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PLANTLET REGENERATION FROM CALLUS OF *Citrullus colocynthis* COLLECTED FROM EL-WADI EL-GADID REGION AS A MEDICINAL PLANT

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ABSTRACT

Regeneration in Citrullus colocynthis collected from EL- Wadi El-Gadid region as a medicinal plant has been achieved through the disorganized culture (callus). Results showed that the best explants for callus formation were; the cotyledon and hypocotyledon when, cultured on MS + 0.1 mg/L kinetin + different concentrations of 2, 4-D + 0.8 % agar + 3 % sucrose at pH 5.8. This medium induced a golden callus at 20 °C and 1000 lux for 8 hr/16 hr light /dark period. The best medium for plantlet regeneration from callus was MS + 0.1 mg/ L IAA + different concentrations of 6 BA + 0.8 % agar + 3 % sucrose at pH (5.7) under conditions of 30°C, 2500 lux and 10 hr light / 14hr dark period. It was shown that maximum regeneration capacity for one callus was 59 R_0 plantlets. Rooting medium consisted of ½ MS + different concentrations of NAA + 0.7 % agar + 3 % sucrose at pH (5.7). The in vitro regeneration of this plant would prevent its eradication due to expansien of housing in its area of natural growth.

Key words: ***Citrullus colocynthis, Callus, Plant regeneration. Root formation***

INTRODUCTION

Bitter apple (*Citrullus colocynthis*) as a medicinal plant is grown as a wild type plant in EL- Wadi El-Gadid Governorate, Egypt. It is used as a popular drug for many human diseases. It contains alkaloids, resins, glycosides (Cucurbitacin E. as anti cancer drug) and many other pharmaceutical substances. On other hand, it is considered as a drought resistant plant in comparison to other cucurbitace plants, such as sweet melon etc. As housing extendes, this plant will be eradicated. *In vitro* plant regeneration from *Citrullus colocynthis* was reported by Frazier and Kute (1991) and Dabuza *et al* (1997). Plant tissue culture technology will facilitate to design a new program for plant breeding depending on *in vitro* culture, which leads to produce the secondary products of medicinal plants through biofermentors, such as anticancer, purgative, carminative, liver disease, Joundice , ascabies , eye disease, antirheumatic (Nag *et al* 1983, Oyolou 1982, Nayab *et al* 2006). This research aimed to find a new method for in vitro rapid clonal propagation through tissue culture techniques.

MATERIALS AND METHODS

The present investigation was carried out in 1996 in the Cell and Tissue Culture Laboratory, Genetics Branch, Botany Department, Faculty of Agriculture, Al-Azhar University.

Plant material

Fruits of the *Citrullus colocynthis* collected from plants grown in EL- Wadi El-Gadid Governorate were provided by Dr. Hussein Salem. Fac.of. Agric. Al- Azhar University.

Tissue culture studies

1-Seed germination: Following steps and trials have been carried out:

a- Seeds were soaked in H_2SO_4 , KNO_3 for 24 hours

b- (1) Seeds were soaked in current tap water for 3 days.
(2) Testa of seed were broken under mechanical stress.

C- Sterile embryos were, cultivated in two different conditions:

(1) Embryos were germinated in peatmos and seedlings were transplanted in the field

(2) Embryos were germinated on 0.8 % water agar medium in laboratory at 25 °C in dark for 3 days.

2-Source of explants

Explants were excised from different organs such as:

c- *In vivo* (cotyledon and hypocotyledon): the explants were cultured after they had been surface sterilized with 15 % clorox for 17 minutes.

b- *In vitro* (cotyledon and hypocotyledon).

3-Chemicals

Effect of different combinations of auxins and cytokinins on callus induction, plant regeneration and root formation was studied. All explants [cotyledon and hypocotyledon] were cultured on MS + auxins and cytokinins at different combinations of (6 BA, 2, 4-D, Kinetin, NAAand IAA). Cultures were incubated at 20, 25, 30 or 35 °C as shown in Tables (1 through 4).

4-Physical factors

Cultures were incubated under different physical conditions such as:

d- Photoperiod at 8hr / 16 hr , 10 hr / 14 hr or 12 hr / 12 hr light / dark period as shown in Tables (1, 3, 4, and 5).

e- Light intensity at 1000, 1500, 2000, 2500 or 3000 lux as shown in Tables (1 through 6).

5-Plant regeneration:

Plant regeneration from callus has been achieved from 4-5 weeks old calli. They were cultured on MS (Murashig and Skoog 1962) medium supplemented with different concentrations and combinations of NAA, IAA, 6 BA and kinetin as shown in Tables (4 and 5).

6-Rooting media

Shoots were cultured on ½ MS + different concentrations of NAA as shown in Table (6).

Table 1. Effect of MS medium supplemented with combination of 0.1 mg/L kinetin and different concentrations of 2,4-D on callus induction at 20°C, 1000 lux and 8 hr/16 hr light/dark period .

N0.	Source of explant	Cotyledon		Hypocotyledon	
	Medium	*In vivo*	*In vitro*	*In vivo*	*In vitro*
1	MS + 0.0	-	-	-	-
2	MS + 0.1 mg/L K + 0.5 mg/L D	-	-	-	-
3	MS + 0.1 mg/L K + 1.0 mg/L D	+ Silver callus formation	-	-	-
4	MS + 0.1 mg/L K + 2.0 mg/L D	+ Silver callus formation	-	-	-
5	MS + 0.1 mg/L K + 3.0 mg/L D	+ Big silver callus formation	+ Small golden callus formation	-	+ Small silver callus formation
6	MS + 0.1 mg/L K + 4.0 mg/L D	+ Big golden callus formation	+ Small golden callus formation	+ Small golden callus formation	+ Small golden callus formation
7	MS + 0.1 mg/L K + 5.0 mg/L D	+ small silver callus formation	+ Small golden callus formation	+ Big golden callus formation	+ Small golden callus formation

* 0.8 % agar and 3% sucrose . The pH (5.8) .
K = Kinetin ' D = 2,4-D ' (+) = callus formation , (-) = no callus formation .

Table 2. Effect of MS medium supplemented with combination of 5.0 mg/L 6BA and different concentrations of NAA on callus induction at 25°C, 1500 lux and 8 hr/16 hr light/dark period.

N0.	Source of explant	Cotyledon		Hypocotyledon	
	Medium	*In vivo*	*In vitro*	*In vivo*	*In vitro*
8	MS + 0.0	-	-	-	-
9	MS + 5.0 mg/L 6BA + 0.1 mg/l NAA	+ Big golden callus formation	+ Small golden callus formation	-	-
10	MS + 5.0 mg/L 6BA + 0.2 mg/L NAA	+ silver callus formation	-	-	-
11	MS + 5.0 mg/L 6BA + 0.4 mg/L NAA	-	-	-	-
12	MS + 5.0 mg/L 6BA + 0.6 mg/L NAA	-	+ Small golden callus formation	-	+ Small golden callus formation
13	MS + 5.0 mg/L 6BA + 0.8 mg/L NAA	-	-	+ Big golden callus formation	-
14	MS + 5.0 mg/L 6BA + 1.0 mg/L NAA	-	-	+ Big golden callus formation	-

* 0.8 % agar and 3% sucrose . The pH (5.8) .
(+) = callus formation ' (-) = no callus formation .

Table 3. Effect of MS medium supplemented with different combinations of NAA ,2,4-D and 6BA on callus induction at 25°C, 2000 lux and 10 hr/14 hr light/dark period .

N0.	Source of explant	Cotyledon		Hypocotyledon	
	Medium	*In vivo*	*In vitro*	*In vivo*	*In vitro*
15	MS + 0.0	-	-	-	-
16	MS + 0.6 mg/L NAA +3.0 mg/L 2,4-D + 0.2mg/L 6BA	-	-	-	-
17	MS + 0.6 mg/L NAA +3.0 mg/L 2,4-D +0.4 mg/L 6BA	Small silver callus formation	+ Small silver callus formation	+ Small silver callus formation	-
18	MS + 0.6 mg/L NAA +3.0 mg/L 2,4-D +0.6 mg/L 6BA	-	-	-	-
19	MS + 0.6 mg/L NAA +3.0 mg/L 2,4-D +0.8 mg/L 6BA	+ Big silver callus formation	-	+ Small golden callus formation	+ Small golden callus formation
20	MS + 0.6 mg/L NAA +3.0 mg/L 2,4-D +1.0 mg/L 6BA	+ Big silver callus formation	+ Small golden callus formation	Elongation of explant and keep viability	Elongation of explant and keep viability

* 0.8 % agar and 3% sucrose . The pH (5.7) (+) = callus formation ' (-) = no calaus formation

Table 4. Effect of different concentrations of 6 BA and 0.1 mg/L IAA on plant regeneration (in vivo) at 30°C, 2500 lux and 10 hr/14 hr light/dark period.

N0	Source of explant	Cotyledon	Hypocotyledon
	Medium		
21	MS + 0.0	-	-
22	MS + 0.1 mg/L IAA + 0.5 mg/L 6BA	- Big golden callus , no shoot	- Silver callus , no shoot
23	MS + 0.1 mg/L IAA + 1.0 mg/L 6BA	- Big golden callus , no shoot	- Silver callus , no shoot
24	MS + 0.1 mg/L IAA + 1.5 mg/L 6BA	- Big greenish callus , no shoot	- Silver callus , no shoot
25	MS + 0.1 mg/L IAA + 2.0 mg/L 6BA	- Golden callus with green colour, no shoot	+ Shoot formation, 3-5 plantlets, plant hight was X=2cm, (4) leaves
26	MS + 0.1 mg/L IAA + 2.5 mg/L 6BA	+ Greenish callus, start regeneration	- Golden callus , no shoot
27	MS + 0.1 mg/L IAA + 3.0 mg/L 6BA	+ Shoot formation, 12-16 plant - lets, plant hight was X=3cm , (6) leaves	- Golden callus , no shoot

0.8 % agar and 3% sucrose. The pH (5.7) (+) = regeneration of plantlet ' (-) = no plantlet regeneration.

* all the in vitro callus had very weak totipotency .

Table 5. Effect of different concentrations of kinetin with 0.05 m g/L NAA and light on callus regeneration (*in vivo*) at 35°C, 3000 lux and 12 hr/12 hr light/dark period.

N0	Source of explant / Medium	Cotyledon	Hypocotyledon
28	MS + 0.0	-	-
29	MS + 0.05 mg/L NAA + 0.5 mg/L Kinetin	+ Shoot formation, 2-3 plantlets ,plant hight was X=2cm and (3) leaves	- Big silver callus , with greenish colour , no plant regeneration
30	MS + 0.05 mg/L NAA + 1.0 mg/L Kinetin	+ Shoot formation, 20-25 plantlets ,plant hight was X=(3-8)cm and (6) leaves	- Silver callus , and greenish colour , no plant regeneration
31	MS + 0.05 mg/L NAA + 1.5 mg/L Kinetin	+ Shoot formation, 7-10 plantlets ,plant hight was X= 4cm and 3 leaves	+ Shoot formation 8 plantlets ,plant hight was X=5cm and 4 leaves
32	MS + 0.05 mg/L NAA + 2.0 mg/L kinetin	- Greenish callus , no plant regeneration	- Greenish callus, no plant regeneration

0.7 % agar and 3% sucrose. The pH (5.7) (+) = regeneration of plantlet ' (-) = no plantlet regeneration.

Table 6. Effect of ½ MS medium supplemented with different concentrations of 6 BA on root formation (*in vivo*) at 25°C, 2000 lux and 12 hr/12 hr light/dark period.

N0	Source of explant / Medium	Cotyledon	Hypocotyledon
33	MS + 0.01/2	-	-
34	MS + 0.1 mg/L NAA 1/2	-	-
35	MS + 0.3 mg/L NAA1/2	+ Few root formation after 45 days	-
36	MS + 0.5 mg/L NAA1/2	+ Large scale root formation	+ Few root formation after 40days
37	MS + 0.7 mg/L NAA1/2	+ Few root formation after 45days	+ Few root formation after 37days
38	1/2MS + 1.0 mg/L NAA	+ Few root formation after 35 days	+ Few root formation after 50 days

0.7 % agar and 3% sucrose. The pH (5.7).

RESULTS AND DISCUSSION

Table (1) showed that the best medium for callus induction was MS + 0.1 mg/L kinetin + 4.0 mg/L 2,4-D at 20 °C, 1000 lux and 8 hrs /16 hrs light /dark period. This callus originated from cotyledon *in vivo* explant . The properties of this callus is very big in size, golden colour, fraible and highly profilirated. While the callus originated from *in vitro* cotyledon was very small in size and golden colour on medium No. (5) (Fig. 2 .a).

In vivo hypocotyledon was very weak in callus induction except when cultured on media No. (6) and (7). While *in vitro* hypocotyledon showed good response on medium No. (6) (Fig.1. a) these data showed that the source of explant (*in vivo*) was better in callus initiation in comparison with that of *in vitro* source. Therefore, it is recommended to use *in vivo* method for rapid response of callus formation and the interaction between 2, 4-D and 0.1 mg/L kinetin by increasing the 2, 4 - D concentration *(Nayab et al 2006)*.

Table (2) showed that there is no response for *in vivo* cotyledon explant except on medium No. (9) which showed big size golden colour callus. While the *in vitro* cotyledon had response with media No. (9) and (12) of small size golden colour callus. The results indicated that *in vivo* gave the best results, on medium No. (13) (Fig 1 b and 2 b) with, big size golden colour callus. This result may be due to the combination between 5.0 mg/L 6-BA and increasing of NAA concentration up to 1.0mg/L

Table (3) showed that the best media for *in vivo* cotyledon were media No. (19) and (20) which gave big size silver colour callus by increasing the concentration of 6-AB (from 0.2 mg/L up to 1.0 mg/L) with 0.6 mg/L NAA and 3mg/L 2,4-D. While the best media for *in vitro* cotyledon were media No. (17) and (20). On other hand the highest responsive media for *in vivo* hypcotyledon were media No. (17), (19) and (20).

We conclude that the increasing of 6-BA concentration could give high response for callus formation when it is combined with 0.6 mg/L NAA + 3.0 mg/L 2,4-D at 25 °C, 2000 lux and 10 hrs/14 hrs. light / dark period.

Finally from Tables (1), (2) and (3) we conclude that the best medium was No. (6) using *in vivo* cotyledon explant at 20 °C , 1000 lux and 8 hr /16 hr. light / dark period . This medium consisted of MS + 0.1 mg/L kinetin + 4.0 mg/L 2,4-D + 3 % sucrose + 0.8 agar at pH (5.8). Those results agree with these reported by Frazier and Kute (1991).

Fig.1.(a)

Fig. 1. (b)

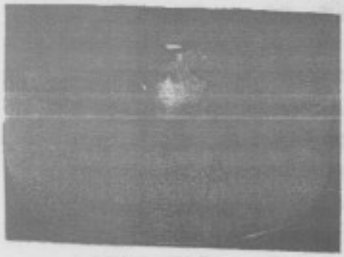

Fig.2.(a)

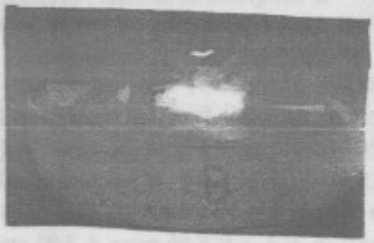

Fig.2.(b)

Fig.1. (a) Callus induction from cotyledon (of cultivated field plant) grown on medium No.(2) (see Table1)

Fig.1. (b) Callus induction from hypocotyledon (of cultivated field plant) grown on medium No.(13) (see Table2)

Fig.2. (a) Callus induction from cotyledon (excised *in vitro)* grown on medium No.(5) . (see Table1)

Fig.2. (b) Callus induction from hypocotyledon (excised *in vitro)* grown on medium No. (13) . (see Table2)

Fig. 3

Fig. 4

Fig. 3. Subcultured callus derived from *in vivo* cotyledon (note galobular shape and starting differentiation) grown on medium No.(26) (see Table4)

Fig. 4. Shoot formation from callus derived from cotyledon (*in vivo)* and grown on medium No. (27) . (see Table 4)

Fig. 5

Fig. 6

Fig .5. Rapid clonal propagation for shoot formation from callus derived from cotyledon *in vivo* grown on medium No. (30) (see Table 5)

Fig. 6. Plant regeneration and root formation on medium No. (36) . (see Table 6)

Tables (4 and 5) indicated that totipotency response of regenerated cotyledon calli from *in vivo* was the best totipotency using media No. (26), (27) and (30). Medium No (26) contains MS + 0.1 mg/L IAA + 2.5 mg/L 6-BA where greenish callus start regeneration from cotyledon *in vivo* (Fig. 3). Medium No. (27) contains MS + 0.1 mg/L IAA + 3.0 mg/L 6-BA + 0.8 % agar + 3 % sucrose at pH (5.7) (Fig . 4).

Callus originated from cotyledon incubated at 30°C, 2500 lux and 10 hr light /14 hr dark period. The totipotency capacity was 12-16 Ro regenerated plantlets. While medium No. (30) showed totipotency capacity of 59 Ro regenerated plantlets. Medium No. (30) contains MS + 0.05 mg/L NAA + 1.0 mg /L kinetin + 0.7 agar + 3 % sucrose at pH (5.7) (Fig 5). This is an indication for the totipotencey power of increasing kinetin concentration as cytokinin and growing at 35 °C , 3000 lux and 12 hr /12 hr light / dark period . On other hand there was no response for *in vitro* hypocotyledon.

In Table (6) the results showed that the best rooting medium was No. (36) (Fig 6). This medium consists of ½ MS + 0.5 mg/L NAA. These results indicated the effictiveness of NAA on rooting formation and agree with those reported by Frazier and Kute (1991).

In Conclusion, this is the best method for regeneration of Egyptian *Citrullus colocynthis* El-wadi El-Gadid, as a medicinal plant. It can be used for preservation of *Citrullus colocynthis* germplasm from eradication as an important medicinal plant.

In future a new method could be developed depending on cell culture / cell suspension for extraction of active constituents by using Biofermentor technology.

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استيـــلاد نباتات من كالوس نبات حنظل الوادى الجديد كنبات طبى

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أجريت هذه الدراسة على نبات الحنظل النامى طبيعيا بمحافظة الوادي الجديـد بهـدف الإكثـار السريع والدقيق لهذا النبات من الكالوس إلي نباتات كاملة من خلال زراعة المنفصلات النباتية المختلفة من الأوراق الفلقية والساق تحت ظروف فيزيقية (الحرارة – الضوء – طـول الفتـرة الـضوئية)، وظـروف كيميائية (تركيزات من الأوكسين والسيتوكينين) . وكانت أفضل النتائج المتحصل عليها لإنتاج الكـالوس من الأوراق الفلقية والساق هي بيئة MS (موراشيج وسكوج 1962) والمضاف اليها 0.1 ملي جم / لتر كينيتين + تركيزات مختلفة من 2-4 -داي كلوروفينوكـسي حـامض الخليـك + 0.8 % أجـار + 3 % سكروز عند درجة PH (5.8) حيث كان الكالوس كبير الحجم ولونه أصفر . وكانت أفضل البيئات لتكشف النباتات من الكالوس هي بيئة MS مضافاً إليها 0.1 ملي جرام / لتر أندول حمض الخليـك + تركيـزات مختلفة من 6- بنزيل اديتين + 0.8 % أجار + 3 % سكروز عند درجـة PH (5.7) ، وأيـضاً أفـضل البيئات للإكثار السريع في زيادة نسبة الإستيلاد هي بيئة MS مضافاً إليها 0.05 ملي جرام نفثالين حمض الخليك + تركيزات مختلفة من الكينيتين ، أما تكوين الجذور فكانت أفضل النتائج هي بيئة1/2MS مـضافاً إليها تركيزات مختلفة من حمض الخليك + 0.7 % أجار عند PH (5.8) .وقد تحصلنا على (59) نبـات من كالوس واحد خلال 29 يوم من إعادة زراعة الكالس وتجزئته.الجدير بالذكر أن هذا النبات يحتوي مواد جايكوسيدية (Cucurbitacin glycoside) تستخدم لعلاج سرطان الثدى وقلويدات ومواد صمغية لهـا فوائد طبية عديدة ذات قيمة اقتصادية عالية تستخدم كعقاقير صيدلانية في أمراض العصر.