Dirasat, Pure Sciences, Volume **38**, No. **1**, 2011

Investigation of the Effect of Some Ultrasonic Waves and Electrotreatment in Callus Initiation and Plant Regeneration of *Ricinus Communis* L. Seedlings

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ABSTRACT

Explants of different parts (shoot tips, leaves, stems, cotyledonary leaves, hypocotyls and roots) of *Ricinus communis* seedlingsin addition to the callus derived from these explants, were exposed to ultrasonic waves at a frequency of 47.6KHz for 5,10, 15, 20, 30, 40, and 60 minutes. For the first time. The explants were cultured on agar solidified Murashige and Skoog medium containing various concentrations of Naphthalen acetic acid (NAA) and Benzyl adenine (BA).The electrotreatment involves the exposure of the above explants and its callus to different voltages (200, 500, 1000 volt) for 1, 5,10 milli second. The Results indicated the encouraging effect of ultrasonic waves in callus initiation and its growth. In fact, the inductive effect was represented by reducing the time needed for callus induction and the fresh weight of callus. Also, the protein content was increased in most samples. In addition, the ultrasonic waves had a stimulating effect on increasing the ability of shoot tips callus for shoots regeneration to reach 77.77% when exposed for 60 minutes. This study showed, in one aspect, the efficient and stimulative effect of electrotreatment in callus initiation, growth, protein content and differentiation of *R. communis.* The positive effect of this technique is reflected on the ability of stems and shoot tips callus for shoot formation as the percentage reached 120 % and 90 % when callus exposed to 200 and 1000 volt for 10msec, respectively.

Keywords: *Ricinus communis*, Ultrasonic Waves,Callus, Electrotreatment.

1. INTRODUCTION

Discoveries of cell cultures capable of producing specific medicinal compounds at a rate similar, or superior to that of intact plants are of increasing importance and, recently have attracted attention of researchers for application on different plants, since the production would be under controlled conditions independent of climatic changes or soil conditions (Vanisree *et al*., 2004).

Of the above mentioned techniques are ultrasound which is a sound of a frequency more than 20 KHz, inaudible to the human ear (Chisti, 2003). In biotechnological processes, ultrasonication method is widely used for laboratory scale and it does not require sophisticated equipment or extensive technical training (Yaldagard *et al*., 2007). It is a novel physical method acts as an alternative stress on cells or tissues. The most common interaction mechanisms which are involved in this case are acoustically induced cavitational activity which causes heat and chemical effects (Yaldagard et al., 2008). In addition to these, acceleration the rate of influx or uptaking of a substance into a seed by ultrasonication can also be caused by mechanical effect (Yaldagard *et al*., 2007). On the other hand, a part of the work of Wu and Lin(2002) indicated that the exposing of cell suspension culture of *Panax ginseng* to ultrasonic waves of frequency 38.5 KHz for 6 minutes, increase the production of saponins from these cultures.

Electroporation, described as the formation of transient pores which are formed in the cell membrane in presence of a strong external electrical field(Purves, 2001). Through these pores, ions and other small water-soluble objects may flow (Ji *et al*., 2006). It is known also as electropermeabilization, which is used in many important biological and medical applications (Pavlin *et al*., 2007; Prudhomme *et al*., 2006).It is the most successful method for introducing DNA into mycobacteria **(**Goude and Parish, 2008). It has been used to increase growth of callus, regeneration capability and protein content of different plant species such as *Matricaria chamomilla* (Salih, 2001), *Solanum nigrum*

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(Al-Mallah and Salih, 2003). It seems, that few information about using ultrasound technique on plant tissue culture, in general, is available. This motivated us to undergo an investigation, as a first trial, on using this technique on tissue culture of caster bean *Ricinus communis* L, a medicinal plant belonging to the family Euphorbiacea, aiming at testing the effect of ultrasonic waves and electrotreatment on callus initiation and plant regeneration of *R.communis*.

2. MATERIALS AND METHODS

Treatments

Explants of different parts (shoot tips, leaves, stems, cotyledonary leaves, hypocotyls, and roots) of *R.communis* seedlings, in addition to the callus which are prepared as mentioned in Al- Obaedi (2007), were divided into two groups. The first group was exposed to ultrasonic waves at a frequency of 47.6KHz for 5, 10, 15, 20, 30, 40 and 60 minutes.The second group was exposed to Electrotreatment using an electroporator (Al-Mallah, 2002) at different voltages (200, 500, 100 volts) for 1, 5, 10 milli second. Both groups were cultured on agar-solidified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing various concentrations of Naphthalen acetic acid (NAA) and Benzyl adenine (BA)as shown below (Al- Obaedi, 2007).

Best media for callus initiation from explant of R. communis L. plant

Explant	Medium
Hypocotyls, stems and roots	MS + 2.0mg /NAA & 1.2mg/LBA
Leaves and cotyledonary leaves	MS + 1.0mg/L NAA & 2.0 mg/LBA
Shoot tips	MS +2.0mg/LBA

Fresh weight of the callus, both that resulted from treated explants and the original one, was determined at 30 and 60 days post - treatment, respectively.

Protein Determination

Total protein content of callus was determined according to Lowery et al.(1951).

Regeneration of Plants from Treated Callus

Stems and shoot tips callus were cultured on selected regeneration medium (MS + 0.1 mg / L NAA and 1.0 mg/ LBA and MS + 2.0 mg/ LBA respectively) which is mentioned in Al- Obeadi (2007) and then exposed to ultrasonic waves of a frequency 47.6 KHz for the same periods mentioned above. Finally, specimens were incubated at 25 ± 2 ºC under alternative light and dark (16 and 8 hrs, respectively) and a light intensity of 1200 Lux. For electrotreatment, stems and shoot tips callus were cut into pieces of 1gm each, cultured on different media (mentioned above) at a rate of 3 pieces/ 20 ml medium. Samples were exposed to the same incubation conditions, mentioned above.

Rooting of the Regenerated Shoots

Shoots, regenerated from treated callus (both ultrasonic and electrotreatment) were transferred to a solidified MS medium, free of growth regulators.

3. RESULTS

Table (1) indicates that callus initiation reached 100% for all parts subjected to the ultrasonic waves accompanied by a reduction in the period of callus initiation, as callus formation was completed at day 15 post culturing. On the other hand, percentage of callus initiation decreased to 45% and 50% at subjection period of 40 and 60 minutes, respectively.

In addition, the ultrasonic waves reflected a positive effect on growth of callus resulted from parts subjected to this treatment too, pointing out an increase in the fresh weight of the resulted callus as the highest fresh weight of the shoot tip's callus was 8.0gm at 40 minut treatment compared to 6.0 gm for control (Fig.1,A).

For callus resulted from leaves, stems, and cotyledonary leaves the highest fresh weight was 9.859, 11.983 and 14.435gm, respectively (Fig.1,B,C,D), whereas the best fresh weight for hypocotyls callus was 12.327gm at 30 minute compared to 10.228gm for roots (Fig.1,E.F).

The results showed a stimulating effect of ultrasonic waves on the fresh weight of callus, exposed to the frequency used in this study. The best exposing period for shoot tips callus was 40 minute as the fresh weight of 1.0 gm of this callus reached 6.5 gm (Fig. 2,A). This period was also suitable for leaves callus since the fresh weight was 10.764gm compared to 6.430 gm to the control (Fig. 2, B). The highest bearing for 60 minute exposition among all callus types was that of stem's callus, the fresh weight was 11.116 gm compared to 10.302 gm for the

control (Fig. 2,C).Interestingly, 40 minute exposing period was optimum for cotyledonary leaves callus as its fresh weight was 7.906 gm (Fig. 2,D). Moreover, all exposing period were not suitable for hypocotyls callus except 30 minute exposing period, as the fresh weight reached 8.637 gm compared to 7.805gm for the control (Fig.2, E). Root callus showed a response similar to that of hypocotyls callus, and its fresh weight decreased to 4.809, 5.797 and 4.463 gm after subjection to 5,10 and 15 minute, respectively, as compared to the control (6.380).But the fresh weight increased to 8.233 at 30 minute exposing period (Fig. 2,F).

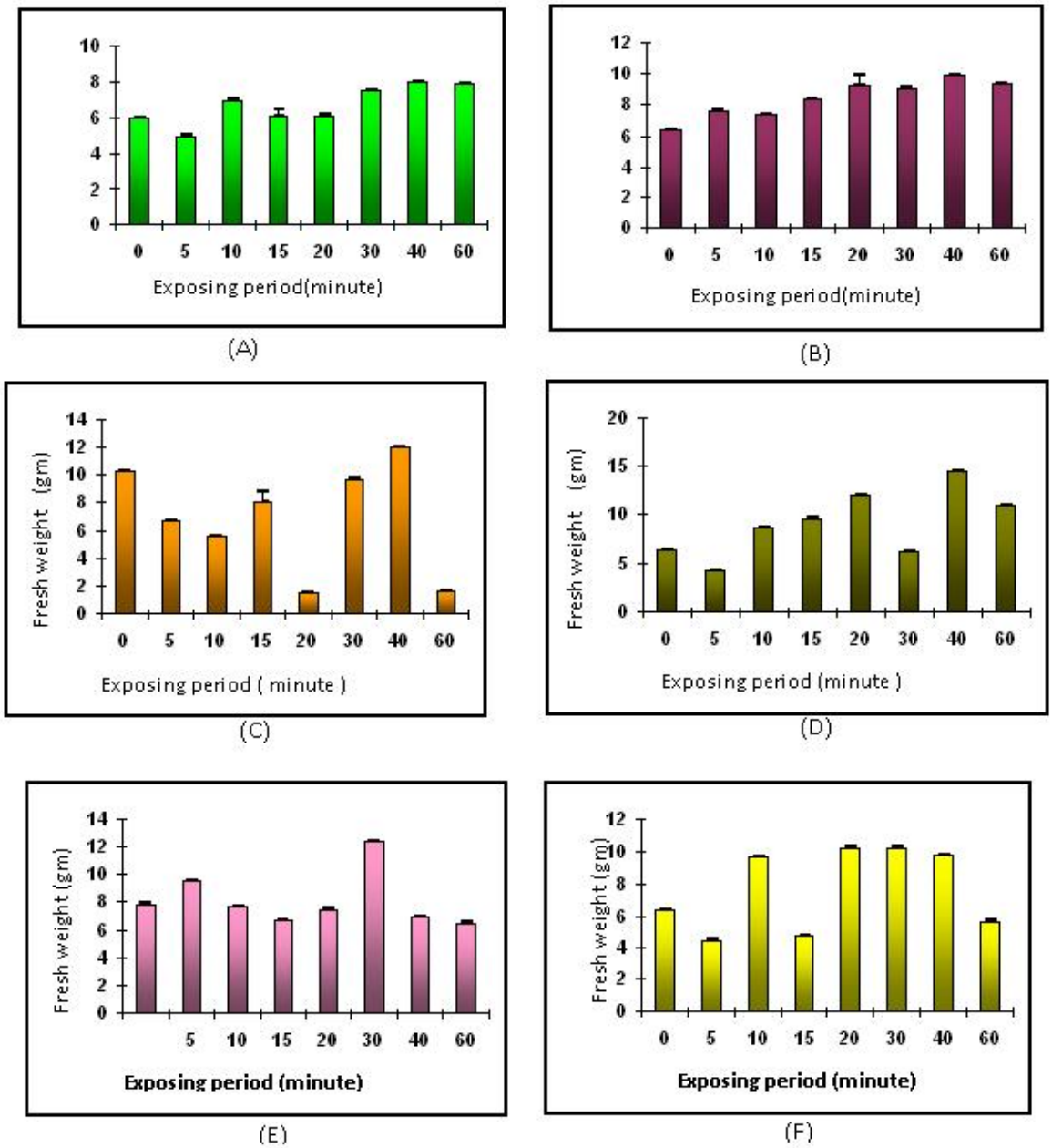

Fig. 1. Effect of ultrasonic waves (frequency47.6KHz) in fresh weight of callus after 30 days

(A): Callus derived from shoot tip subjected to ultrasonic period.

(B): Callus derived from leaves explants exposed to ultrasonic waves

(C): Callus derived from stem explants exposed to ultrasonic waves.

D): Callus derived from cotyledonary leaves exposed to ultrasonic waves.

(E): Callus derived from hypocotyls explants exposed to ultrasonic waves.

(F): Callus of roots explants exposed to ultrasonic waves.

Callus Maintenance

Callus which is subjected to ultrasonic waves grew faster than that of control, and it needed only two weeks for sub-culturing compared to 3-4 weeks for the control callus.

Effect of Ultrasonic Waves on Shoot Regeneration

It is obvious, that shoot formation from stem callus was not stimulated by ultrasonic waves (Table 2). In contrary, these waves promoted shooting from shoot tips callus, and shoot formation began 20 days post exposing to ultrasonic. At the beginning of shooting small singled leaves appeared (Fig. 3,A).followed by appearance of very short stem formation (Fig. 3,B). The percentage of shoot formation was 77.77 at 60 minute exposing period (Table3).All the shoots regenerated from ultrasonic subjective callus failed in rooting.

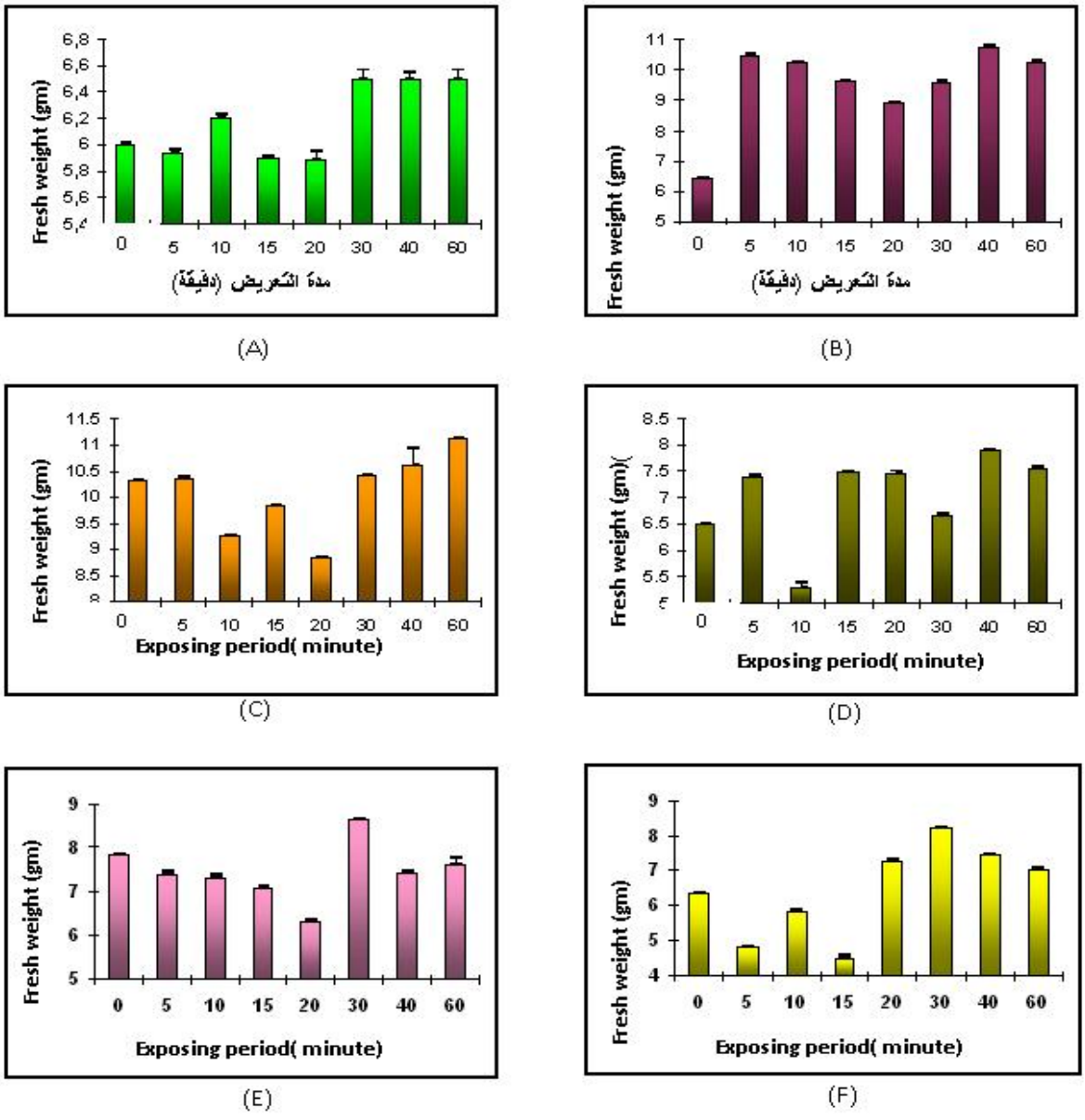

Fig. 2. Fresh weight of *R. communis* Callus after 30 days of exposing to ultrasonic waves

(A): callus of soot tips exposed to frequency 47.6KHz of ultrasonic waves

(B): Leaves callus.

(C): Callus of stems.

(D):Callus of cotyledonary leaves.

(E): Callus of hypocotyls (F): Callus of roots.

Table 1. Callus initiation from different parts of *R. communis* subjected to ultrasonic waves (frequency 47.6KHz)

Beginning of callus initiation(days)						callus initiation (%)					
Exposing period (minutes)	**ST*****	**CL****	**S***	**L****	**H***	**R****	**ST*****	CL ******	**S***	**L** **	**H***
5	9	13	7	15	9	100	100	100	100	100	100
10	9	12	7	13	9	100	100	100	100	100	100
15	8	12	8	12	8	100	100	100	100	100	100
20	8	10	8	12	6	100	100	100	100	100	100
30	7	10	5	10	6	100	100	100	100	100	100
40	7	10	5	10	5	100	100	100	100	45	100
60	7	10	5	10	5	100	100	100	100	50	100
control	8	14	8	18	7	100	100	100	100	100	100

***ST**: Shoot tip, **CL**: cotyledonary leaves, **S**: Stem, **L**: Leaves, **H**: Hypocotyls, **R**: Root.*

Number of cultured explants is 20 / treatment

**Explants cultured on MS medium containing 2.0 mg/L NAA and 1.2mg/L BA.*

***Explants cultured on MS medium supplemented with 1.0 mg/L NAA and 2.omg/L BA.*

**** Explants cultured on MS medium containing 2.0 mg /L BA.*

Table 2. Shoot regeneration from stems callus of R. communis exposed to ultrasonic waves (frequency 47.6KHz)in MS medium containing 1.0 mg/LNAA and 1.0mg/L BA

Exposing period (minute)	No. of regenerated shoots	Shoot formation(%)
5	0	0
10	0	0
15	2	20
20	0	0
30	0	0
40	0	0
60	0	0
Control	9	90

**No. of cultured pieces is 10/ treatment.*

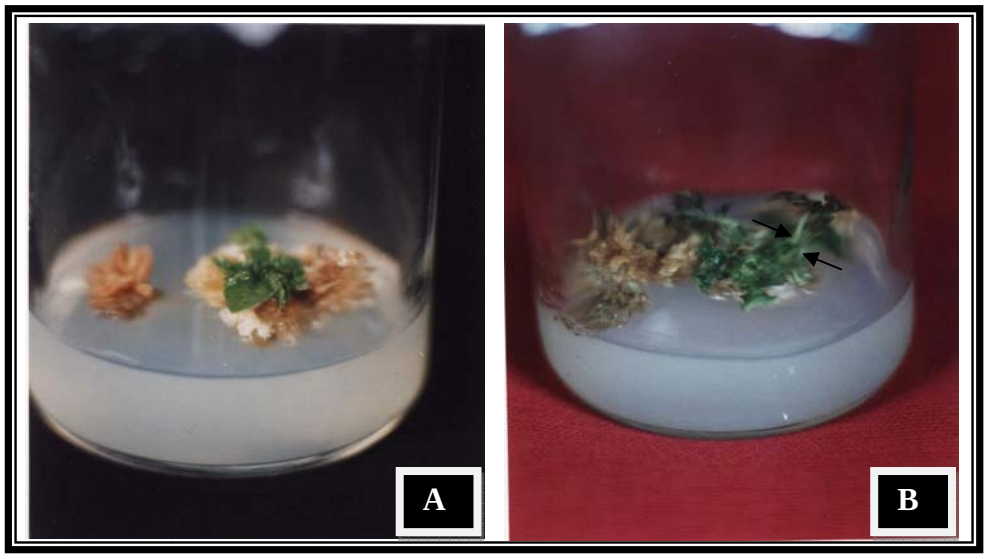

Fig. 3. Shoots regeneration from shoot tips callus exposed to ultrasonic waves (frequency 47.6 KHz) in MS medium containing 2.0mg/LBA

***(A):** Shoot tips callus after 20 days of exposing to above frequency for 10 minutes.*

***(B):** Callus in (A) after 30 days of culturing in the differentiation medium.*

Table 3. Shoots regeneration from shoot tips callus exposed to ultrasonic waves (frequency 47.6 KHz) in MS medium supplemented with 2.0 mg/LBA

Exposing period	No. of Shoots	Shoots formation (%)
5	5	55.55
10	4	44.44
15	5	55.55
20	6	66.66
30	5	55.55
40	6	66.66
60	7	77.77
Control	6	66.66

**No. of cultured pieces is 9/ treatment.*

Table 4. Protein content of *R. communis* callus exposed to frequency 47.6KHz of ultrasonic waves

Exposing period	Protein content mg/gm ± SD					
	Hypocotyls pocotyls	Leaves	Stems	Cotyledonary leaves	Shoot tips	Root
0	1.68±0.01	1.52±0.02	1.92±0.01	1.53±0.02	1.500±0.01	1.50±0.01
5	1.84±0.01	1.64±0.02	1.54±0.0	1.31±0.01	1.40±0.02	1.32±0.02
10	1.67±0.02	1.61±0.01	1.43±0.01	1.73±0.01	1.60±0.02	1.84±0.02
15	1.55±0.02	1.72±0.01	1.70±0.02	1.83±0.02	1.51±0.02	1.35±0.02
20	1.63±0.02	1.81±0.01	0.0±0.0	2.07±0.02	1.506±0.02	1.90±0.02
30	2.12±0.02	1.79±0.02	1.84±0.02	1.50±0.02	1.65±0.04	1.99±0.02
40	0.04±1.58	1.87±0.01	2.01±0.01	2.21±0.02	1.70±0.02	1.85±0.02
60	0.02±1.54	1.82±0.01	0.0±0.0	1.98±0.02	1.68±0.02	1.44±0.02

No. of replicates 3 / treatment

Determination of Protein Content

Results showed a trace increase in the protein content of callus derived from shoot tips subjected to ultrasonic waves, and its highest level was 1.7 mg/ gm in 40 minute exposing period, compared to 1.5 mg/ gm in the control. Beside that the content of stem callus, cotyledonary leaves and leaves was 2.01, 2.21 and 1.87 mg/gm respectively (Table 4). It is important to mention, that the protein content of stem callus decreased to 0.0 mg/gm in both 20 and 60 minute exposing period.

Effect of Electrotreatment in Callus Initiation of *R. communis*

Results showed the stimulating effect of different voltages (200, 500, 1000V) for different periods (1, 5, and 10msec) on callus initiation of R. communis. This effect is reflected in reducing the time needed for callus formation (Table 5). Also, the electrotreatment effect was obvious on increasing the fresh weight of callus exposed to different voltages. The response of callus to those treatments varied according to the source of the callus (Table 6).

Effect of Electrotreatment on Shoot Formation

The positive effect of electrotreatment was obvious in enhancing the ability of shoot tips and stems callus for shoot production. It appeared that exposing stem callus to 200V / 10msec increased the shoot formation up to 120 % (Table 7). Single leaves formation was the first step in shooting (Fig.4,A), followed by short stem formation (Fig.4,B),which developed to complete shoot afterv30 days. At the same time, the electrotreatment encouraged shoot tips callus to produce shoots, and the shoot appeared after 20 days.(Fig. 4,C).The best treatment was 1000V/10msec(Fig. 4,D), as the percentage of shoot formation reached 90 % (Table 8). The rooting of the regenerated shoots was very easy in solidified MS medium free from growth regulators which started 7 days post transferring to rooting medium. (Fig. 4, E). The regenerated plants were characterized by their fast and good growth (Fig.4, F).

Fig. 4. Ability of electroporated stem's and shoot tip's callus of R. communis on shoot formation

(A)*: beginning of shoot formation from stem's callus exposed to 500 V/ 1msec after 12 days of transferring to MS medium containing 0.1 mg/ LNAA and 1.0 mg/L BA.*

(B)*: Shoots in (A) after 18 days.*

(C)*: Shoot tips callus exposed to 1000 V/ 10msec after 20 days of culturing in MS medium containing 2.0 mg /L BA.*

(D)*: Shoot tips callus in (C) after 25days.*

(E)*: Shoot regenerated from electrotreted callus after 7 days in rooting medium (MS medium free from growth regulators).*

(F)*: Regenerated R. communis plant after 30 days from transferring to rooting medium.*

Table 7. Shoot formation from electrotreated stem's callus of *R. communis* after 30 days of culturing in MS medium containing 1.0 mg /LNAA and 1.0 mg /L BA

Treatments	Callus differentation	
	No. of shoots	Shoot formation (%)
200v/1msec	3	30
200v/5msec	5	50
200v/10msec	12	120
500v/1msec	11	110
500v/5msec	0	0
500v/10msec	0	0
1000v/1msec	0	0
1000v/5msec	0	0
1000v/10msec	0	0
control	9	90

**No. of cultured pieces 10 / treatment.*

Table 8. Effect of electrotreatment on shoot regeneration from shoot tips callus after 30 days of transferring to mS medium containing 2.0 mg/L BA

Treatments	Callus differentiation	
	No. of regenerated Shoots	Shoot formation (%)
200v/1msec	6	60
200v/5msec	7	70
200v/10msec	7	70
500v/1msec	8	80
500v/5msec	7	70
500v/10msec	7	70
1000v/1msec	8	80
1000v/5msec	8	80
1000v/10msec	9	90
Control	7	70

** No of cultured pieces 10/ treatment.*

Table 9. Protein content in *R. communis* callus exposed to electrotreatment

Treatments	∗Protein content (mg/ gm) ± SD					
	Hypocotyls hoot tips	Coteledonary leaves	Stems	Leaves	Hypocotyls	Roots
200V/1msec	0.02 ±1.742	0.04 ± 1.903	0.01 ± 1.910	0.02 ± 1.882	0.04 ± 1.713	0.01 ± 1.77
200V/5msec	0.02 ± 1.731	0.02 ± 1.980	0.01 ± 1.931	0.02 ± 1.800	0.07 ± 1.830	0.03 ± 1.809
200V/10msec	0.02 ± 1.810	0.05 ± 1.970	0.02 ± 1.983	0.03 ± 1.890	0.02 ± 1.796	0.02 ± 1.83
500V/1msec	0.02 ± 1.82	0.04 ± 1.86	0.03 ± 1.929	0.04 ± 1.851	0.04 ± 1.863	0.01 ± 1.81
500V/5msec	0.04 ± 1.66	0.02 ± 1.932	0.02 ± 1.851	0.02 ± 1.921	0.05 ± 1.681	0.02 ± 1.805
500V/10msec	0.04 ± 1.703	0.04 ± 1.88	0.03 ± 1.873	0.02 ± 1.859	0.01 ± 1.78	0.02 ± 1.883
1000V/1msec	0.02 ± 1.712	0.03 ± 1.909	0.03 ± 1.810	0.01 ± 1.856	0.04 ± 1.73	0.04 ± 1.920
1000V/5msec	0.04 ± 1.735	0.04 ± 1.820	0.03 ± 1.903	0.04 ± 2.001	0.02 ± 1.642	0.02 ± 1.922
1000V/10msec	0.04 ± 1.831	0.07 ± 1.921	0.04 ± 1.705	0.02 ± 1.940	0.01 ± 1.601	0.01 ± 1.923
Control	0.01 ± 1.500	0.02 ± 1.540	0.02 ± 1.930	0.04 ± 1.532	0.02 ± 1.670	0.01 ± 1.532

**No. of replicates 3 / treatment /plant part.*

Protein Content Determination

Results indicated the stimulating effect of electrotreatment on increasing the protein content of *R. communis* callus. The highest level of protein was 2.001 mg/gm in leaves callus exposed to 1000V/5msec, whereas the lowest level was 1.601mg/ gm in hypocotyls callus exposed to 1000V/ 10msec (Table 9).

4. DISCUSSION

Ultrasonic waves has attracted the attention of researchers in different aspects, and had many application on prokaryotic and eukaryotic cells (Wu and Wu, 2006).

In the present study, the ultrasonic waves had an obvious effect on callus initiation from different parts of *R. communis*, expressed by reducing the period required for callus initiation. This may be due to stimulation of auxins biosynthesis (Katrina and Milan, 2000). The effect of ultrasonic waves on viability of cell suspensions have been shown with *Petunia hybrida* (Bohm *etal*., 2000, 2002) which differ according to different exposing period. The stimulating effect of ultrasonic waves on fresh weight and protein content may refer to formation of small pores or cavities known as Acoustic cavitations (Suslik, 1988).The biomass and protein content of *Senedesmus acutus* increased when exposed to ultrasonic waves (Bozhkova and Dencheva, 1995). On the other hand, the decrease of fresh weight and protein content of stem callus exposed to these waves for 20 and 60 minute may refer to the mechanical effect of ultrasonic waves which, some times, lead to degeneration and death of cells (Lui *etal*., 2005). Suslik (1988) pointed out that these waves have inhibited DNA, RNA, and protein synthesis in *Pissum sativum*.

The obvious inhibitory effect of ultrasonic waves on shoot formation, noticed in most treatment in the present study except when shoot tips callus was exposed to 47.6 KHz for 60 minute may refer to the fact that these waves stimulate auxin synthesis (Byers, 1984) which lead to a disturbance in the equilibrium between cytokinines and auxins, then a decrease, and sometimes inability, of callus to produce shoots.The negative effect of ultrasonic waves has been reflected on rooting of shoots which may refer to the weakness and small- sized leaves, or to the above mentioned reasons.

In the present study, the positive and efficient effect of electrotreatment has been shown on growth and differentiation of callus through promoting the explants to form callus, which may be due to increase, or stimulation of DNA synthesis and cell division (Rech *etal.*, 1988).This is in accordance with the results of AL-Mallah (1993, 1994) on tobacco and Salih (2001) on chamomile. The positive effect of electrotreatmen on increasing the number of shoots formed from stem and shoot tips callus may refer to the stimulation of temporary pore formation in the cell membrane, enhancing the uptake of ions, nutrients, growth regulators and amino acids from the medium to the cell (Phansiri etal.,1994; Purves, 2001). On the other hand, the reduction of the ability of stem callus on shoot formation when voltage and exposing period was increased may refer to inability of callus cells to bear high voltages; or to the formation of large pores which can not be repaired by the cells leading a disturbance in the ionic equilibrium (Weaver, 1995).

The increase in the protein content in the callus exposed to electrotreatment may be due to the same reasons mentioned above. This result is in accordance with that of Joresbo and Brunsted (1991) on carrot, tobacco and sugar beat exposed to electrotreatment.

5. CONCLUSIONS

From the results of the present study it was clear that both ultrasonic waves and electrotreatment express a positive effect on callus initiation, growth and differentiation of *R. communis*, suggesting that both techniques have the potential to be used in plant biotechnology to improve other plant systems.

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تأثير الأمواج فوق السمعية والمعاملة الكهربائية في استحداث وتمايز كالس بادرات الخروع *Ricinus communis* L.

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ملخـص

في الدراسة الحالية عرضت القطع المختلفة (القمم النامية، الاوراق، السيقان، الاوراق الفلقية، السيقان تحت الفلقية والجذور) لبادرات الخروع *Ricinus communis* لاضافة الى الكالس الناتج من هذه القطع الى الامواج فوق السمعية بتردد 47.6 KHz لمدد مختلفة (5، 10، 15، 20، 30، 40، 60 دقيقة). زرعت القطع على وسط موراشيج وسكوك (MS) الصلب الحاوي تراكيز مختلفة من نفثالين حامض الخليك (NAA) والبنزايل أدنين (BA). كما تضمنت الدراسة الحالية تعريض القطع أعلاه والكالس الناتج منها الى فولتيات مختلفة (200، 500 و1000) لمدد 1، 5 و10 ملي ثانية. وأشارت النتائج الى التأثير المشجع للامواج فوق السمعية في نمو كالس الاجزاء المختلفة لبادرات الخروع، وقد تمثل التأثير التحفيزي في اختزال المدة الزمنية اللازمة لاستحداث الكالس اضافة الى زيادة الوزن الطري للكالس. كما أظهرت النتائج زيادة قابلية كالس القمم النامية على انتاج الافرع الخضرية لتصل إلى 77.77% عندما عرض للتردد أعلاه مدة 60 دقيقة. كما أظهرت هذه الدراسة في أحد جوانبها التأثير المحفز للمعاملة الكهربائية في استحداث كالس بادرات الخروع ونموه وتمايزه حيث بلغت نسبة تكوين الافرع الخضرية من كالس قطع السيقان والقمم النامية 120% و90% على الترتيب اضافة الى زيادة محتواه البروتيني.

الكلمـات الدالة: الخروع، الامواج فوق السمعية، الكالس، المعاملة الكهربائية.

* قسم علوم الحياة، كلية التربية، جامعة الموصل، الموصل، العراق. تاريخ استلام البحث 2010/1/5 وتاريخ قبوله 2010/12/26.