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Micro Propagation Protocol for Umbrella Papyrus (*Cyperus alternifolius* L.) Plants by Using Tissue Culture Technique

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Abstract: The objectives of this research were to improve an efficient and applicable protocol for large scale propagation for high quality of umbrella papyrus (*C. alternifolius* L.) using tissue culture techniques in a short time, to optimize and establish best conditions for stable regeneration syst pharmaceutical industries. The research was carried out at the Tissue Culture Laboratory, Horticulture Research Institute, Agriculture Research Center, during 2018 and 2019 seasons. The highest percent of shoot formation (30.25%), the highest percent of aseptic cultures and survival of explants (100%) were obtained as a result of using Clorox at 25% for 15 min. Throughout the multiplication stage, the highest percent of shoot formation reached 100 % with repeating culture of explants (three times) on MS medium supplemented with BA at 1.0 mg/l and kin at 1.5 mg/l. The highest numbers of shootlets/explant and the longest shootlets were obtained when 1.0 mg/l of BA + 1.5 em, to be used in the phytoremediation technology for sustainable environmental development and the mg/l of kin were added to MS culture medium of 3/4 strength .During the rooting stage. IBA and NAA had promotive effects on rooting formation of shootlets. The highest numbers of roots/explant and the longest roots were obtained when 0.5 mg/l IBA + 1.5 mg/l kin. The best results of plant acclimatization traits after 8 weeks then after 6 months were obtained by using peatmoss + hundz-soil+ sand (1:1:1v/v/v/). The amplified DNA fragments using SCoT 1, SCoT 12 and SCoT 14 primers for mother and micropropagated plants indicated that the produced pattern by primer SCoT 12 had a maximum number of 6 bands of DNA fragments with molecular size ranging between 200 and 8000 bp, micropropagated plants showed similarity in relation to mother plant.

Key words: *Cyperus alternifolius* L · MS (Murashige and Skoog) medium · Clorox: Sodium hypochlorite · Rhizome explants · *In vitro* regeneration · Multiplication · Rooting and SCoT –PCR technique

INTRODUCTION

Cyperus alternifolius L. plants belongs to the Family Cyperaceae . It is considered from semiaquatic plants and with the common names of umbrella plant, umbrella sedge or umbrella palm and umbrella papyrus. It is native to northern and tropical Africa, used as beautiful accent in water gardens and at the margins of pools or ponds and in a wide variety of landscaping i.e., patio, pot plant and tropical looking accent plant. It has a valuable usage as wonderful unusual cut flower. The aerial parts of *C. alternifolius* possess significant protective effect against hepatotoxicity. Numerous secondary metabolites are isolated from *Cyperus* sp. including quinones, essential oils, flavonoids and sesquiterpenes. In folk medicine the stems, leaves and roots of *C. alternifolius* are used as aphrodisiac. Umbrella sedge plays a significant function in remove and uptake the pollutant through a physical, chemical and biological mechanism in the phytoremediation treatment, the plant selection for phytoremediation criteria includes the high growth rate plant, able to uptake the contaminant and tolerance in the toxic pollutant, adapt in the climate and control in dispersion [1-9].

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Nowadays plant tissue culture is a deep-rooted knowledge which has made significant contributions to the propagation and development of agricultural yields in general. Superior contribution is envisaged from this technology in years to come, both in its own true and as an assistant to the application of molecular biology. Understanding of the biological processes that permit the manipulation of *in vitro* morphogenesis and investigations on various physiological, biochemical and molecular aspects of plant hormones will seriously advance our information and offer knowledge that will help address the issues of *in vitro* recalcitrance or *in vitro* plant growth and improvement [10].

Cytokinins are a group of plant growth hormones that stimulates cell division, cell differentiation, shoot initiation and reproductive growth in many plants and BA continues to be a key role player on promoting the growth of stout seedlings during seed germination and explant preparation and for the formation of desired adventitious multiple shoots during *in vitro* regeneration [11, 12].

Therefore, the present experiment will greatly advance our knowledge, provide information that will help address the issues of umbrella papyrus (*C. alternifolius* L.) plants, because it has historical importance in our Pharaonic civilization in addition, there is no sufficient researches about tissue culture on this plant and because of an increasing demand on phytoremediation technology.

MATERIAL AND METHODS

To achieve the goal of this investigation, the present study was carried out during two successive seasons of 2018 and 2019 at the Tissue Culture Laboratory, Horticulture Research Institute, Agriculture Research Center to study the influence of some factors on the behavior of *in vitro* consecutive micropropagation stages containing those of culture establishment, shootlets multiplication, rooting and acclimatization stages.

Plant Materials: Healthy young rhizomes of 2-3 cm length were excised from *Cyperus alternifolius* L. plants grown at El-Orman Botanical Garden, Giza Governorate and Egypt as explant source for this work.

Culture Establishment Stage: Rhizomes explants were washed in a tap water for 30 min. to remove soil and other unwanted contaminants. They were then rinsed in septol soap (soapy water) with shaking for 30 minutes, at that time washed with running tap water for one hour. They were kept in double distilled water for 3-4 hours to eliminate phenolic substances and leach out of explants. They were immersed for 5, 10 and 15 min. in 10, 20 and 25% clorox solution (5.25 % sodium hypochlorite). Each treatment consisted of 7 jars, each jar contained five nodal explants. After receiving the disinfection treatments, the explants were rinsed three times with a sterile distilled water and the explants were cultured on a basal MS medium supplied with 30 g/l sucrose, 7 g/l agar and +BA1.0 mg/l+kin. 2.0 mg/l. Decontamination, survival and shoot formation percentages were recorded after 4 weeks of incubation for this stage.

Culture Medium and Incubation Condition: MS culture medium Murashige and Skoog [13]. This medium contained vitamins, 30 g/l sucrose and solidified by 0.7% agar previous to autoclaving. It was then adjusted to pH 5.80 by addition of 0.1 N KOH or 0.1 NHCl. It was distributed in glass jars (11.5 cm height × 6.5 cm diameter with their polypropylene caps) and autoclaved at 121°C for 20 min. under 1.05 kg/cm^2 pressure, left to cool and stored at 25±2°C for one week before being used. During experiment, jars were incubated in growth chambers at 25°C under 16 hrs. photoperiod using Phillips cool-white fluorescent tubes (120 cm long) at light intensity of 3000 Lux till data were collected. All cultures were incubated for 4 weeks under controlled conditions.

Shootlet Multiplication Stage: To optimize best condition for shoot formation system two experiments were designed the first was designed to study the effect of different concentrations of benzyl adenine (BA) and (kinetin) kin at 0.5, 1.0 and 1.5 mg/l on growth behavior. The second was designed to study the effect of the best concentration of cytokinins from the first experiment combined with various strengths of MS-medium (½, ¾ and full strength) supplied with 30 g/l sucrose, 7 g/1 agar on shoot formation percentage, number of shootlets/explant, shootlet length (cm) and number of leaves formed per shootlet. Micro-shoots (1.0-1.5 cm long) produced in the establishment stage were removed and vertically cultured in the culture jars on shootlet multiplication stage through three successive subcultures. The first experiment included 9 treatments and the second experiment included 3 treatments and each treatment combination was replicated 6 times (one jar/replicate) in a completely randomized design and the explants were re-cultured three times, at 30 days interval. The healthy produced shoots were subcultured in the best medium known from the current experiment and maintained to rooting experiments.

Table 1: Chemical analysis of peatmoss according to Noor el-deen [16]

Chemical Components	
Organic matter	92-94%
Density (Vol. Dry)	84-92 g/l
Ash	6-10%
Water relation capacity	65-75%
Salinity	300 mg/l
pH value	3-5
N	1.06 %
P	0.23%
K	1.74 %
Fe	420 ppm
Mn	23 ppm
Zn	40 ppm
Cu	7.20 ppm

Table 2: Chemical analysis of organic soil conditioner (Hundz-soil) according to Youssef and Awad [17]

Chemical Components	
kg/M	216.0
Humidity %	3.00
pH (1-10)	7.60
EC dSm^{-1} (1-10)	1.40
Total nitrogen%	1.30
$NH4NO_3$ µg g^{-1}	1628.0
NO_3µg g^{-1}	68.00
C:N	35.40:1
Total P%	0.08
Total K%	0.11
Organic matter (%)	78.20
Organic carbon (%)	45.30

Rooting Stage: In this stage, the experiments were conducted to study the effects of numerous hormones IBA and NAA at 1.0, 2.0 and 4.0 mg/l plus kin at 0.5 mg/l on rooting behavior of the grown shootlets. The produced uniform *in vitro* shootlets from the multiplication stage were individually separated and cultured in 6 replicates and incubated for 6 weeks. After that: rooting (%), number of roots/plantlet, root length (cm) were recorded.

***Ex vitro* Acclimatization Stage:** Healthy, homogenous and well rooted plantlets (about 8.0 cm long, 4.0 roots/plantlet of 4.0 cm long) obtained from the rooting experiment, were removed from culture medium. To remove the remains of medium and agar, the roots were carefully washed with a distilled water. Plantlets were individually cultured in pots (25 cm) containing different sterilized moist potting media, watered with water containing a fungicide (Topsin at 0.1%). Pots were tightly covered with clear plastic bags to maintain high humidity and kept in a shaded greenhouse. One week later, ventilation of the plantlets gradually started by making small hole in the plastic bags. This procedure increased with time by increasing the number and size of the holes. After 4 weeks, plastic bags were completely removed. The research was conducted to study the influence of seven soil mixtures on the characteristics of the acclimatization stage. The potting mixture was peatmoss only (the analysis of which is shown in Table (1), hundz-soil only the analysis of which is shown in Table (2) sand, peatmoss + hundz-soil (1:1, v/v) peatmoss + sand (1:1, v/v) , hundz-soil + sand (1:1, v/v) and peatmoss + hundz-soil +sand (1:1:1, v/v/v).The acclimatized plantlets were water irrigated twice a week for four weeks before transplanting out-door. This experiment consisted of four treatments, four replicates/treatment and six pots/replicate. After 8 weeks the following data were recorded: survival %, plant height (cm) and root length. Also, after 6 months data recorded for vegetative growth parameters, included; plant height (cm), leaf length (cm), number of leaves / stem, number of stems/ plant, fresh weight / plant (g) and rhizomes fresh weight (g) were recorded. Chemical analyses of chlorophyll a, b and total (mg/g f.w.) were determined according to Moran [14]. And total carbohydrates in rhizomes (mg/g d.w.) was determined according to Herbert *et al*. [15].

Molecular Diversity Assessment:

DNA Isolation: Genomic DNA was isolated from freshly leaves by DNeasy plant mini kit (bio basic). DNA quantity was checked by means of absorbance ratios A_{260}/A_{280} through a UV-spectrophotometer where DNA is pure with a ratio A_{260}/A_{280} from 1.8-2.0. Moreover, using electrophoresis in 1.5 % agarose gel with ethidume bromied, a qualitative check for DNA samples was done.

Polymerase Chain Reaction: Genomic DNA were used for a template for Polymerase Chain Reaction (PCR) amplification using 8 SCoT primers. On the other hand, SCoT primers were designed from consensus sequence derived from the previous studies by Joshi *et al.* [18]; Sawant *et al.* [19]; Collard and Mackill [20] and procured from Biobasic Com. All ScoT primers were 18-mer and were from Dataset I based on highly expressed genes as described by Sawant *et al.* [19]. For SCoT primers design, the start codon ATG (+1, +2 and +3), 'G' at position +4, 'C' at position +5 and A, C, C and A at positions +7, +8, +9 and +10, respectively, were fixed (5´-----ATGGCTACCA---3´).

Polymerase Chain Reaction: Amplification reactions for SCoT technique were achieved as described by Xiong *et al*. [21] and Fathi *et al*. [22] respectively and were carried out in Techni TC-512 Thermal Cycler as follows: One cycle at 94°C for 4 min followed by 40 cycles of 1 min at 94°C, 1 min at annealing temperature 57°C and 2 min at 72°C, followed by 72°C for 10 min, the reaction was finally stored at 4°C.

Gel Electrophoresis: Amplified products were loaded and separated on a 1.5% agarose gel with 100 bp to 1.5 kb ladder markers. The run was carried out for about 30 min at 100 V in mini submarine gel BioRad.

Gel Reading and Analysis: DNA banding pattern photos were photographed using Bio-1D Gel Documentation system and were analyzed by GelAnalyzer3 software scoring clear amplicons as present (1) or absent (0) for each primer and entered in the form of a binary data matrix. From this matrix, DNA-profiles were performed for SCoT techniques according to Adhikari *et al*. [23]. Polymorphic Information Content (PIC) and DI (Diversity Index) were calculated according to, PIC = $1-p^2-q^2$, DI=average PIC value as described by Gorji *et al.* [24] where p is frequency of present amplicon and q is frequency of absent amplicon. Also from binary data, the capability of each primer to differentiate among studied was evaluated according to Resolving power (Rp) value calculated as described in Prevost and Wilkinson [25].

According to binary data matrix, Molecular distances MD were calculated by Nei and Li coefficient and cluster analysis was performed using XLSTAT.7 software [26].

Statistical Analysis: The lay-out of the research attained on shoot establishment, rooting and acclimatization stages were designed as one factor in a completely randomized design. However, that of experiments conducting on shootlets multiplication stage was arranged as a two factorials in a completely randomized design as described by Snedecor and Cochran [27] at 5% probability level. Data obtained were statistically analyzed using MSTAT Computer Program [28]. Means were compared by Duncan's multiple range test as described by Duncan [29] to verify differences among means of various treatments.

RESULTS AND DISCUSSION

Culture Establishment: Data presented in Table (3) proved that the highest percent of decontamination for cultures and survival of explants (100 %) were obtained as a result of using clorox 25% for 15 min. Also, the same treatment led to the highest shoot formation (30.28 %) followed by clorox 20 % for 15 min. as gave 28.15%. Data showed that the use of disinfectant retarded the formation of shoots to establish *in vitro* culture and this may be attributed to the increased its toxic effect, it on the explants tissue. However, Ancy *et al.* [30]; Farsi *et al*. [31] and Teixeira da Silva *et al*. [32] reported that disinfection of plant material is the most important step of the tissue culture protocol, the most frequently chosen sodium or calcium hypochlorite was one of the most frequently chosen surface disinfection agents.

In vitro Shooting Capability

Effect of Cytokinin Type and Concentrations: Data illustrated in Table (4) indicated that the *in vitro* shooting induction throughout multiplication stage of *Cyperus alternifolius,* L. plants under influence of two types of cytokinins (BA and Kin.) at three concentrations (0.5, 1.0 and 1.5 mg/l) for three repeated subcultures. The results discovered the promotive effect of BA at 1.0 mg/l and kin. at 1.5 mg/l. on the percent of shootlets formed /explant which was highest (94.0 %) at the second subculture as a result of this treatment comparing with other treatments. Using 1.0 mg/l of BA for the second subculture led to 45.0 % of shootlets formation. The number of shootlets formed/explant as a result of the above mentioned treatments behaved the same trend as shown in Table 4. It is observed that the highest numbers of shootlets/explant were obtained from BA 1.0 mg/l + kin.1.50 mg/l as gave 2.68 shootlets while using 0.5 mg/l of BA. for the second subculture which led to 1.11 of number of shootlets formed/explant.The behavior of both *in vitro* shootlets length and number of leaves formed/shootlet was paralleled as revealed in Table 4. Culturing the explants on MS with BA 1.0 mg/l + kin.1.5 mg/l resulted in the longest shootlets (13.11 cm) as well as the highest number of leaves formed/shootlet (18.23) as compared to other treatments. These results may be due to that BA continues to be a key role player on the formation of desired adventitious multiple shoots during *in vitro* regeneration [11, 12].These results were in agreement with those achieved by Yew et *al.* [33].

Effect of Cytokinin Type and MS Medium Strength: Actually, the results in Table (5) proved that the promotive effect of MS medium supplemented at 3/4 strength when combined with BA at 1.0 mg/l and kin. at 1.5 mg/l. gave the best results of the percent formation of shootlets/explant, shootlet number, shootlet length and leaves number as compared with other treatments.

Table 3: Effect of disinfectants concentration % and soaking time (min.) on stem nodal explants of (*Cyperus alternifolius* L.) plants during culture establishment stage

	Measurements											
	Decontamination (%)				Survival (%)				Shoot formation (%)			
	Soaking time (min.)				Soaking time (min.)				Soaking time (min.)			
Disinfectants %	5	10	15	Mean	5	10	15	Mean	5	10	15	Mean
Clorox 10 %	40.12 i	58.14 f	82.51 c	60.26 C	52.13 g	56.13 f	86.40 d	64.89 C	17.15 h	24.01 f	26.14 c	22.43 C
Clorox 20 %	42.16 h	60.61 e	94.13 b	65.63 B	45.61 i	69.14 e	94.43 b	69.73 B	15.16 i	25.12 d	28.15 b	22.81 B
Clorox 25%	46.12 g	76.12 d	100.00 a	74.08 A	59.40 h	91.11 c	100.00 a	83.50 A	22.14 g	24.22 e	30.28 a	25.55A
Mean	42.80 C	64.96 B	92.21 A		52.38 C	72.13 B	93.61A		18.15 C	24.45 B	28.19 A	

Means within a column having the same letters are not significantly different according to Duncan's multiple range test (DMRT) at 5% level

Table 4: Effect of cytokinin type and concentration on *in vitro* growth behavior during shoot multiplication stage of *Cyperus alternifolius* L. plants

		Subculture			
Measurements	Growth regulators (mg/l)	Sub 1	Sub 2	Sub 3	Mean
Shootlet formation (%)	BA+ Kin (0.5 + 0.5) mg/l	77.00 b	62.00 l	45.00 r	F61.33
	BA + Kin (0.1 + 0.5) mg/l	66.00 h	58.00 o	71.00 e	C65.00
	BA+ Kin (1.5 + 0.5) mg/l	54.00 q	59.00 n	69.00 g	G60.67
	BA+ Kin (0.5 + 1.0) mg/l	60.00 m	45.00 r	62.00 l	H55.67
	BA+ Kin (1.0 + 1.0) mg/l	63.00 k	60.00 m	63.00 k	D62.00
	BA+ Kin (1.5 + 1.0) mg/l	59.00 n	62.00 l	65.00 i	D62.00
	BA+ Kin (0.5 + 1.5) mg/l	65.00 i	56.00 p	64.00 j	61.67 E
	BA+ Kin (1.0 + 1.5) mg/l	82.00 a	77.00 b	59.00 n	B68.67
	BA+ Kin (1.5 + 1.5) mg/l	70.00 f	76.00 c	74.00 d	A77.33
	Mean	66.22 A	61.67 C	63.56 B	
Shootlet number	BA+ Kin (0.5 + 0.5) mg/l	1.50 gh	1.15 j	1.60 fg	H1.42
	BA + Kin (0.1 + 0.5) mg/l	1.68 efg	1.96 cd	1.72 defg	D1.79
	BA+ Kin (1.5 + 0.5) mg/l	1.68 efg	1.60 fg	1.96 cd	E1.75
	BA+ Kin (0.5 + 1.0) mg/l	1.92 cde	1.86 def	1.84 def	1.87 C
	BA+ Kin (1.0 + 1.0) mg/l	2.50 a	2.16 bc	2.22 b	2.29 A
	BA+ Kin (1.5 + 1.0) mg/l	1.56 g	1.64 fg	1.22 b	F1.47
	BA+ Kin (0.5 + 1.5) mg/l	1.85 def	1.23 ij	1.26 ij	G1.45
	BA+ Kin (1.0 + 1.5) mg/l	2.68 a	1.54 g	1.62 fg	B1.95
	BA+ Kin (1.5 + 1.5) mg/l	1.68 efg	2.16 bc	1.45 ghi	1.76 E
	Mean	1.89 A	1.70 B	1.65 C	
Shootlet length (cm)	BA+ Kin (0.5 + 0.5) mg/l	8.12 m	3.96 p	5.14 o	H5.74
	BA + Kin (0.1 + 0.5) mg/l	7.52 n	9.14 k	9.65 ij	8.77 G
	BA+ Kin (1.5 + 0.5) mg/l	11.14 f	12.00 d	8.14 m	C10.43
	BA+ Kin (0.5 + 1.0) mg/l	10.13 h	9.12 k	10.69 g	9.98 D
	BA+ Kin (1.0 + 1.0) mg/l	10.52 g	8.23 m	10.22 h	9.66 E
	BA+ Kin (1.5 + 1.0) mg/l	8.22 m	9.67 ij	9.51 j	F9.13
	BA+ Kin (0.5 + 1.5) mg/l	12.14 d	12.80 bc	11.64 e	B12.19
	BA+ Kin (1.0 + 1.5) mg/l	13.00 ab	12.65 c	13.11a	12.92 A
	BA+ Kin (1.5 + 1.5) mg/l	9.15 k	8.77 l	9.80 i	F9.24
	Mean	9.99 A	9.59 C	9.77 B	
Number of leaves	BA+ Kin (0.5 + 0.5) mg/l	8.12 j	7.98 j	8.10 j	8.07 F
	BA + Kin (0.1 + 0.5) mg/l	8.16 j	8.65 i	7.21 k	8.01 F
	BA+ Kin (1.5 + 0.5) mg/l	9.65 g	10.12 ef	9.65 g	9.81 D
	BA+ Kin (0.5 + 1.0) mg/l	9.65 g	10.00 ef	9.15 h	9.60 D
	BA+ Kin (1.0 + 1.0) mg/l	9.10 h	8.65 i	9.10 h	8.95 E
	BA+ Kin (1.5 + 1.0) mg/l	13.12 a	12.63 b	11.20 c	12.32 A
	BA+ Kin (0.5 + 1.5) mg/l	10.23 e	11.20 c	9.89 f	10.44 C
	BA+ Kin (1.0 + 1.5) mg/l	13.12 a	10.98 c	10.65 d	10.94 B
	BA+ Kin (1.5 + 1.5) mg/l	11.20 c	10.20 e	10.20 e	11.17 B
	Mean	10.26 A	10.05 B	9.46 C	

Sub1st = subculture, Sub2 = 2nd subculture and Sub3 = 3rd subculture. Means within a column having the same letters are not significantly different according to Duncan's multiple range test (DMRT) at 5% level

Table 5: Effect of cytokinin type and MS medium strength on *in vitro* growth behavior during shoot multiplication stage of *Cyperus alternifolius* L. plants

		Subculture			
Measurements	Growth regulators (mg/l) and MS (g./l)	Sub 1	Sub 2	Sub 3	Mean
Shootlet formation (%)	1.0 mg/l BA+ 1.5 mg/l Kin.+ MS 1/2 strength	90.00 e	76.00 h	86.00 f	84.00 C
	1.0 mg/l BA+ 1.5 mg/l Kin.+ MS 3/4 strength	92.00 d	100.00 a	97.00 b	96.33 A
	1.0 mg/l BA+ 1.5 mg/l Kin.+ MS full strength	86.00 f	82.00 g	94.00 c	87.33 B
	Mean	89.33 B	86.00 C	92.33A	
Shootlet number	1.0 mg/l BA+ 1.5 mg/l Kin.+ MS 1/2 strength	2.36 cd	2.60 bc	2.55 bc	2.50 B
	1.0 mg/l BA+ 1.5 mg/l Kin.+ MS 3/4 strength	2.40 cd	2.90 a	2.94 a	2.75 A
	1.0 mg/l BA+ 1.5 mg/l Kin.+ MS full strength	2.26 d	2.43 bcd	2.68 ab	2.46 B
	Mean	2.34 C	2.64 B	2.72 A	
Shootlet length (cm)	.0 mg/l BA+ 1.5 mg/l Kin.+ MS 1/2 strength	10.26 g	12.03 d	11.52 c	11.27 C
	1.0 mg/l BA+ 1.5 mg/l Kin.+ MS 3/4 strength	14.29 b	14.23 b	15.12 a	14.55 A
	1.0 mg/l BA+ 1.5 mg/l Kin.+ MS full strength	11.26 f	13.23 c	12.02 d	12.17 B
	Mean	11.94 B	13.16 A	12.89 A	
Leaves number	1.0 mg/l BA+ 1.5 mg/l Kin.+ MS 1/2 strength	17.04 e	19.26 b	17.05 e	17.78 C
	1.0 mg/l BA+ 1.5 mg/l Kin.+ MS 3/4 strength	18.69 c	20.23 a	19.22 b	19.38 A
	1.0 mg/l BA+ 1.5 mg/l Kin.+ MS full strength	18.32 d	19.43 b	18.00 d	18.55 B
	Mean	17.99 B	19.64 A	18.09 B	

Sub1st = subculture, Sub2 = 2nd subculture and Sub3 = 3rd subculture. Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level

These results may be due to that cytokinin is one of the important plant hormones for plant growth and development that is known to promote cell division and differentiation. These results were in agreement with those achieved by Seyyedyousefi *et al*. [34] as they declared that the physiological role of cytokinin has been attributed to the activation of RNA, enzyme activity and protein synthesis as was studied by different kinds of cytokinin. It can also stimulate lateral bud growth and thus can cause multiple shoot formation through breaking apical dominance of shoots.

***In vitro* Rooting Capability:** Data presented in Table (6) indicate the influences of IBA, NAA and kin. on *in vitro* rooting capability of *Cyperus alternifolius,* L. The highest root length and root numbers were obtained from IBA+kin (1.0+0.5) mg/l treatment followed by NAA+kin. (2.0+0.5) mg/l treatment. The results may be due to that cytokinins as a plant growth regulator causes shoot initiation by stimulating cell division and reducing apical dominance. Using dilute media formulations improved root formation, since the high concentration of salts may prevent root growth, even in the presence of auxins in culture media similar results were gained by Seyyedyousefi *et al.* [34] and Ghareeb and Taha [35].

***Ex vitro* Acclimatization:** Data illustrated in Table (7) and Fig. D studied the effect of seven soil mixtures on the characteristics of the acclimatized plantlets after 8 weeks. Data indicated that the best results for plant survival and plant height of acclimatized plantlets were obtained from using peatmoss + hundz-soil+sand (1:1:1v/v/v) treatment. This increment may be due to the stimulatory influence of applied hundz-soil on the vegetative growth by Eissa *et al*. [36] Also, the soil mixture of peatmoss + sand (1:1v/v) appeared promotive effect on the length of roots formed per plantlet. Similar results were obtained by Ghareeb and Taha [35]. Also, the results are summarized in Tables (8 and 9) and Fig. E. showed the effect of the same soil mixtures on vegetative growth traits and chemical composition of *Cyperus alternifolius,* L. plants after 6 months from planting, the highest value was recoded from peatmoss + hundz-soil+sand (1:1:1v/v/v) treatment. This results may be attributed to that hundz- soil contains essential nutrients needed for the plants, retains water longer than regular soil , so plants develop strong root system while reducing water loss and provides a perfect distribution of nutrients to growing plants. Results of the present experiment were in line with many researchers who revealed that the beneficial effects of hundz-soil for vegetative growth, as observed by Youssef and Awad [17] and Eissa *et al.* [36].

Molecular Genetic Assessment Using SCoT –PCR Technique: The eight SCoT primers succeeded in amplifying DNA fragments for the mother plant and the micropropagated plant genotypes of *Cyperus alternifolius* (Fig. 2). Polymorphism levels differed from one primer to another, i.e. SCoT 8 primer exhibited high level of polymorphism (50%), while, SCoT 4 and SCoT 12

Table 6: Effect of IBA, Kin. and NAA on rooting %, roots numbers and length of *Cyperus alternifolius* L. plants

Growth regulators (mg/l)	Rooting (%)	Roots number	Root length (cm)
IBA +Kin.(1.0+ 0.5) mg/l	85.00 a	2.57 a	10.60 a
IBA+Kin.(2.0+0.5) mg/l	65.00 c	1.69 d	8.14 c
IBA +Kin. (4.0+0.5) mg/l	55.00 d	1.32 e	6.68 e
NAA.+Kin.(1.0+0.5) mg/l	45.00 f	2.07 c	7.90 d
NAA+Kin.(2.0+0.5) mg/l	79.00 b	2.43 b	10.54 b
NAA Kin.(0.4+ 0.5) mg/l	53.00 e	1.20 f	5.16 f

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

Table 7: Effect of soil mixtures on *Cyperus alternifolius* L. plantlets growth behavior after 8 weeks during *ex vitro* acclimatization stage

	Measurements		
Soil mixtures	Survival (%)	Plant Height(cm)	Root length (cm)
Peat moss	60.00 f	21.14 f	13.15 c
Hundz-soil	64.02 e	24.10 e	11.05 e
Sand	54.14 g	19.20 g	11.15 e
Peat moss+ Hundz-soil (1:1v/v)	69.14 b	27.23 b	14.31 b
Peatmoss + Sand (1:1v/v)	68.12 c	26.14 c	15.50 a
Hundz-soil+Sand (1:1v/v)	66.14 d	25.15 d	11.26 e
Peatmoss + Hundz-soil+Sand (1:1:1v/v/v)	72.52 a	30.23 a	11.85 d

Means within a column having the same letters are not significantly different according to Duncan's multiple range test (DMRT) at 5% level

Table 8: Effect of soil mixtures on vegetative growth traits after 6 months in *Cyperus alternifolius* L. plants during *ex vitro* acclimatization stage

	Measurements					
Soil mixtures	Plant height (cm)	Leaf length (cm)	N. of stems/ plant	N. of leaves/ stem	Fresh weight/plant (g)	Rhizome fresh weight/ plant (g)
Peat moss	42.00 g	16.01 f	8.14 e	14.62 d	65.14 f	44.17 d
Hundz-soil	56.20 d	18.26 d	8.64 d	14.25 e	78.25 c	60.20 a
Sand	45.14 f	15.20 g	7.14 g	12.15 f	63.14 g	21.20 g
Peat moss+ hundz-soil (1:1v/v)	60.14 c	19.24 c	7.62 f	16.23 b	67.20 d	45.20 c
Peatmoss + Sand (1:1v/v)	65.16 b	16.22 e	9.50 c	14.67 c	66.26 e	40.10 f
Hundz-soil+Sand(1:1v/v)	45.24 e	20.63 b	10.20 b	14.67 c	79.26 b	43.20 e
Peatmoss+hundz-soil+Sand (1:1:1v/v/v)	68.52 a	24.14 a	12.20 a	18.69 a	83.24 a	56.12 b

Means within a column having the same letters are not significantly different according to Duncan's multiple range test (DMRT) at 5% level.

Table 9: Effect of soil mixtures on chlorophyll a, b and total chlorophyll content (mg/g.f.w.) and total carbohydrates in rhizomes (Mg/g d.w.) after 6 months in *Cyperus alternifolius, L.* plants leaves

	Measurements			
Soil mixtures	Chlorophyll a (mg/g.f.w.)	Chlorophyll b (mg/g.f.w.)	Total Chlorophyll (mg/g.f.w.)	Total carbohydrates in rhizomes (mg/g d.w.)
Peat moss	1.32 b	0.48 c	1.80 bc	19.14 e
Hundz-soil	1.49 a	0.66 a	2.15 a	27.22 a
Sand	1.14 c	0.44 c	1.58 c	15.14 g
Peat moss+ Hundz-soil (1:1v/v)	1.20 c	0.52 bc	1.72 c	22.25 c
peatmoss + sand (1:1v/v)	1.32 b	0.52 bc	1.84 bc	17.25 f
hundz-soil+sand(1:1v/v)	1.40 ab	0.62 ab	2.02 ab	20.61 d
peatmoss + hundz-soil+Sand (1:1:1 v/v/v)	1.43 a	0.60 ab	2.03 ab	25.16 b

Means within a column having the same letters are not significantly different according to Duncan's multiple range test (DMRT) at 5% level

primers exhibited moderate level of polymorphism (33.33%) each one. On the other hand, SCoT 3, SCoT 6 and SCoT 10 have no polymorphism as exhibited in Table (10).The number of total bands, polymorphic bands, unique bands and percentage of polymorphism for each primer of the eight primers are shown in Table (10) SCoT1 Primer showed 4 DNA fragments with molecular size ranging from 185 to 475 bp (Fig. 2 and Table 10), one band was polymorphic (25 %) and one of them was unique band at 475 bp for the treated sample genotypes. SCoT3 primer showed three DNA fragments with molecular sizes ranging from 235 to 540 bp, they all were monomorphic bands. SCoT4 primer showed three DNA fragments with molecular size ranging from 280 to 460 bp, one fragment was polymorphic (33.33 %%) and one of them was unique band at 460 bp for the mother plant genotype. SCoT6 primer showed three DNA fragments with molecular size of ranging from 335 to 425 bp, they all were monomorphic bands. SCoT8 primer showed two DNA fragments with molecular size 210 to 360 bp, one fragment

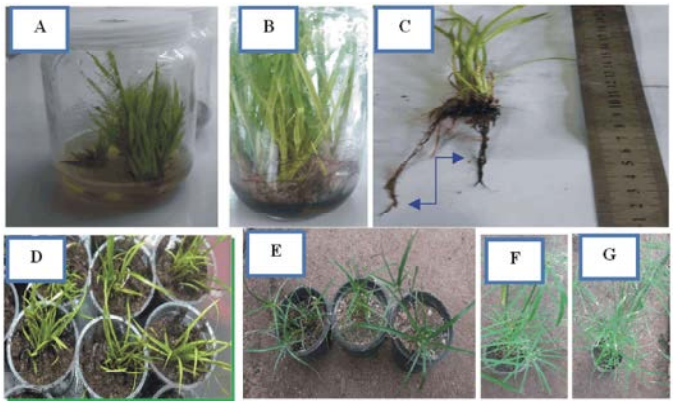

Fig. 1: Micropropagation stages of *Cyperus alternifolius* L plant. A: *In vitro* culture establishment stage, B, C: Rooting of *in vitro* microshoots, D, E, F and G: *ex vitro* acclimatization stage

Table 10: Molecular genetic data estimated from banding patterns combination of SCoT technique for the mother plant and the micropropagated plant genotypes of *Cyperus alternifolius*

Primer Name	Primer sequence	Molecular size range bp	Total Band	Monomorphic Band	Polymorphic band	Unique Band	Polymorphic %
ScoT 1	ACG ACA TGG CGA CCA CGC	185 - 475	4	3	1	1	25%
SCoT 3	ACG ACA TGG CGA CCC ACA	235 - 540	3	3	-	-	-
SCoT 4	ACC ATG GCT ACC ACC GCA	280 - 460	3	2	1	1	33.33%
SCoT 6	CAA TGG CTA CCA CTA CAG	335 -425	3	3	-	-	-
SCoT 8	CAA TGG CTA CCA CTA CAG	210 - 360	2	1	1	1	50%
SCoT 10	ACA ATG GCT ACC ACC AGC	245 - 500	3	3	-	-	-
SCoT 12	ACGACATGGCTACCAACG	200 -8000	6	4	2	2	33.33%
SCoT 14	ACC ATG GCT ACC AGC GCG	240 - 500	4	3	1	1	25%
Total		185 - 8000	28	22	6	6	21.42%

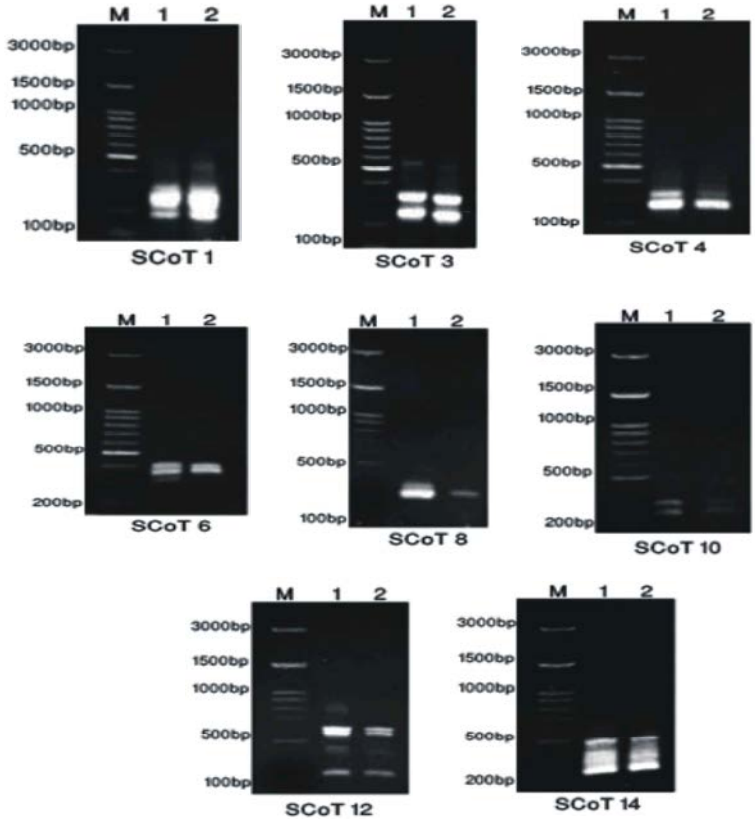

Fig. 2: Banding patterns of SCoT -PCR products for *Cyperus alternifolius* L. plants
1= Mother plant 2= Micropropagated plant

was polymorphic (50%) and one of them was unique band at 360 bp for the treated sample genotypes. SCoT10 primer produced three fragments with molecular size ranging from 245 to 500 bp (Fig. 2 and Table 10) as they all were monomorphic bands. SCoT12 primer resulted in six DNA fragments with molecular size ranging from 200 to 8000 bp, two fragments were polymorphic (33.33%) in which two of them were unique bands at 460 bp and 8000 bp for the mother plant genotype. SCoT14 primer resulted in four DNA fragments with molecular size ranging from 240 to 500 bp, one fragment was polymorphic (25%) and one of them was unique band at 430 bp for the mother plant genotype, while the other two fragments are considered as common fragments.

The tissue culture is an effective process of clonal propagation, though there are number of somaclonal differences in the obtained regenerated plants. These results are in agreement to those of Ghareeb and Taha [35] as they reported that somaclonal differences are usually caused by the generated mutations from tissue culture process. The mutation effects in tissue culture has been attributed to many stress aspects such as exposure to sterilization methods , wounding, changing of media balance as a result of increasing the concentration of plant growth regulators, sugars as a replacement of leaves photosynthesis, light condition and imperfection the relationship between humidity and transpiration. Also, Mangena [12] reported that BA may lead to abnormal plant responses (particularly, during germination and seedling development), habituation and other developmental aberrations fostered by prolonged exposure of plant materials to such *in vitro* conditions.

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CONCLUSION

Umbrella papyrus *(Cyperus alternifolius* L.) plants micropropagation protocol was established with a rapid proliferation of shoots and facility of microshoots rooting as well as plantlets acclimatization to the external environment. Using this protocol is suitable to produce a uniform source for umbrella papyrus plants and thus more applications in a short time to be used in the phytoremediation technology for sustainable environmental development and the pharmaceutical industries. This study were found that the highest percent of shoot formation reached 100 % on MS medium supplemented with BA at 1.0 mg/l and kin at 1.5 mg/l after culture three, throughout the multiplication stage. The highest numbers of shootlets/explant and the longest shootlets were obtained when 1.0 mg/l of BA + 1.5 mg/l of kin were added to MS culture medium of 3/4 strength. IBA and NAA had promotive effects on rooting formation of shootlets during the rooting stage .The highest numbers of roots/explant and the longest roots were obtained when 0.5 mg/l IBA + 1.5 mg/l kin. The best results of plant acclimatization traits after 8 weeks then after 6 months were obtained by using peatmoss + hundz-soil+ sand (1:1:1v/v/v/). The amplified DNA fragments using SCoT 1, SCoT 12 and SCoT 14 primers for mother and micropropagated plants indicated that the produced pattern by primer SCoT 12 had a maximum number of 6 bands of DNA fragments with molecular size ranging between 200 and 8000 bp, micropropagated plants showed similarity in relation to mother plant. The authors aimed in the future to use this micropagation protocol in genetic transformation systems or in the biotechnology studies.

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