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Original Paper

Effect of medium strength and activated charcoal on *in vitro* shoot multiplications and growth of jojoba

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Abstract

In vitro grown plant materials of jojoba plant (*Simmondsia chinensis* L.) were used to study the effect of medium strength and activated charcoal (AC) on shoot multiplication and growth. Reduction in shoot multiplication was detected when explants were cultured on half strength MS medium. The highest shoot multiplication was obtained on the full strength MS medium containing 3.0 mg/l BAP and 0.1 mg/l NAA. Increase the osmotic pressure of the medium by increasing the concentrations up to double strength MS medium resulted in an increase of the growth parameters. Jojoba multiplication and *in vitro* growth was improved using activated charcoal; the best results were obtained on MS medium supplemented with 1 gm/l activated charcoal, 3 mg/l BAP and 0.1 mg/l NAA. While gene expression was stable under the influence of activated charcoal, it expressed some variation under the influence of medium strength. On the other hand, full medium strength resulted in maximum shoot multiplication and expressed several polypeptides with different molecular weights (150, 102.6, 65 and 42 KDa) and new peroxidase isoform (POX-1). Double strength MS medium expressed one polypeptide (8.6 KDa). Increased staining intensity of peroxidases and stimulate shoot growth.

Keywords: Charcoal, esterase, micropropagation, peroxidase, protein pattern.

Introduction:

*Simmondsia chinen*sis (Link) Schneider is the sole species in the family *Simmondsiaceae* and commonly known as jojoba. It is long-lived evergreen perennial woody desert shrub native to Sonoran desert of Arizona, South Western USA and Northern Mexico (Hogan, 1979; Mills *et al*., 1997). Due to its ability to tolerate several stresses and the economical importance of its liquid wax, jojoba is grown commercially in several countries such as Australia, Argentina, Egypt and India. Jojoba wax is used as replacement for sperm whale in several manufactures (Mills *et al.,* 1997; Benzioni *et al.,* 1999). Jojoba wax is used as an efficient lubricant over a wide range of temperatures (Yermanos, 1979: Low and Hackett 1981; Wang and Janick 1986). Jojoba oil is also used as anti-foaming agents and resins (Bashir *et al.*, 2008).

Propagation of jojoba can be established using seeds, seedlings and stem cuttings. While the conventional vegetative propagation using stem cuttings is widely used, some difficulties and constrains of this procedure makes the application of *in vitro* technique is the most interesting one for jojoba propagation. For example, jojoba is dioecious plant species and true-to-type by sexual propagation is not guaranteed (Chaturvedi and Sharma, 1989). In addition, vegetative propagation in jojoba was established by rooting of semi-hardwood cuttings but the maximum number of possible propagules is limited by plant size (Low and Hackett, 1981; Lee, 1988; Cao and Gao, 2003). Tissue culture obtained plants free from pathogens and grow more vigorously than others obtained from seedlings or vegetative propagation (Lee, 1988; Mohasseb *et al.,* 2009).

Different ingredients of inorganic and organic salts as well as inert supportive materials were used to culture plant cell, tissue or organ under *in vitro* conditions (Huang and

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Murashige, 1977). These ingredient as a chemical factors with other physical factors determine the success of cultures. In plant tissue culture techniques, activated charcoal is recommended (Lu *et al*., 1990). due to its structure with a very fine network of pores creating large inner surface area where many substances can be adsorbed (Pan and van staden 1998). Consequently, activated charcoal improves cell growth and development.

Gene expression can be studied by SDS PAGEs and isoenzymes to explain how plants are affected by developmental stages and stress agents. Implication of specific polypeptides in metabolism of plants under stress and tissue culture conditions was suggested (Radic and Pevaler-kozlina, 2010). Increase or decrease in the staining intensity of isoenzyme band represents a negative or positive change in enzyme activity (Khavkin and Zabrodina 1994; Hassanein *et al*., 1999_{a}). indicating that the gene involved in the synthesis of this isoenzyme form is differentially activated under specific condition (Chawla, 1991; Hassanein *et al*., $1999_{a, b}$; EI- Tayeb and Hassanein 2000).

Gene expression is influenced by biotic and abiotic factors. In jojoba as oil producing plant and tolerates stress conditions, gene expression is not fully understood. Consequently, the aim of this work was to study the effect of medium strength and activated charcoal on gene micropropagation and gene expression.

Materials and methods:

Plant materials:

Jojoba seeds were obtained from the Egyptian Natural Oil Co. S. A. E., Ismailia Farm, Salam Zone, Manayef, Ismailia, Cairo, Egypt. The farm was planted in Ismailia in 1991 and it was used for research and production. To establish shoot culture of jojoba, seeds were germinated in plastic pots under room condition. Nodeal segments were collected from one year old plants and subjected for sterilization in 5% clorox solution for four minutes followed by ethyl alcohol 70% contained drops of Twin 20 for further four minutes, then nodes were washed by sterilized distilled water three times, three minutes each. After sterilization, the ends of each nodal segment were discarded to reach maximum 1.5 cm long with one node prior to transfer to MS medium (Murashige and Skoog 1962).

The used MS medium was supplemented with 3% sucrose, 3 mg/l δ-benzylaminopurine (BAP) and 0.1 mg/l Naphalene acetic acid (NAA). The medium was solidified with 0.8 % agar at pH 5.8. Medium sterilization was done by autoclaving at 121°C for 20 minutes. Cultures were incubated in tissue culture room (28 ± 2°C with 16-h photoperiod at 100 µmol m-2 s-1). Thirty explants were used for each treatment. Regeneration frequency (%), number of shoots per explants, length of shoots (cm), number of leaves per shoot, and number of nodes per shoot were estimated.

Jojoba shoots multiplication and growth as influenced by medium strength:

Shoots (1.5 cm) obtained from *in vitro* grown plant materials were cultured on MS medium at different salt strengths: half strength, full strength, one and half full strength and double full strength. All the used media were supplemented with 3 mg/l BAP and 0.1 mg/l NAA. Cultures were incubated under tissue culture condition (16 h daily light at 100 µmol m-2 s-1) at 28 ± 2°C. After incubation period, length of shoots, number of leaves per shoot, and number of nods per shoot were determined.

To study gene expression of the jojoba cultivars, two isoenzymes were visualized; they were peroxidase (POX) and esterase (EST). In addition, shoots grown on different media strengths were collected, and subjected for SDS-PAGE analysis. Protein bands were visualized using Coomassie blue dye.

Effect of activated charcoal and several growth regulator concentrations on jojoba shoots growth and multiplication:

Jojoba microshoots were cultured on MS medium contained 1gm activated charcoal and several concentrations of phytohormones: MS medium without growth regulators, MS with 4 mg/l BAP and MS with 3 mg/l BAP and 0.1 mg/l NAA. Shoot length, leaves number per shoot, node number per shoot, fresh weight,

and number of shoots per explant were estimated after six weeks. Shoots were incubated under tissue culture conditions (16 h daily light at 100 μmol m-2 s-1) at 29 ± 1°C.

Effect of low concentrations of BAP and NAA as well as different concentrations of activated charcoal on shoot multiplication and shoot growth:

Microshoots were transferred to half strength MS medium supplemented with 1.25 mg/l BAP + 1.3 mg/l NAA and 0.5, 1, 2, and 3 g/l activated charcoal. The shoots were incubated at 30°c and 250 lux. After four weeks, shoots number per explants, shoot length, number of leaves, and number of node per shoot were determined.

Isoenzyme analysis:

One gram of the plant material was ground on ice in a mortar using 1 cm3 0.04 M Tris-HCl, pH 7.0, containing 0.002 M cysteine and was used for esterase detection. In case of SDS-PAGE the buffer contained 0.25 M Tris-HCl, pH 8.5, and 0.3 % (v/v) 2-mercaptoethanol. In both cases, the homogenate was centrifuged at 15000 g at 4℃ for 15 min. native PAGE was performed in 7.5 % acrylamide slab gels. Protein samples were loaded onto the gel wells for electrophoresis. Gels were run at 10 mA per gel for 6 h at 4 ℃ with 0.025 M Tris-HCl + 0.192 M glycine buffer, pH 8.9

Peroxidase detection:

The peroxidase bands were stained by phosphate buffer, Guaiacol and H_2O_2 as described by Siegel and Galston 1967.

Esterase detection:

The esterase bands were stained by α- and β-naphthyl acetate with blue RR salt as described by Brewer (1970) and Chibbar *et al*., (1988).

SDS PAGE Analysis:

Approximately 1 gram fresh plant materials were ground in a mortar and pestle in liquid nitrogen. Crashing continued until the plant materials were completely homogenized. The crushed samples were mixed with 1 ml extraction buffer (50 mM Tris-HCl buffer, pH 6.8, glycerol 10% w/v, ascorbic acid 0.1%, cysteine hydrochlode 0.1% w/v). The samples were subjected for centrifugation at 15000 rpm for 30 min. Electrophoresis was performed using 11% polyacrylamide gel at 10 °C and 60 mA according Laemmli (1970). Coomassie blue staining method for the SDS PAGE was performed.

Statistical analysis:

Treatments were compared by LSD test at the 5% level of significance (p = 0.05).

Results and Discussion

One year old plants were used as a source of explants in this work. Nodal explants were sterilized as described in materials and methods section and transferred to induce shoot multiplication on MS medium supplemented with 3 mg/l BAP and 0.1 mg/l NAA.P. Then, these *in vitro* grown plant materials were used to study the effect of medium strength on shoot multiplication and growth. The obtained data indicated that the number of formed shoots was significantly decreased when the concentrations of MS components were more than or less than full strength MS components (Table 1). The highest shoot multiplication was obtained on the full strength MS medium containing 3.0 mg/l BAP and 0.1 mg/l NAA as was reported previously by Hassanein *et al*., (2015). Generally, the calculated growth parameters increased when the explants were cultured on MS medium with chemical components higher than that of the normal basal MS medium (full strength). In jojoba as stress tolerant plant species (Al-Ani *et al*., 1972), increase the osmotic pressure of the medium by increasing the concentrations of medium components stimulated jojoba growth. In general, decrease the concentration of MS components to half strength resulted in a decrease in the values of the measured parameters. On the other hand, (Mohasseb *et al*., 2009). found that half strength MS medium containing growth regulators established multiple shoot formation in jojoba but the used medium did not support shoot growth where they remained compact and stunted, and they needed full strength MS medium to continue normal growth.

The highest leaf number/shoot was obtained when the jojoba shoots were cultured for 8 weeks on double strength MS medium supplemented with 3 mg/l BAP and 0.1 mg/l

NAA (Fig. 1). Under the high concentration of MS components more than one leaf/ node was formed, where two or three leaves/ node were detected. On the other side, when the concentration of MS components were reduced to less than double strength MS medium, sometimes one leaf/ node was formed. Therefore the number of leaves/ node was not in accordance with the number of nodes/shoot.

MS strength	Number of shoots/ ex4plant	Length of shoot (cm.)	Number of leaves/ shoot	Number of nodes/ shoot	Shoot fresh weight (g)
Half	4*	3.6*	10*	5*	0.18*
Full	12	6	12	7	0.3
One and half full	7*	7*	10*	5*	0.36
Double full	7*	7.8*	15*	6	0.38

Table (1): Effect of medium strength on *in vitro* grown shoots on MS medium supplemented with 3mg/l BAP and 0.1 mg/l NAA for eight weeks.

* Means significantly different (t-test) from jojoba shoots cultured on full MS medium supplemented with 3mg/l BAP and 0.1 mg/l NAA at $P < 0.05$.

Fig. (1): Photograph showing *in vitro* shoot growth under the influence of medium strength (MS) containing 3 mg/l BAP and 0.1 mg/l NAA: (a) shoot was incubated in glass jar containing full strength MS medium; (b) one and half strength MS medium and (c) double strength MS medium for eight weeks.

Gene expression was studied under the influence of medium strength. SDS-PAGE pattern of jojoba shoots grown for one week on different salt strengths of MS medium was studied (Fig. 2). Gel analysis indicated that, about 30 polypeptides with apparent molecular weight ranging from 8.6-200 KDa were detected. The synthesis of polypeptides in jojoba shoots was not strongly influenced by the concentration of MS components. The highest staining intensity of polypeptide bands was detected when jojoba shoots were subcultured on full strength MS medium under tissue culture condition (Lane 2). On contrast, the lowest staining intensity of polypeptide bands was detected when jojoba shoots were subcultured on one and half full strength MS medium (Lane 3). Bands with different molecular weights (150, 102.6, 65, and 42.6 KDa) were detected when jojoba shoots were subcultured on half or full strength MS medium. On the other hand, the polypeptide of molecular weight of 8.6 KDa was detected when shoots were subcultured on double full strength MS medium (Lane 4) and it was not detected in shoots subjected to the other treatments.

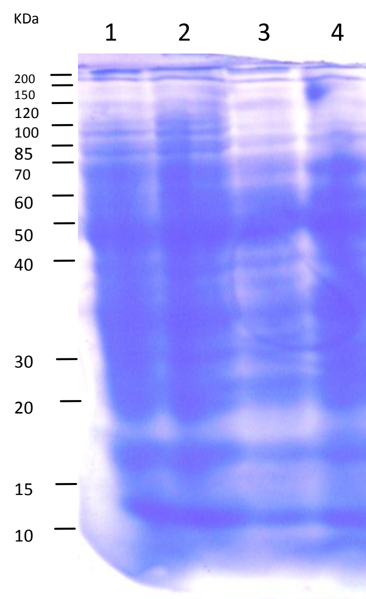

Fig. (2): SDS-PAGE of jojoba shoots were grown under the influence of different medium strength containing 3 mg. BAP and 0.1 mg. NAA for one week. Lane 2: jojoba shoots on full strength MS medium, Lane 1, 3, and 4 represent jojoba shoots on half, one and half, and double full MS strength, respectively.

To study the effect of medium strength on peroxidase expression, jojoba shoots were

subcultured on different MS salt strengths for one week and subject to isoperoxidase analysis (Fig.3). When conditions stimulated shoot multiplication on full strength MS medium or more were used, one new peroxidase band (POX-1) was detected (Fig. 3). In addition, staining intensity of several isoenzyme bands were progressively increased with the increase of MS strength. The highest staining intensity was detected when plant materials were cultured on one and half or double strength MS medium. It indicated that increase of peroxidase activity was necessary for establishment of shoot multiplication and improvement of shoot growth. The increase of the enzyme activity was illustrated by increase the number of isoenzyme forms and/or the increase in the staining intensity of isoenzyme bands (Khavkin and Zabrodina 1994, Hassanein, 1998; 2004 $_{a,\ b}$). The regeneration potential of the cultured tissue was dependent on both scavenging potential and the ability to produce H_2O_2 through NADH-POX activity. Phenol oxidases use phenolic compounds to scavenge the reactive oxygen species (Nicholson and Hammerschmidt 1992; Benson and Roubelakis-Angelakis 1994; Elstner and Osswald 1994).

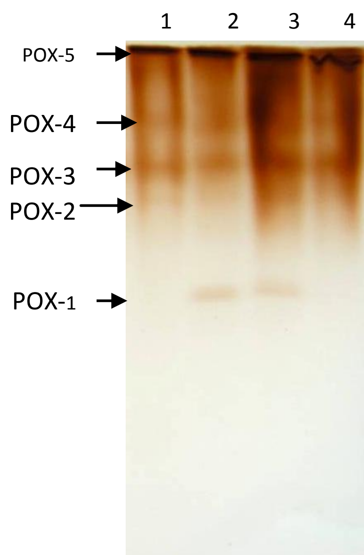

Fig. (3): Native gel electrophoresis of POX isoenzyme pattern of jojoba shoots grown under the influence of different MS medium strength containing 3 mg/l BAP and 0.1 mg/l NAA for one week. Lane 2: jojoba shoots on full strength MS medium, lanes 1, 3, and 4 jojoba shoots on half, one and half, and double full MS strength, respectively.

Esterase (EST) expression under the influence of different medium salt strengths was visualized in Figure 4. A total of 16 different EST isoenzyme forms were detected. Each MS strength expressed identical pattern. One isoenzyme forms (EST-1) was detected when jojoba shoots were subcultured on full MS strength medium supplemented with 3 mg/l. BAP and 0.1 mg/l NAA for one week (Lane 2), this band could not be detected under the other applied conditions. While distinct pattern of EST was expressed when the shoot cuttings were cultured on full or double strength of MS medium, where two EST bands (EST-7 and EST- 16) were detected (lane 2 and 4), the staining intensity of bands of shoots cultured on double strength MS medium was higher than that under full strength MS medium. In addition, one isoenzyme form (EST-9) was only detected when jojoba shoots were subcultured on double MS strength (Lane 4**),** this isoenzyme form could not be detected under the other applied conditions. Distinct isoenzyme form (EST-15) could only be detected when the plant materials were subcultured on 1.5 MS strength.

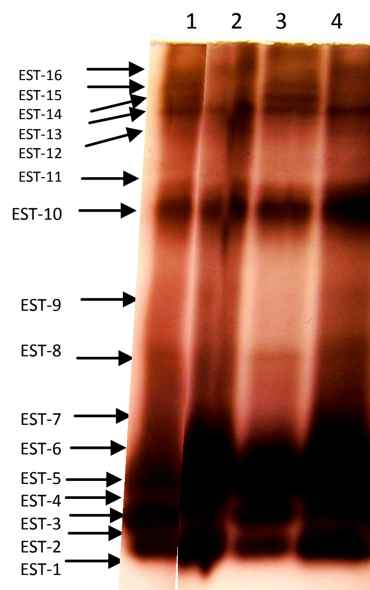

Fig. (4): Native gel electrophoresis of esterase isoenzyme pattern of jojoba shoots grown under the influence of different MS medium strengths containing 3 mg/l BAP and 0.1 mg/l NAA for one week. Lane 2: jojoba shoots on full strength MS medium supplemented,

lanes 1, 3, and 4 jojoba shoots on half, one and half, and double full MS strength, respectively.

Activated charcoal was often used in plant tissue culture to improve cell growth and differentiation (Thomas 2008; Agrawal *et al.,* 2002). In general, activated charcoal improved jojoba multiplication and *in vitro*

growth. In six weeks, activated charcoal stimulated shoot multiplication and growth parameters. The positive effect of charcoal application was registered in other works (Abdulwahed, 2013). These values of growth parameters increased when the medium was supplemented with activated charcoal with BAP alone or in combination with low concentration of NAA. The best data was recorded when jojoba explants were cultured on MS medium supplemented with 1 gm/l activated charcoal in combination with 3 mg/l BAP and 0.1mg/l NAA. Thomas (2008) explained the positive effect of activated charcoal on cultured plant tissues. He reported that activated charcoal is characterized by fine network of pores with large inner surface area where many substances can be adsorbed. This processes exert positive effects on morphogenesis, it may be due to its ability to absorb of inhibitory compounds in the culture medium and decrease its toxic effect. In addition, activated charcoal has an important role in the release of natural substances which promote growth, increase the darkening of culture media, and adsorb of vitamins, metal ions and plant growth regulators including abscisic acid and ethylene (Thomas 2008).

BAP (mg/l)	NAA (mg/l)	Activated Charcoal	Shoot length (cm)	Leaves number/ shoot	Number of nods / shoot	Shoot fresh weight (gm)	Number of shoots /explants
--	--	--	3*	6*	3*	0.1*	1*
--	--	1 gm/l	5	10	5	0.17	2
4	--	1 gm/l	5.5	10	5	0.3*	3
3	0.1	1 gm/l	5	13*	9*	0.3	5*
3	0.1	-	5	12*	8*	0.2	4*

Table (2): Effect of 1 gm/l activated charcoal in combination with growth regulators on *in vitro* multiplication of jojoba shoots growth on MS medium for six weeks.

* Means significantly different (t-test) from jojoba shoots cultured on MS medium supplemented with 1gm/l activated charcoal at $P < 0.05$.

When low concentrations of growth regulators (1.25 mg/l BAP and 1.3 mg/l NAA) were used, the estimated parameters were significantly affected by the concentration of activated charcoal in the medium (Table 3). Shoot length and number of nodes/shoot increased with increase the concentration of activated charcoal. On the other side, the number of leaves decreased significantly with the increase of activated charcoal more than 0.5 gm/l medium. It was also registered when the strength of medium was reduced to half strength of MS medium. It was confirmed that the components of medium may be reduced due to the presence of activated charcoal as was reported by several authors. They reported that activated charcoal adsorb hormones (Nissen and Sutter,1990; Ebert *et al.,* 1993), vitamins (Pan and van Staden,1998; Thomas 2008), or metal ions (VanWinkle *et al.,* 2003). Drastic dip in concentration of PGRs and other organic supplements were reported (Thomas, 2008).

Ac. Conc. (gm)	Number of shoots/ explants	Shoot length (cm)	Number of leaves/ shoot	Number of nods/ shoot
0.5	2	4.6	14	5.3
1	1	6*	11.3	5.6*
2	1	7*	11*	5.6*
3	1	7*	12.3*	7*

Table (3): Effect of activated charcoal on shoot multiplication and growth of *in vitro* grown shoots on half strength MS medium for four weeks.

* Means significantly different (t-test) from jojoba shoots cultured on half strength MS medium supplemented with 1.25 mg/l BAP and 1.3 mg/l NAA and 0.5gm/l activated charcoal at $P < 0.05$.

SDS PAGE of jojoba microshoots under the influence of different concentrations of activated charcoal for one week expressed the same protein patterns (Fig. 5). Variation among the treatments could not be detected. Activated charcoal did not exert significant change in shoot multiplication and protein pattern when the concentration of growth regulators was low (1.25 mg/l BAP and 1.3 mg/l NAA) than reqiured for maximum multiplication (3 mg/l BAP and 0.1mg/l NAA).

Staining for peroxidase isoenzyme (POX) of jojoba microshoots under the influence of different activated charcoal concentrations for one week is shown in Figure 6. Generally, five isoenzyme forms were detected. No variation in the peroxidase isoenzyme pattern was detected among jojoba microshoots cultured on MS medium supplemented with (1.25 mg/l BAP and 1.3 mg/l NAA) and different concentrations of activated charcoal (0.5, 1, 2, and 3 gm/l). Both peroxidase and activated charcoal have an important role to prevent the accumulation of brown exudates resulted from phenolic oxidation (Fridborg *et al*., 1978; Thomas, 2008). Consequently, the applied culture conditions did not stimulate obvious change in peroxidase expression.

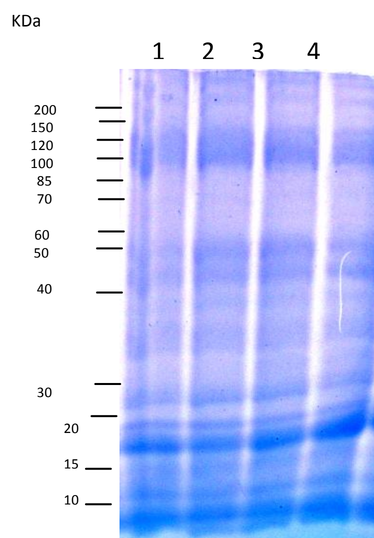

Fig. (5): SDS-PAGE of jojoba shoots cultured for 7 days on MS medium supplemented with 1.25 mg/l BAP and 1.3 mg/l NAA and 0.5, 1, 2, and 3 gm/l activated charcoal, lanes 1, 2, 3 and 4, respectively.

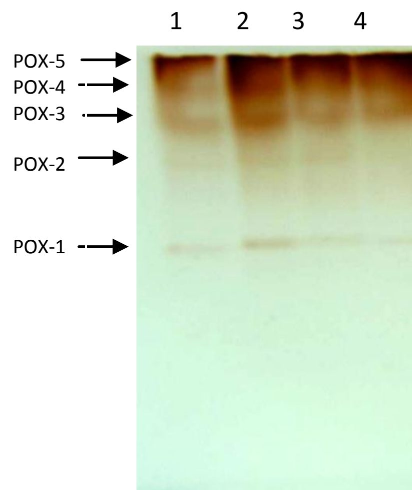

Fig. (6): Native gel electrophoresis of POX isoenzyme pattern of jojoba shoots cultured for 7 days on MS medium supplemented with 1.25 mg/l BAP and 1.3 mg/l NAA and 0.5, 1, 2, and 3 gm/l activated charcoal, lanes: 1, 2, 3 and 4, respectively.

Esterase expression of jojoba microshoots under the influence of different concentrations of activated charcoal was also studied. A total of 16 EST isoenzyme forms were detected (Figure 7). On contrast of SDS-PAGE and peroxidase pattern, the native esterase pattern showed some variation among the used microshoots under the influence of activated charcoal concentrations. It was clear that, culturing of jojoba microshoots on MS medium supplemented with 1.25 mg/l BAP + 1.3 mg/l NAA and 1gm/l activated charcoal (lane 2) resulted in disappearance of 8 isoenzyme forms (EST-1, -6, -7, -8, -9, 10, 11 and -12) in comparison to those of other treatments. Furthermore, staining intensity of bands of jojoba microshoots subcultured on MS medium supplemented with 1.25 mg/l BAP + 1.3 mg/l NAA and 2 gm/l activated charcoal (lane 3) was higher than those of the other treatments. Such changes in isoenzyme expression in jojoba and other plant species suggest that the genes involved in the synthesis of these isoenzymes are differentially activated during development (Chawla, 1991, Hassanein, 2004_a). In addition, the intensity of isoenzyme bands gives indication about the activity of these isoenzymes as was reported previously (Khavkin and Zabrodina 1994; Hassanein, 1998).

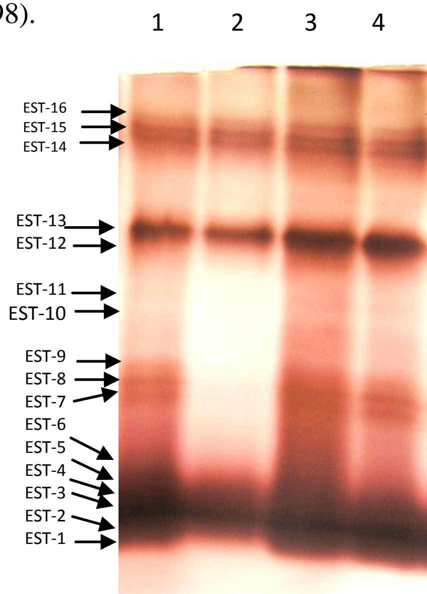

Fig. (7): Native gel electrophoresis of EST isoenzyme pattern of jojoba shoots cultured for 7 days on MS medium supplemented with 1.25 mg/l BAP + 1.3 mg/l NAA and 0.5, 1, 2, and 3 gm/l activated charcoal, lane 1, 2, 3 and 4, respectively.

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الملخص العربى

تأثير قوة الوسط الغذائي والفحم المنشط علي التضاعف الخارجي والنمو للأفرع الخضرية لنبات الجوجوبا

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ملخص

استخدمت الأفرع الخضرية لنبات الجوجوبا والنامية تحت ظروف المعمل لدراسة تأثير قوة الوسط الغذائي (MS) والفحم المنشط علي التضاعف والنمو الخارجي للأفرع الخضرية. تم تسجيل معدل منخفض من تضاعف الأفرع الخضرية عندما وضع مستقطع الأفرع الخضرية علي وسط غذائي بتركيز نصف قوة (half strength MS). تم الحصول علي افضل تضاعف لمستقطعات الأفرع الخضرية، عندما تم زراعتها علي وسط غذائي بتركيز قوة كاملة ومحتوية علي ٣ ملليجرام/ لتربنزيل أمينو بيورين و ٠،١ ملليجرام ام / لتر نفثالين اسيتيك. زيادة الضغط الاسموزي للوسط الغذائي بزيادة قوته إلي الضعف نتج عنه زيادة في قياسات النمو. تحسن التضاعف باستخدام مستقطعات من الأفرع الخضرية عندما زود الوسط الغذائي بالفحم المنشط، حيث كانت أفضل النتائج عندما زرعت المستقطعات النباتية علي وسط غذائي بتركيز قوة كاملة (MS) يحتوي علي ٣ ملليجرام / لتر بنزيل أمينو بيورين و ٠،١ ملليجرام / لتر نفثالين اسيتيك و ١ جرام / لتر فحم منشط. بينما شهد التعبير الجيني حالة من الثبات تحت تأثير الفحم النشط، شهد حالة من التغاير تحت تأثير التركيزات المختلفة من الوسط الغذائي. الوسط الغذائي بتركيز قوة كاملة نتج عنه افضل تضاعف للأفرع الخضرية مصحوباً بالتعبير لعدد من عديدة الببتيدات بالأوزان الجزيئية مختلفة (١٥٠و ١٠٢،٦ و ٦٥ و ٤٢ كيلو دالتون)، وكذلك التعبير لطرتز جديد من البيركسيديز (POX-1). تضاعف تركيز الوسط الغذائي نتج عنه التعبير لعديدة ببتيد بوزن جزيئي ٦،٨ كيلو دالتون، كما عمل ذلك علي زيادة النمو والصباغة لطرز انزيم البيروكسيديز.