected after 11 d under other conditions. The effect of light on regeneration of *S. nigrum* was subject of separate report (Hassanein *et al.* 1999b).

Root formation: Root formation in *S. nigrum* was induced by 1 mg dm^{-3} IBA and other auxins. In general, like shoot formation, the formation of root was proceeded by slight enlargement at the base of explants before the root emergence (Fig. 4). De Klerk *et al.* (1997) reported that rhizogenesis must undergo three phases: induction, initiation and elongation, but for the difficulties to isolatethe induction phase in most experiments, this phase has been included into the initiation phase (Hu and Wang 1984). In general, an exogenous cytokinin/auxin ratio > 1 was needed to establish shoot formation and < 1 for root formation. In *S. nigrum* cytokinins was not needed for root induction. It's worthy to mention that some cytokinin residue is virtually present in the regenerated shoots due to their initiation on shoot induction medium with cytokinins.

Fig. 4. The enlargement and the appearance of adventitious roots at the base of the explant cultured for one week on half strength MS medium supplemented with 1 mg dm^{-3} IBA.

Within 8 d, the stem internodes or microshoots showed adventitious root formation when they were cultured on half-strength MS medium supplemented with different concentration of IBA or without phytohormones (Table 5). Root formation on auxin-free medium was not enough to transfer the rooted plantlets to the soil because only one or two roots are formed. IBA in concentration of 1 mg dm^{-3} was most effective. Increasing the concentration of IBA more than 1 mg dm^{-3} decreased the number and growth of the formed roots (Table 5). The segments which did not form roots, formed callus (Lane 1979). The percentage of root formation of cultured microshoots (100 % using 1 mg dm^{-3} IBA) was higher than that of stem internode segments (55 %) under all the tested concentrations of IBA, but the number of roots was nearly the same. This indicate that the presence of leaves and shoot tips as a system for regulation of endogenous phytohormones and a receiver for light was very important for root organogenesis of *S. nigrum* (Hassanein *et al.* 1999).

Table 5. Effect of different concentrations of IBA on root organogenesis of *S. nigrum* from microshoot segments on half strength MS medium. Each value was recorded after three weeks on root induction medium (* - means significantly different from roots on MS/2 medium supplemented with 1 mg dm^{-3} IBA at $P < 0.05$).

IBA [mg dm^{-3}]	Root formation [%]	Number of roots [explant^{-1}]	Length of root system [cm]
0	53	2*	1.5
1	100	9	1.4
3	48	5*	0.5*
5	13	4*	0.1*

While the roots were formed in the presence or absence of auxins, differences in the number and the morphology of the formed roots were observed under the influence of different auxins (Table 6). When NAA was used hairy roots were observed. In other works, NAA was the most effective auxin for induction of root formation (Hu and Wang 1984). The growth of roots after root initiation was not significantly influenced by the effective concentration of IBA (1 mg dm^{-3}) in comparison to plants rooted on phytohormone-free-medium (Table 5).

Table 6. Root organogenesis of *S. nigrum* from microshoots cultured on MS medium supplemented with different types of auxins. Each value was recorded after three weeks on root induction medium (* - means significantly different from roots MS/2 medium supplemented with 1 mg dm^{-3} IBA at $P < 0.05$).

Auxin [mg dm^{-3}]	Number of roots [shoot^{-1}]	Root formation [%]
0	2	100
1 (IBA)	9	100
1 (IAA)	7	77
1 (NAA)	4*	64

All the tested media with 1 mg dm^{-3} IBA induce root formation on stem internode or microshoot segments but the number of the initiated roots were influenced (Fig. 5). When the salt concentration of the root induction medium was lowered to one-half (MS/2, MSC/2) or using SH medium, rooting was better (9 roots per shoot) than on the high salt media such as B5 and MS (6 and 7 roots per shoot, respectively). Sometimes roots are unable to initiate in such high salt concentration regardless of the type of hormone present (Hu and Wang 1984, Endress 1994). It is worthy to mention that sucrose concentration of MS medium has no effect on root formation of *S. nigrum*.

Darkness decreased the percentage of stem internode segments which were responded to form roots on IBA containing medium. Also, the number of roots per explant and root growth were retarded (Hassanein *et al.* 1999b). The retardation in root formation was accompanied with an increase of callus formation on the base of explant. On the other side, root growth was more better when the culture containers were wrapped with aluminum foil (Hennen and Sheehan 1978). Light induced phenol compounds synthesis which synergised the role of IBA in root formation of *S. nigrum* (Hu and Wang 1984, Hassanein 1999b). Therefore, the number of formed shoots on microshoots increased with the increase the light irradiance. While, microshoots cultured at darkness formed only 4 roots, microshoots cultured at 16-h photoperiod with irradiance of 20 μmol m^{-2} s^{-1} (dim light) formed 7 roots, and microshoots cultured at 16-h photoperiod with irradiance of 100 μmol m^{-2} s^{-1} formed 9 roots.

Protoplast culture: Protoplast were easily isolated from shoot tips with the youngest three leaves or from old leaves (6-weeks-old). Most of the released protoplasts (90 %) were highly cytoplasmic and spherical, especially when the protoplasts were isolated from shoot tip. In V-KM medium the protoplasts began to divide 2 d after culture. In two weeks microcalli imbedded in agarose lens were easily detected using inverted microscope. These microcalli were transferred to MS, B5 or B5C medium containing 0.5 mg dm^{-3} BAP to get shoot formation. B5C was the most suitable medium for shoot formation (60 %) in comparison to B5 (51 %) or MS (34 %). The number of the regenerants obtained from protoplasts isolated from shoot tips (110 shoots from 0.5 g shoot tips) was higher (six fold) than that obtained from protoplasts isolated from the same mass of mature leaves (18 from 0.5 g of leaves). Binding (1985) reported that shoot organization

was appeared in *S. nigrum* in 14 d after protoplast isolation when cell colonies were transferred to the regeneration media 5 d after plating.

The regenerated shoots were rooted on half strength medium supplemented with 1 mg dm^{-3} IBA and transferred to the soil. All these transferred plants were fertile. The high regeneration ability of *S. nigrum* protoplasts and presence of marker gene (atrazine resistance on pt-DNA) make *S. nigrum* as suitable partner in somatic hybridization experiments to produce hybrid and cybrid plants (Hassanein 1993, Gressel *et al.* 1984, Binding *et al.* 1982, 1987, 1988a, 1992).

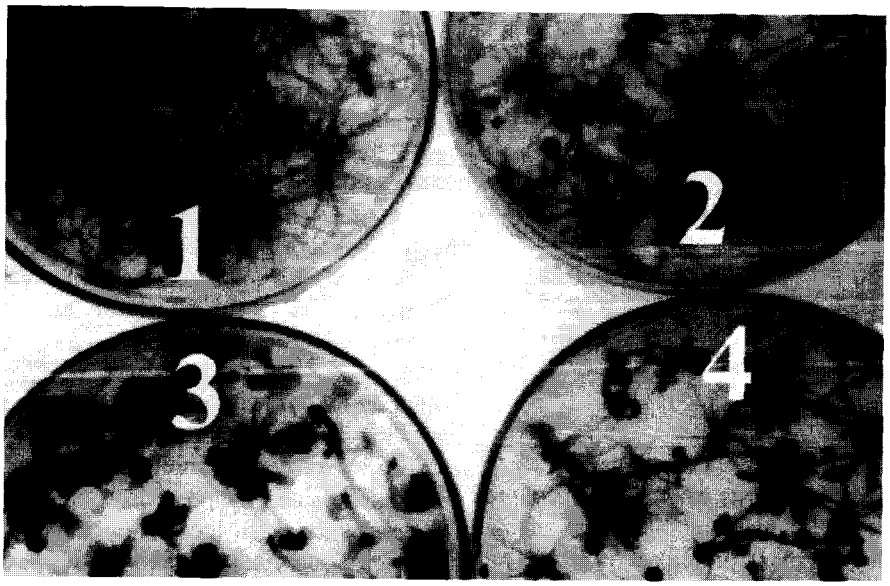

Fig. 5. Root formation from the base of the microshoot cuttings cultured on different types of basal medium for five weeks: half strength MS with 1.5 % sucrose (dish 1); half strength MS with 3 % sucrose (dish 2); SH (dish 3) and MS (dish 4).

Table 7. Effect of different protocols on callus formation obtained from 0.5 g of young and old tissues. Each value was recorded after six weeks of culture (* - means significantly different from values obtained by culturing shoot tip protoplasts on V-KM and callus induction medium at $P < 0.05$).

	Protoplast young shoot tip	old expanded leaves	Tiny explant young stem internode	old stem internode	Large explant young stem internode	old stem internode
Number of calli	109	66 *	84 *	73 *	53 *	44 *
Mass of calli [g]	10.8	7.4 *	14.8*	13.4*	7.8 *	9 *
Regenerated calli [%]	66	53.3	40.1	11.7	28.0	12.3

Callus formation: After 5 d on callus induction medium the base of stem or the edge of the leaf segments showed continuos increase in size leading to callus formation. The most suitable combination for callus formation was MS basal medium containing 0.5 mg dm^{-3} BAP, 1 mg dm^{-3} NAA and 0.1 mg dm^{-3} 2,4-D, it termed MTH (Table 8). In general, high auxin and low cytokinin concentrations stimulate cell division and callus formation (Endress 1994). Addition of BAP into the callus induction medium increased the callus mass. Callus could be divided and transferred to new medium in 2 - 3 weeks intervals.

In agarose sheets and liquid V-KM medium, the inverted microscope examination showed that all the tiny-explants or even cell aggregates excised from *S. nigrum* leaves or stems showed high cell division and microcalli formations on the cut ends of the tiny-explants. Using this method, callus could be established in high quantity when the microcalli were transferred from V-KM into MTH. The mass of calli obtained from tiny-young-stems in 6 weeks (14.8 g of calli per 0.5 g of cultured tissue) was higher than those obtained from tiny-mature-stems (13.4 g). The number of calli and their mass as well as their ability to regenerate shoots of young tissues was higher than that of old tissues (Table 7).

Callus could be established from cultured protoplasts upon their transfer from V-KM (after the formation of microcalli in 2 - 3 weeks) into MTH. It is well known that the transferred fragment of callus must be of sufficient

size (20 - 100 mg) to ensure renewed growth on the fresh medium (Street 1969, Dodds and Roberts 1995). The microcalli of *S. nigrum* during their transfer from V-KM into MTH was very small (2 - 5 mg), nevertheless their size was sufficient to establish callus formation. These calli increased in size continuously when they subcultured every two weeks on MTH medium. The mass of calli obtained by culturing of plant protoplast isolated from shoot tips was higher than from that obtained from fully expanded leaves. The mass of calli obtained from culture of tiny explants was higher than that obtained from the same mass of other types of explants irrespective their age and the used protocols (Table 7).

Table 8. Effect of different combinations of phytohormones on callus formation and callus growth of normal size explants in ten weeks on MS medium (* - means significantly different from calli on callus induction medium at $P < 0.05$).

BAP [mg dm^{-3}]	NAA [mg dm^{-3}]	2,4-D [mg dm^{-3}]	Callus formation [%]	Calli fresh mass [g]
1.00	1	-	54	0.22
0.25	1	0.1	79	0.50 *
0.50	1	0.1	100	0.30
0.75	1	0.1	46	0.36 *
-	1	0.1	88	0.11 *
-	-	0.1	83	0.17 *
-	-	1.0	82	0.04 *

Transfer of the calli from MTH media onto B5C medium with 0.5 mg dm^{-3} BAP resulted in shoot regeneration of *S. nigrum*. On shoot induction medium, shoot regeneration of callus obtained from protoplast cultured on V-KM and MTH media (66 %) was higher than of callus obtained from tiny (40 %) or large stem (28 %) segments (Table 7). In general, if the microcalli obtained from shoot tip protoplasts were transferred directly from V-KM to shoot induction medium, 80 % of the formed calli formed shoots. It means that by changing the culture conditions suitable for callus formation, the meristematic cells developing during callus formation are stimulated to shoot organogenesis (Hu and Wang 1984, Eddress 1994). When the calli of *S. nigrum* were left for more than 4 weeks on MTH without subculture most of them formed roots (63 %) but few (3.5 %) initiated one or two shoots. Endress (1994) reported that delay transfer the *S. nigrum* calli from callus induction to regeneration medium is sufficient to reduce the differentiation potential.

Transfer of the plantlets to the soil: Since tissue-cultured plantlets grown in a sheltered environment at 100 % relative humidity, the shock of transfer to a nonfavorable atmosphere results in immediate desiccation. Transfer the rooted microshoots of *S. nigrum* from tissue culture condition to plastic pots covered by plastic bags (10 d), maintained high humidity, was enough for successful transfer. The transferred shoots grew well in soil:sand:peat mixture of 1:1:1. The shoots rooted under the influence of IBA or IAA were successfully transferred to the soil, but shoots rooted under the influence of NAA never able to grow under open culture condition.

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