CATRINA (2008), 3 (3): 25 -32

Micropropagation of *Capparis cartilaginea* Decne.

Raifa A. Hassanein[1], Madiha F. Gabr[2], Ahmed M. Ahmed[2], Ghada A. Hegazi[2*]
[1]Botany Department, Faculty of Sciences, Ain Shams University, Cairo, Egypt
[2]Ecology and Dry Land Agriculture Division, Desert Research Center, Cairo, Egypt

ABSTRACT

Micropropagation was used to propagate the wild economic important plant; *Capparis cartilaginea* which is difficult to propagate in normal conditions. For establishment and multiplication, MS medium containing 0.1 mg/L NAA + 3 mg/L BA was the most suitable medium for *Capparis cartilaginea* shoot tip explants. This medium gave the highest percentage of survival (100%), axillary shoot formation (76.74%), mean number (3.2) and length (0.564 cm) of axillary shoots per explant. The concentrations of 0.5 and 1 mg 2iP/L were suitable for the elongation of one (3.82 cm) and three shoots (3.114 cm) explants, respectively. The highest rooting percentage (22.22%) was obtained on half strength MS agar gelled medium supplemented with NAA at 2 mg/L under six days of dark incubation. A comparative study through the nucleic acids content and the protein banding pattern was carried out between the *in vitro* produced plantlets and the mother plant in autumn and spring seasons. The concentrations of RNA and DNA as well as the number of protein bands in the plantlets produced from tissue culture were increased over those of the newly growing parts of the mother plant.

Key words: *Capparis cartilaginea*, micropropagation, medicinal plants, nucleic acids, and protein profile.

INTRODUCTION

The conservation of natural populations of plants is very important for maintaining biological diversity. These plants have a current or potential economic value either in themselves or as donor of genes. *In vitro* propagation is one of the approaches used to conserve these plants and to avoid their loss (Laliberté, 1997). *Capparis cartilaginea* is a shrub belonging to the family *Capparidaceae*. It is difficult to propagate in normal conditions and is an important species as a source of active substances which can be used for several medicinal and other purposes. Phytochemical studies revealed that this species contains glycosides, tannins, flavonoids, alkaloids, saponins, unsaturated sterols and coumarins (Al-Gohary, 1987). Leaves and stems are used for bruises, childbirth, earache, headache, paralysis, snakebite, swelling and itching. The fruit is sweet when ripe, with strong fruity smell (Ghazanfar, 1994). Also, *Capparis cartilaginea* is used as a diuretic, tonic, expectorant, anthelmintic and emmenagogue, and to treat rheumatism, gout, tuberculosis and blood pressure (Auh and Aftab, 1994).

The present work is concerned to study the response of *Capparis cartilaginea* to micropropagation and consequently the production of identical true to type plantlets. There is no previous literature on the micropropagation of *Capparis cartilaginea* species. Although *Capparis spinosa*, Ancora and Cuozzo (1985) and Rodriguez *et al* (1990) developed multiple shoots from nodal shoot segments of field grown *Capparis spinosa* plants. Surface sterilization of (1-2 cm) nodal segments was accomplished by dipping the explants in 80% ethanol for 3 min. and subsequently in 20% commercial bleach (sodium hypochlorite 5.6%) plus 0.05% Tween-20 for 20 min. Tissues were then thoroughly rinsed several times with sterile distilled water. By culturing nodal shoot segments of *Capparis spinosa* on a modified MS medium in the presence of BA (4 μM) plus IAA (0.3 μM) and GA_3 (0.3 μM) a 20-fold multiplication every 20 days was achieved. Regeneration was induced on single shoots taken from proliferating clusters subcultured for 20 days on a medium with reduced BA (2 μM) without auxin and gibberellin. Higher rooting responses (70%) for *Capparis spinosa* were obtained after 20-day incubation period in darkness on solid half-strength MS1 medium (MS mineral salts+0.5 μM myo-inositol+1μM thiamine) plus IAA (30 μM), following by a subsequent 20-day culture period on half-strength MS1 basal medium (Rodriguez *et al*, 1990). Ancora and Cuozzo (1985) reported that primary shoots of *Capparis spinosa* were developed on MS medium, and axillary shoots were developed on transfer to a medium containing BA, GA_3 and IAA.

Moreover, Deora and Shekhawat (1995) developed a method for micropropagation of mature trees of *Cappparis decidua* by obtaining multiple shoots from nodal explants on MS medium+0.1 mg/L NAA+5 mg/L BAP+ additives (50 mg/L ascorbic acid and 25 mg/L each of adenine sulfate, L-arginine and citric acid). The shoots were multiplied by subculturing nodal shoot segments onto MS+0.1 mg/L IAA+1 mg/L BA+ additives, at intervals of 3 weeks. 60-70% of the shoots of *Capparis decidua* rooted when pulse treated with 100 mg/L IBA on half strength MS liquid medium for 4h, and then transferred onto hormone-free half strength agar-gelled MS basal salt medium. Incubation in the dark at ± 2?C for 6 days favored root induction.

* *Corresponding author: hegazighada@yahoo.com*

MATERIALS AND METHODS

(A) Micropropagation experiments

Capparis cartilaginea shrubs grow in the upstream parts of Wadi Sudr in South Sinai on rocky grounds and slopes. Healthy shoots with terminal buds were collected during the active growing seasons, spring (April) and autumn (October), moistened and wrapped. Explants were prepared and washed under running tap water for 10 min. for shoot tips and 30 min. for stem node sections.

1. Explant sterilization

The two explant types (shoot tips and stem node sections) were subjected to different sterilization treatments as following:

- Shoot tip explants were treated with different concentrations of sodium hypochlorite solution (1.5, 1 and 0.75% for 20 and 15 min. for each concentration).
- Stem node sections were treated with the following treatments:
  a. Treating with different concentrations of sodium hypochlorite solutions (2.5, 1.5, 1 and 0.75 %) for different periods (20, 15, 10 and 5 min.), respectively, in addition to 0.5% sodium hypochlorite solution for 5, 3, 1 min. and few seconds.
  b. Submerging the explants into 40% ethyl alcohol for 1 min., and then soaking in 0.75% sodium hypochlorite solution for 1 min.
  c. Treating with sodium hypochlorite solution for stem node sections covered with paraffin wax on the two ends to prevent the penetration of the solution to the internal tissues of the explant because of its high sensitivity to sterilization. Sodium hypochlorite solution at 1% used for surface sterilization was tried at different periods (20, 15, 10 and 5 min).
  d. Dipping the explants in 0.1% w/v mercuric chloride ($HgCl_2$) solution for few seconds.
  e. Sterilizing by several rinses with sterilized distilled water.

After sterilization, the two ends of the stem node sections were discarded and some explants were cut longitudinally, while the axillary buds were isolated from others.

2. The basic nutrient medium

Basal medium of Murashige and Skoog (1962) salts and vitamins supplemented with 100 mg/L myo-inositol and 30 g/L sucrose was used. Growth regulators such as benzyl adenine (BA), Δ²-isopentenyladenine (2iP), 2-naphthalene acetic acid (NAA), 3-indolebutyric acid (IBA) and indole-3-acetic acid (IAA) were used independently or in combination at different concentrations. All media were adjusted to pH 5.7-5.8 using either 0.1 N NaOH or 0.1 N HCl before gelling with 8 g/L agar in the establishment and rooting stages and 2.5 g/L phytagel in the proliferation stage. 15 ml volumes of media were dispensed into 25×150 mm culture tubes, and for proliferation; 30 ml volumes into baby food jars. All were closed with autoclavable polypropylene caps and autoclaved for 15 min. at 121?C and 1.1 Kg/cm² pressure for sterilization, then left to cool.

3. Culture conditions

The sterilized explants were cultured on the media under complete aseptic conditions in the Laminar Air Flow Hood. Tissue culture tubes and jars were then placed in an air conditioned incubation room at a temperature of 26±2?C under a photoperiod of 16 hour with a light intensity of 2 K Lux, provided by cool white day light fluorescent tubes.

4. Effect of growth regulators on the different growth stages of the explants

(a) Establishment and Multiplication stage

The media were supplemented with NAA (0.1 mg/L) in combination with different concentrations of BA (1, 3 and 5 mg/L), in addition to the control (nutrient medium without growth regulators). For multiplication, obtained axillary shoots were excised and cut into 1-2 bud segments then cultured on the best establishment medium without auxin. All treatments consisted of 17 replicates. Data were taken after 8 weeks of date of culture.

(b) Elongation stage

To enhance the elongation of the proliferated shoots, four different concentrations of 2iP (0.5, 1, 1.5 and 2 mg/L) were used, and two different types of explants (1-2.5 cm average of length) were used; one shoot explant and three shoots together. Each type of explant for each 2iP concentration had seven replicates, and the explants were cultured in tissue culture tubes and data were taken after 8 weeks of incubation.

(c) Rooting stage

Elongated shoots were tested for rooting on root induction media which were taken from the literature of different strengths of MS salts and vitamins in addition to 100 mg/L myo-inositol, 30 g/L sucrose and different treatments of auxins (IBA, IAA and NAA). Two explant types, one shoot explant and a cluster of shoots of at least 3 cm long were used. Two dark incubation periods, 6 days and 12 days were tested for each treatment.

Statistical analysis

Variance analysis of data was done using ANOVA test for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using Duncan (1955) new multiple range test. Means followed by the same letter are not significantly different at $p \leq 0.05$.

(B) Comparative studies

A comparison between the *in vitro* produced plantlets of *Capparis cartilaginea* and the mother plant in the two seasons (spring and autumn) was made through the nucleic acid content and the protein banding pattern.

1. Determination of nucleic acid content

The method applied for total RNA and DNA extraction was similar to that described by Morse and Carter (1949). RNA content was estimated by the orcinol reaction according to Dishe (1953) and DNA content was determined applying the diphenylamine (DPA) colour reaction as described by Burton (1956).

2. Protein banding pattern

Discontinuous Sodium Dodecyl Sulphate Polyacrylamide Slab Gel Electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970), as modified by Studier (973). It is used in the fractionation of total soluble protein. The molecular weight in rate of flow (Rf) of samples was calculated using a special computer program by Media cybernetics (1993-1997).

RESULTS

(A) Micropropagation experiments

1. Explant sterilization

The highest survival percentage from the sterilization treatments was 100% for shoot tip explants by using 1% sodium hypochlorite for 15 min followed by 3-4 times rinsing with sterile distilled water. For the stem node sections, all sterilization treatments applied caused whiteness of the explants after culturing them, even with the application of paraffin wax at the two ends of the stem cuttings. This means that such explants are very sensitive and may have a special tissue absorbs the sterilizing agent even through its epidermis.

2. Establishment and multiplication stage

Concerning the establishment and multiplication of shoot tip explants of *Capparis cartilaginea*, Table (1) indicates that the MS medium containing 0.1 mg/L NAA+3 mg/L BA was the most suitable medium (Photo 1). This medium gave the highest percentage of survival (100%) and axillary shoot formation (76.47%) as well as mean number (3.2) and length (0.564 cm) of axillary shoots per explant compared with the other used treatments.

Capparis cartilaginea axillary shoots produced from the establishment stage when cultured in auxin free medium (MS+3 mg/L BA) they gave 9.466 increase in number of axillary shoots/explant as shown in Photo (2). This stage of proliferation was repeated at regular intervals of 8 weeks for each subculture.

3. Elongation stage

The elongation experiment revealed that there is in general a negative correlation between the mean increase in number and length of axillary shoots; the treatment which gave more numbers of axillary shoots, their lengths were small and vise versa (Table 2). It can also be recommended the use of 2 mg/L 2iP for increasing the axillary shoot numbers for both one and 3

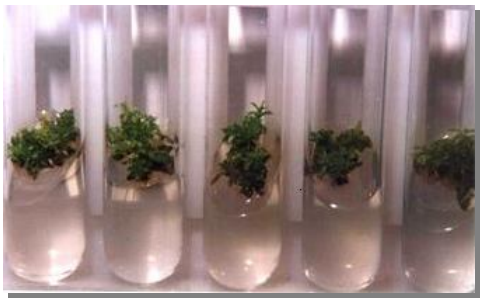

Photo (1): Establishment of *Capparis cartilaginea* shoot tip explants on MS medium+0.1 mg/L NAA+3 mg/L BA.

Photo (2): Proliferation of *Capparis cartilaginea* shoots on MS medium+3 mg/L BA.

Table (1): *In vitro* establishment and multiplication of shoot tips of *Capparis cartilaginea* cultured on MS nutrient medium supplemented with NAA and BA.

Conc. (mg/L)		% of survived explants	% of explants forming axillary shoots	Mean number of axillary shoots/explant	Mean length of axillary shoots (cm)
NAA	BA				
0.0	0.0	88.24	33.33	1.0 d	0.320 b
0.1	1.0	100.0	29.41	1.8 c	0.280 b
0.1	3.0	100.0	76.47	3.2 a	0.564 a
0.1	5.0	94.12	31.25	2.4 b	0.380 b

Table (2): Elongation of *in vitro* proliferated shoots of *Capparis cartilaginea* cultured on MS nutrient medium supplemented with different 2iP concentrations using two types of explants.

2iP conc. (mg/L)	Type of explant			
	One shoot		3 shoots together	
	Increase in		Increase in	
	mean number of axillary shoots/explant	mean length of axillary shoots (cm)	mean number of axillary shoots/explant	mean length of axillary shoots (cm)
0.5	1.8 b	3.82 a	3.8 a	1.760 b
1.0	3.6 a	2.44 b	1.4 b	3.114 a
1.5	1.2 b	2.00 b	4.2 a	1.800 b
2.0	3.8 a	0.68 c	4.0 a	0.486 c

shoots explants. While for axillary shoots elongation, the concentration of 0.5 and 1 mg/L are suitable for one and three shoots explants, respectively (Photo 3).

4. Rooting stage

The highest rooting percentage (22.22%) was obtained on half strength MS agar gelled nutrient medium supplemented with NAA at 2 mg/L under 6 days of dark incubation as shown in Table 3 (Photo 4).

(B) Comparative studies

1. Nucleic acids content

The results in Table (4) show clearly that during autumn, the concentrations of RNA as well as the ratio of RNA/DNA were considerably increased than those recorded during spring. On the contrary, the concentration of DNA was slightly higher in spring than in autumn. However, the concentration of both RNA and DNA in plantlets produced from tissue culture were considerably higher as compared with their contents in the newly growing parts of the mother plants. While the ratio of RNA/DNA in the *in vitro* plantlets was more or less the same as in the newly growing shoots of the native plants during spring. However, during autumn the ratio was greatly lower in plantlets than in the newly growing shoots.

2. The protein banding pattern

Table 5 and photo 5 show that the molecular weights expressed in rate of flow (Rf) ranged between (0.013) and (0.66). It can be also observed that 12 bands were appeared in autumn and spring on the same rows, except the band (R 11) of 0.3 Rf and (R 6) of 0.15 Rf value which presented in autumn and in spring, respectively. In the *in vitro* produced plantlets, the number of protein fractions was higher than those of the mother plant (18 bands).

Photo (3): Elongation of *Capparis cartilaginea* shoots on MS medium supplemented with different concentrations of 2iP using: one shoot explant (the above photo), 1= 0.5 mg/L 2iP, 2 = 1 mg/L 2iP, 3 = 1.5 mg/L 2iP and 4 = 2 mg/L 2iP and a cluster of 3 shoots (the photo below), 1=1 mg/L 2iP, 2=1.5 mg/L 2iP,3=2 mg/L 2iP and 4=0.5 mg/L 2iP.

Photo (4): Poor rooting of *Capparis cartilaginea.*

DISCUSSION

Micropropagation experiments

The results of the establishment and multiplication stage is in agreement with that obtained in *Acacia mangium* by Bhaskar and Subhash (1996). Such results suggest that the elimination of NAA from the medium produce higher rates of proliferation.

From the results obtained from the rooting experiments, it is noticed that all explants which gave

Table (3): Percentages of *in vitro* rooted explants of *Capparis cartilaginea*.

Nutrient medium	Auxin concentration (mg/L)			% of rooting	% of callusing
	NAA	IBA	IAA		
MS	0.0	0.0	0.0	0.000	0.000
	2.0	0.0	0.0	0.000	20.00
1/2 MS	0.0	0.0	0.0	16.67	0.000
	0.5	0.0	0.0	0.000	0.000
	2.0	0.0	0.0	22.22	22.22
	0.0	2.0 liquid soaking into 100 mg/L solution then transferred to auxin free ½ MS medium	0.0	0.000	0.000
	0.0	½ hr soaking	0.0	0.000	0.000
		2 hr soaking	0.0	0.000	0.000
		4 hr soaking	0.0	14.29	35.71
	0.0	0.0	5.0 for 6 days and 20 days then transfered to auxin free ½ MS medium	10.00	10.00
¼ MS	0.0	0.0	0.0	0.000	0.000
	0.0	0.5	0.0	0.000	18.18
Agar/water	0.0	0.0	0.0	0.000	0.000

Table 4. RNA and DNA contents and its ratio in the newly growing shoots of *Capparis cartilaginea* mother plant during autumn and spring and the *in vitro* produced plantlets.

RNA (mg/g.f.wt.)			DNA (mg/g.f.wt.)			RNA/DNA		
Autumn	Spring	Plantlets	Autumn	Spring	Plantlets	Autumn	Spring	Plantlets
2.825	0.866	7.392	0.535	0.635	3.250	5.330	1.360	2.100

positive results were those cultured on half strength MS nutrient medium, this agreed with the results of Sriskandarajal and Mullines (1981) who stated that reduction of the inorganic salts concentrations may be important for various plants in root initiation stage. Also, Nemeth (1986) reported that the use of mineral nutrients at half strength generally promoted rhizogenesis in woody plants. Torres (1989) added that concentration of macro and micro nutrients are frequently reduced to half their normal values during rooting phase, although this varies with plant species. Half strength MS medium free from hormones may give results according to Jones and Murashige (1974) who reported that in some species all what is required induce rooting is to transfer obtained shoots to a cytokinin free medium. NAA gave higher rooting percentage than IBA which followed by IAA which gave the least rooting percentage, this may be due to that NAA is much more active than IAA in promoting rooting while IBA has intermediate rooting activity for its intermediate susceptibility to various kinds of oxidative destruction (Kenton, 1955). The dark treatment promoted rooting and enhanced callus formation at the explant base which

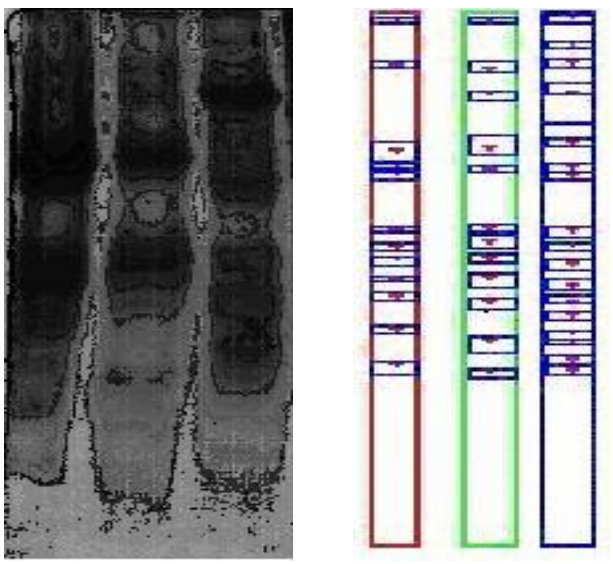

Photo (5): Photo and diagram of SDS-PAGE of protein in *Capparis cartilaginea* during autumn (Lane 1) and spring (Lane 2) as well as the *in vitro* produced plantlets (Lane 3).

Table (5): Rf values of the different protein fractions of *Capparis cartilaginea* during autumn and spring as well as the *in vitro* produced plantlets.

Protein fractions	Autumn	Spring	Plantlets
R1	0.013	0.013	0.013
R2			0.061
R3			
R4	0.1	0.1	0.096
R5			
R6		0.15	0.15
R7			
R8			0.2
R9	0.26	0.25	0.24
R10	0.29	0.29	0.29
R11	0.3		0.3
R12			
R13			
R14	0.41	0.4	0.4
R15	0.44	0.42	0.43
R16	0.46	0.45	0.46
R17	0.5	0.48	0.5
R18	0.53	0.53	0.53
R19			0.56
R20			0.59
R21	0.6	0.6	0.61
R22			0.64
R23	0.66	0.66	0.66
Total no. of bands	*12*	*12*	*18*

is common with woody plants (Ochatt *et al*, 1990) and was attributed to the rapid photodegradation of IAA and IBA (Epstein and Ludwig-Müller, 1993). This may be due to the rapid decrease in peroxidase activity, which controls the endogenous auxin concentration, or to the increase in phenolic compounds, which act on synergy with auxins, modulating auxin oxidase activity or protecting auxin action (Belaizi *et al*, 1989).

It could be concluded however, that *Capparis cartilaginea* gave very promising results in proliferation and elongation stages, its *in vitro* produced explants didn't succeed in high percentage of root initiation using the tested treatments, and it needs more studies to enhance the rooting percentage (Photo 4). In this regard, Hartmann and Kester (1975) stated that once a plant becomes mature, the ability to root may be diminished. In addition, Westwood (1972) explained this point and mentioned that juvenile tissues contain more rooting promoters than adult trees and lack flower buds which inhibit rooting.

Comparative studies

There is no doubt that nucleic acids play an important role in the different biochemical and physiological processes which place plant development. In the present results, during autumn the higher amounts of RNA and the activity of DNA to produce RNA (as recorded in the ratio of RNA/DNA), indicate the active synthesis of RNA during the period before new growing induction. It may be also refer to synthesis of different types of proteins as well as hydrolytic enzymes which protect the plants against the adverse conditions of drought and salinity. In this concern, Short *et al*. (1969) confirmed that in different plant species the high content and high rate of nucleic acid synthesis were associated with the beginning of the period of obvious growth. He found that both DNA and RNA contents of cell suspension culture of *Acer pseudoplatanus* rise early in the period of culture and before any significant increase in cell number with the onset rapid cell division, there is a rapid decline in DNA content per cell to reach a value which persists during sequent period of growth. On the other hand, the lower amounts of RNA during the active growing season might be attributed to the increase in dry weight of the cells at a more rapid rate than that of RNA (Hegazi, 1974).

From the protein banding battern, it can be concluded that the protein fractions in the *in vitro* produced plantlets were considerably more than that occur on the newly growing parts of the natural plants. This indicates that many proteins appeared to be synthesized in greater abundance during the tissue culture (Bon *et al.*, 1995). Also, some differences could be detected in protein fractions (bands) in the newly growing parts during autumn against spring. This indicates that the plants under dry seasons may synthesize some proteins to compete the adverse conditions. In this respect, Pareek *et al*. (1995) reported that specific proteins may accumulate in response to a number of different biotic or abiotic stress conditions. For instance, HSP 90 (a group of HSPs with molecular weights in the range of 80 to 90 KDa) accumulates in response to drought stress, heat stress, salt stress, cold stress and pathogen infection.

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Received November 1, 2008
Accepted December 20, 2008

الإكثار الدقيق لنبات اللصف

رئيفة حسانين[1]، مديحة جبر[2]، أحمد مرسى[2]، غادة حجازى[2]
[1]قسم النبات، كلية العلوم، جامعة عين شمس، مصر
[2]شعبة البيئة وزراعات المناطق الجافة، مركز بحوث الصحراء، مصر

الملخص العربـــى

يتلخص موضوع البحث في دراسة استجابة نبات اللصف، و هو نبات بري ذو قيمة طبية، للإكثار الدقيق لصعوبة إكثاره بالطرق العادية. أوضحت النتائج أن انسب بيئة للمرحلة البادئة و التضاعف للقمم النامية لنبات اللصف هي بيئة موراشيجي وسكوج التي تحتوي على 0.1 مجم/لتر من نفثالين حامض الخليك و3 مجم/لتر من البنزيل أدينين، حيث أعطت هذه البيئة أعلى نسبة تكوين نموات جانبية (76.74%)، 3.2 متوسط لعدد النموات الجانبية المتكونة لكل منفصل نباتي و0.564 سم متوسط لطول الفرع. وجد أن معاملة النموات الجانبية بأيزوبنتينيل أدينين له تأثير جيد على استطالتها، حيث أدى تركيز 0.5 مجم/لتر من الأيزوبنتينيل أدينين إلى حدوث استطالة مقدارها 3.82 سم لكل منفصل نباتي مكون من نمو واحد، أما تركيز 1 مجم/لتر أدى الى استطالة قدرها 3.114 سم لكل منفصل نباتي مكون من 3 نموات. أعلى نسبة تجذير (22.22%) وصلت إليها نموات نبات اللصف عند استخدام نصف قوة بيئة موراشيجي و سكوج مع إضافة الأجار و 2 مجم/لتر من نفثالين حامض الخليك، و تعريض المنفصلات النباتية الى 6 أيام من الإظلام. كذلك تم مقارنة النباتات الطبيعية بالنباتات ناتج زراعة الأنسجة من حيث محتوى الأحماض النووية و الأنواع البروتينية. لوحظ زيادة تركيز الحامض النووي الديؤكسي و الريبوزي و كذلك عدد البروتينات في النباتات الناتجة من الزراعة المعملية مقارنة بالنموات الحديثة للنبات الأم.