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In vitro Propagation of Purple Nutsedge (*Cyperus rotundus* L.) for Useful Chemical Extraction

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***Abstract*—**The *in vitro* culture procedure of purple nutsedge (*Cyperus rotundus* L.) for multiple shoot induction and tuber formation was established. Multiple shoots were significantly induced from a single shoot of about 0.5 – 0.8 cm long, on Murashige and Skoog (MS) medium supplemented with 4.44 µM 6-benzyladinine (BA) alone or in combination with 2.85 µM 1-indoleacetic acid (IAA), providing 17.6 and 15.3 shoots per explant with 31.2 and 27.5 leaves per explant, respectively, within 6 weeks of culturing. Moreover, MS medium supplemented with 4.44 µM BA and 2.85 µM IAA was suitable for tuber induction, obtaining 5.9 tubers with 3.4 rhizomes per explant. In combination with ancymidol and higher concentration of sucrose, 11.1 µM BA and 60 g/L sucrose or 11.1 µM BA, 7.8 µM ancymidol and 60 g/L sucrose induced 3.5 tubers with 1.6 rhizomes or 3.5 tubers without rhizome, respectively. However, MS medium containing 3.9 or 7.8 µM ancymidol in combination with either 60 or 80 g/L sucrose enchanced significant root formation at 20.9 – 23.6 roots per explant.

***Keywords*—**Purple nutsedge, *Cyperus rotundus*, multiple shoot induction, tuber formation

I. Introduction

PURPLE nutsedge (*Cyperus rotundus* L.), a member of the family Cyperaceae, is one of the traditional medicinal plants found in many regions of the world. The rhizome is commonly used as traditional medicines to treat a variety of diseases [1]. Moreover, potential sources of useful chemicals were also found such as natural antioxidants [2], [3], weight control agent [4], allelopathic agents [5] Juvinile hormone III (methyl-10R,11-epoxy-3,7,11-trimethyl-2E,6E-dodecadienoate) at significant levels [6], [7], and flavoniods [8]. Juvenile hormone III was extracted from *in vitro C. aromaticus* plantlets [7] and four important flavoniods for pharmaceutical industry were found high contents from *in vitro* callus culture [8].

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In vitro culture techniques have been developed for potential mass propagation for plant species in the sedge family Cyperaceae in order to produce plants on a large-scale. Callus of *C. rotudus* was initiated from young rhizome and tuber then developed into shoot and root in order to studying growth and development of the species and for flavonoid extraction from callus [9], [8]. Multiple shoots were induced from nodes of *C. alternifolius* [10], inflorescences and tuber buds of *C. pangorei* [11], callus derived from roots of *in vitro* plantlets of *C. aromaticus* [4], [12], callus of *Caustis blakei* [13] and seeds of *Carex lurida* [14].

The development of *in vitro* culture techniques offers the best route to develop tissue for germplasm preservation, rapid propagation, and crop improvement to increase productivity and profitability, leading to the supply of purple nutsedge for demand and development. However, no report was found in *C. rotundus* for multiple shoot, tuber and rhizome induction through a single shoot.

This study reports a method for *in vitro* propagation of purple nutsedge from tuber axillary bud derived shoots as explants.

II. Materials and Methods

A. Plant Materials

Purple nutsedge plants with underground stems (tuber), grown in the greenhouse (Fig. 1 (a)), were washed with tap water then all leaves were removed and surface sterilized. A single sterilized tuber was excised and cultured on Murashige and Skoog (MS) medium [15] for 2 weeks (Fig. 1 (b)) and 4 weeks (Fig. 1 (c)). An individual shoot, regenerated from tuber about 0.5 – 0.8 cm. long (Fig. 1 (d)), was used as an initial explant for multiple shoot induction and tuber induction.

B. Surface Sterilization of Tuber

After washed with tap water, tubers were washed again with 10% detergent in an ultra-sonic cleaner for 10 min, rinsed with tap water and soaked in 70% ethanol for 5 min. Tubers, in the first method of surface-sterilization, were, firstly surface-sterilized in 5% Physan 20 (10% dimethyl benzyl ammonium chloride, 10% ethylbenzyl ammonium chloride and 80% inert ingredients), then 20% Clorox (6.0% w/w sodium hypo-chlorite) and finally 15% Clorox with 0.02% (v/v) Tween 20 for 10 min each time, then rinsed three times with sterile

distilled water. The second method was the same as the first method and continued with further steps of sterilization. The tubers were then soaked in Antibiotic Antimycotic Solution (Sigma) followed by three rinses in sterile distilled water, and left overnight in the sterile container. All procedures of surface-sterilization were repeated once more for the second method (Table I). The surface-sterilized tuberss were cultured on MS medium for 4 weeks.

C. Media and Culture Conditions

The medium used for multiple shoot and tuber induction was MS medium containing different concentrations of 6-benzyladenine (BA) at 4.44 – 22.20 µM, 30 g/L sucrose and 5.0 g/L agar. For media supplemented with 1-indoleacetic acid (IAA), 2.85 µM IAA was used in combinations with 4.44, 13.32 or 22.20 µM BA. For media supplemented with ancymidol and higher concentrations of sucrose, 3.9 and 7.8 µM ancymidol were used in combinations with 60 or 80 g/L sucrose and 11.1 or 22.20 µM BA. All media were adjusted to pH 5.7, prior to adding agar and autoclaving. Explants were cultured in 240-mL glass jars containing 50 mL of culture medium for 8 weeks. All cultures were incubated at 24 ± 1^0C with a 16-h photoperiod of 35 – 40 µmol.m^{-2}.s^{-1} provided by cool white fluorescent lights.

D. Statistical Analysis

Number and length of shoots, number of leaves, roots, tubers and rhizomes were recorded. Each treatment was replicated 10 times. The completely randomized design (CRD) was used as the experimental design and data were analyzed by Duncan's New Multiple Range Test at $P = 0.05$ [16].

III. Results

A.Surface Sterilization of Purple Nutsedge

Tubers, from plants grown in the glasshouse, were surface-sterilized with 2 methods as described above. For the first method, 18.0% of treated rhizomes were sterile. However, 48.9% of rhizomes treated with the second method were found sterile (Table I).

B.Effects of BA and IAA on Multiple Shoot and Tuber Induction from a Single Shoot of Purple Nutsedge

Individual single shoots about 0.5 – 1.0 cm. long, regenerated from *in vitro* tuber culture, were cultured for 8 weeks on MS medium supplemented with BA (0.00 – 22.20 µM) alone or in combination with IAA (0.00 – 2.85 µM). It was found that there was no significant difference among number of shoots and number of leaves induced by BA at the concentration of 4.44 – 22.20 µM alone and 4.44 – 13.32 µM in a combination with 2.85 µM IAA providing 15.2 – 17.6 shoots per explant with 23.9 – 31.2 leaves (Table II; Fig. 2 (b) and (c)). However, BA at the concentration of 4.44 µM gave the highest number of shoots and leaves per explant (17.6 shoots and 31.2 leaves). The significant greater number of tubers, 5.9 tubers per explant with 3.4 rhizomes, was obtained from MS medium supplemented with 4.44 µM BA and 2.85 µM IAA (Fig. 3 (b)). Compared to MS medium without plant growth regulator, 1.0 shoot with 4.1 leaves per explant and 1.0 tuber without rhizome were produced (Table I; Fig. 2 (a)). However, the highest number of root per explant (12.9 roots) was found from MS medium without growth regulator. (Table 2; Fig 3 (a)).

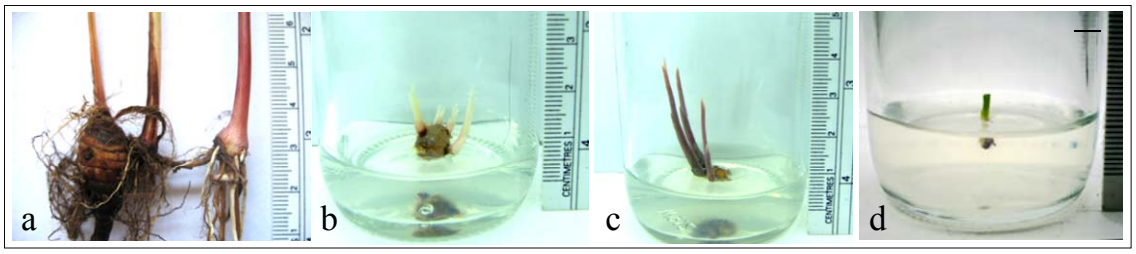

Fig. 1 Sources of explants for *in vitro* culture of purple sutsedge
a) tubers and rhizomes; b) a tuber cultured on MS medium for 2 weeks; c) a tuber cultured on MS medium for 4 weeks; d) a regenerated shoot from tuber used as an explant

TABLE I
PERCENTAGE OF STERILE EXPLANTS (TUBER) AND CONTAMINATION WITH BACTERIA AND FUNGI FROM DIFFERENT SURFACE STERILIZATION METHODS

Surface-sterilization methods	No. of explants	% Sterile explants	Contamination (%)	
			Bacteria	Fungi
5% Physan 20 (10 min), 20% Clorox (10 min), 15% Clorox (10 min)	50	18.0	48.0	34.0
[5% Physan 20 (10 min), 20% Clorox (10 min), 15% Clrox (10 min), Antimycotic Antibiotic Solution (10 min),]* left 24 hrs, repeated [*] once more	90	48.9	26.7	24.4

TABLE II
EFFECT OF BA AND IAA SUPPLEMENTED IN MS MEDIUM ON SHOOT AND TUBER FORMATION OF PURPLE NUTSEDGE SHOOTS AFTER CULTURING FOR 8 WEEKS

PGR (μM)		No. shoot size		growth and development (No. per explant) [1]				
BA	IAA	<1.5 cm	≥1.5 cm	total shoot	leaf	root	tuber	rhizome
0.00	0.00	0.0±0.0 a	1.0±0.0 a	1.0±0.0 a	4.1±0.2 a	12.6±1.7 d	1.0±0.0 a	0.0±0.0 a
4.44	0.00	8.6±0.9 c	9.0±1.1 c	17.6±1.9 c	31.2±2.3 c	5.0±1.1 ab	4.3±0.6 b	3.1±0.7 b
4.44	2.85	7.8±0.8 c	7.6±1.0 bc	15.3±1.8 c	27.5±3.1 c	7.7±1.1 bc	5.9±0.2 c	3.4±0.5 b
13.32	0.00	8.3±1.1 c	6.9±0.9 bc	15.2±1.7 c	24.6±2.1 bc	4.2±0.8 a	3.9±0.3 b	0.7±0.3 a
13.32	2.85	9.5±1.6 c	6.2±0.4 bc	16.3±2.3 c	23.9±1.3 bc	5.1±0.7 ab	4.5±0.2 b	2.7±0.6 b
22.20	0.00	8.9±1.5 c	8.5±1.7 bc	17.4±3.0 c	25.7±3.8 bc	2.3±1.1 a	2.3±0.8 a	3.4±0.5 b
22.20	2.85	3.6±0.6 b	5.8±1.0 b	9.4±1.4 b	19.3±2.8 b	2.7±0.6 a	3.8±0.4 b	2.7±0.6 b

[1] Values are mean ± SE (n = 10). Means followed by the same letters within the same column are not significantly different at *P = 0.05* by Duncan's new multiple range test

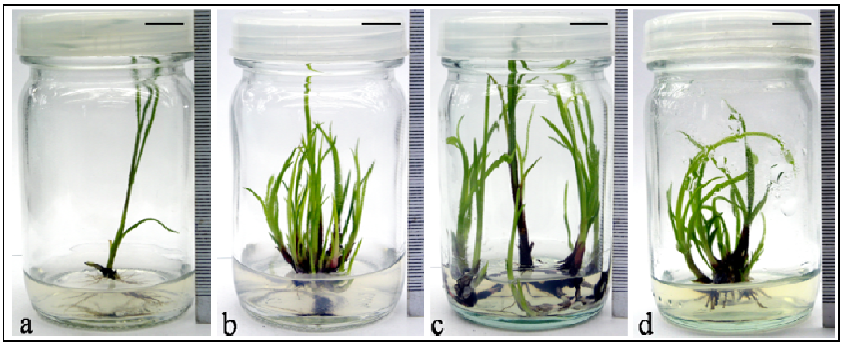

Fig. 2 Effects of plant growth regulators and sucrose supplemented in MS medium on growth of purple nutsedge shoot explants after culturing for 8 weeks (side view)
a) no plant growth regulator; b) 4.44 μM BA; c) 4.44 μM BA and 2.85 μM IAA; d) 60 g/L sucrose and 11.1 μM BA

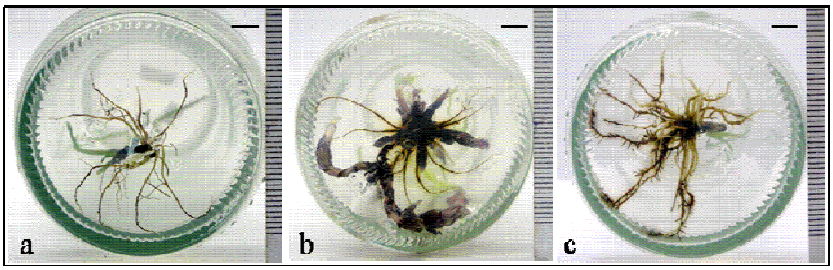

Fig. 3 Effects of plant growth regulators and sucrose supplemented in MS medium on rhizome and root formation of purple nutsedge shoot explants after culturing for 8 weeks (bottom view)
a) no plant growth regulator; b) 4.44 μM BA and 2.85 μM IAA; c) 60 g/L sucrose and 3.9 μM ancymidole

C.Effects of BA, Ancymidol and Sucrose on Shoot, Root, Tuber and Rhizome Induction from a Single Shoot of Purple Nutsedge

Individual single shoots about 0.5 – 1.0 cm. long, regenerated from *in vitro* tuber culture, were cultured on MS medium supplemented with ancymidol (3.9 and 7.8 μM) alone or in a combination with higher concentration of sucrose (60 or 80 g/L) in the presence or absence of BA (11.1 or 22.2 μM). It was found that ancymidol alone, in MS medium with 30 g/L sucrose, did not promote shoot and tuber formation (1.3 – 1.6 shoots per explant with 6.3 – 7.0 leaves per explant and 1.1 – 1.2 tuber per explants) but slightly promoted root formation (17.2 – 18.5 root per explant) as compared to the control medium (Table III). MS medium containing 60 g/L sucrose supplemented with BA at the concentrations of 11.1 or 22.2 μM alone or in a combination with ancymidol at the concentration of 3.9 or 7.8 μM significantly enhanced shoot multiplication, providing 9.6 – 11.4 shoots per explant (Table III; Fig. 2 (d)). However, 11.1 μM BA alone or in a combination with 7.8 μM ancymidol with 60 g/L sucrose resulted in the highest number of tubers with 3.5 tubers per explant with or without 1.6 rhizomes, respectively (Table III). In the presence of higher concentration of sucrose, either 60 or 80 g/L, ancymidole at the concentration of 3.9 or 7.8 μM enhanced root formation, providing 20.9 – 23.6 roots per explant (Table III; Fig. 3 (c)).

TABLE III
EFFECT OF BA, ANCYMIDOL AND SUCROSE SUPPLEMENTED IN MS MED IUM ON SHOOT, ROOT AND TUBER FORMATION OF PURPLE NUTSEDGE AFTER CULTURING FOR 8 WEEKS

Supplements			No. shoot size [1]		Growth and development (No. per explant) [1]				
Su g/L	Amd µM	BA µM	<1.5 cm	≥1.5 cm	total shoot	leaf	root	tuber	rhizome
30	0.0	0.0	0.0±0.0 a	1.0± 0.0a	1.0±0.0 a	4.5±0.2 a	13.3±1.5 bcdef	1.0±0.0 a	0.0±0.0 a
30	3.9	0.0	0.0±0.0 a	1.3±0.1 ab	1.3±0.1 a	6.3±0.4 ab	17.2±0.9 fgh	1.1±0.1 ab	0.0±0.0 a
30	7.8	0.0	0.3±0.1 a	1.6±0.1 abc	1.6±0.3 a	7.0±0.5 ab	18.5±0.8 ghi	1.2±0.1 ab	0.0±0.0 a
60	0.0	0.0	0.1±0.1 a	1.2±0.1 ab	1.3±0.1 a	5.6±0.6 a	16.1±0.9 efg	1.1±0.1 ab	0.0±0.0 a
60	3.9	0.0	0.3±0.2 a	1.9±0.3 abcd	2.2±0.3 abc	8.2±0.8 ab	23.6±1.7 j	1.2±0.1 ab	0.0±0.0 a
60	7.8	0.0	0.3±0.2 a	1.6±0.2 abc	1.9±0.2 ab	7.8±0.6 ab	23.3±1.5 j	1.2±0.1 ab	0.0±0.0 a
60	0.0	11.1	6.6±0.9 de	4.2±0.5 fghi	10.8±1.1 f	18.1±1.7 efg	11.5±0.7 abcde	3.5±0.3 f	1.6±0.5 b
60	0.0	22.2	6.2±0.9 de	4.4±0.7 ghi	10.6±1.3 f	18.6±2.5 efg	9.1±1.3 ab	2.5±0.2 de	0.0±0.0 a
60	3.9	11.1	4.6±1.2 bcd	5.0±0.8 hi	9.6±1.5 f	23.6±2.9 gh	11.4±1.1 abcde	3.2±0.3 ef	0.6±0.3 a
60	3.9	22.2	7.3±1.3 ef	3.6±0.9 efgh	10.9±2.1 f	20.7±3.0 fg	8.2±0.8 a	2.4±0.2 cde	0.0±0.0 a
60	7.8	11.1	6.0±0.8 de	5.4±1.2 i	11.4±1.7 f	26.4±3.0 h	14.4±1.7 cdefg	3.5±0.4 f	0.0±0.0 a
60	7.8	22.2	7.1±1.1 e	4.1±0.7 fghi	11.2±1.8 f	23.1±2.3 gh	7.6±1.1 a	2.3±0.3 cd	0.0±0.0 a
80	0.0	0.0	0.1±0.1 a	1.5±0.2 abc	1.6±0.2 a	5.5±0.5 a	14.9±1.3 defg	1.0±0.0 a	0.0±0.0 a
80	3.9	0.0	0.4±0.2 a	1.9±0.2 abcd	2.3±0.3 abc	7.2±0.5 ab	20.9±1.2 hij	1.2±0.1 ab	0.0±0.0 a
80	7.8	0.0	0.5±0.2 a	2.0±0.3 abcd	2.5±0.3 abcd	9.1±0.6 abc	22.0±1.7 ij	1.6±0.2 abc	0.0±0.0 a
80	0.0	11.1	3.1±0.5 bc	2.7±0.4 bcdef	5.6±0.4 cde	10.2±1.2 abcd	13.5±1.3 bcdef	2.3±0.1 cd	1.6±0.5 b
80	0.0	22.2	9.7±1.1 g	5.4±1.0 i	15.1±1.9 h	19.4±3.3 efg	15.1±2.5 efg	2.2±0.5 cd	0.0±0.0 a
80	3.9	11.1	2.3±0.4 ab	3.0±0.5 cdefg	5.3±0.9 bcde	14.6±2.1 cde	10.1±1.2 abc	2.6±0.4 de	0.6±0.3 a
80	3.9	22.2	5.3±1.4 cde	3.3±0.5 defg	8.6±1.6 ef	15.6±1.8 def	12.1±2.1 abc	2.4±0.2 cde	0.0±0.0 a
80	7.8	11.1	3.1±0.5 bc	2.4±0.5 abcde	5.5±0.9 cde	11.8±1.7 bcd	12.2±1.1 abcde	2.4±0.5 cde	0.0±0.0 a
80	7.8	22.2	3.2±0.4 bc	2.4±0.4 abcde	5.6±0.7 cde	12.0±2.0 bcd	10.3±1.7 abcd	1.9±0.3 bcd	0.0±0.0 a

[1] Values are mean ± SE (n = 20 with 5 explants/container). Means followed by the same letters within the same column are not significantly different at $P = 0.05$ by Duncan's new multiple range test

IV. DISCUSSIONS

In the process of surface sterilization of purple nutsedge tuber, 5% Physan for 10 min was used before treated with 20% and 15% Clorox for 10 min each, followed by Antimycotic Antibiotic Solution for 10 min. and the whole process was repeated once after 24 hr providing 48.9% of sterile explants. For *C. aromaticus* explants, young shoots near the base of rhizome were surface sterilized by 1.0 g/L mercuric chloride for 10 min. and followed by 300 mg/L sodium dichloroisocyanurate for 24 hr. [7].

In the experiments, 4.4 µM BA alone or 4.4 µM BA and 2.85 µM IAA were effective for multiple shoot, tuber and rhizome induction from purple nutsedge young shoots regenerated from tuber culture *in vitro* providing 15.3 – 17.6 shoots with 27.5 – 31.2 leaves and 4.3 – 5.9 tubers with 3.1 – 3.4 rhizomes. However, high concentration of sucrose at 60 g/L and 3.6 or 7.8 µM ancymidol was suitable for root induction with 23.3 – 23.6 roots. Similar results were obtained from multiple shoot induction of *C. aromaticus* young shoots using 4.4 µM BA and 2.46 µM 3-indolebutyric acid (IBA) but modified MS medium (three strength of MS nutrients, ion and vitamins) was applied [7]. For shoot multiplication induced from tuber buds of *C. pangorei*, MS medium supplemented with 10 µM BA was appropriate, providing 10 shoots per explant while the same concentration of zeatin and kinetin gave only 1 shoot per explant [11].

For many species of Cyperaceae, organogenesis was induced from calli in *Caustis blakei* (a cut foliage plant) for mass propagation [13], and cell suspension culture was established in *Cyperus aromaticus* for Juvenile hormone III production [12].

The achieved results were found to be advantage for mass propagation of *C. rotundus* in order to supply enough uniform raw materials with yields of useful chemicals. Moreover, it may provide a basis for genetic improvement in the future.

In summary, 4.44 µM BA alone or in combination with 2.85 µM IAA supplemented in MS medium were suitable for shoot induction while 4.44 µM and 2.85 µM IAA in the same medium enhanced tuber induction. For rooting, 3.9 or 7.8 µM ancymidol in combination with 60 or 80 g/L were suitable.

V. CONCLUSIONS

The procedure in this investigation demonstrated the culture medium for *in vitro* induction of multiple shoots, roots, tuber and rhizome of purple nutsedge (*Cyperus rotundus*) from a single shoot derived from axillary buds of tuber. The development of appropriate techniques for *in vitro* culture and micropropagation of purple nutsedge is necessary for mass propagation to obtain uniform plants for chemical extraction, germplsm collections and breeding program for high yield production of useful chemicals.

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REFERENCES

[1] I. A. Ross, *Medicinal plants of the world Vol. 1: Chemical constituents, traditional and modern medicinal uses*. Humana Press Inc., New Jersey. pp. 469, 2003.
[2] K.R. Nagulendran, S. Velavan, R. Mahesh, V. Hazeena Begum, "*In vitro* antioxidant activity and total polyphenolic content of *Cyperus rotundus* rhizomes." *E-J. Chem*. Vol. 4, pp. 440-449, 2007.
[3] R. Yazdanparast, and A. Ardestani, "*In vitro* antioxidant and free radical scavenging of *Cyperus rotundus.* " *J. Med. Food.*, vol. 10, pp. 667-674, 2007.
[4] B. Lemaure, A. Touche, I. Zbinden, J. Moulin, D. Courtois, K. Mace, and C. Darimont, "Administration of *Cyperus rotundus* tubers extract prevents weight gain in obese Zucker rats. *Phytotherapy Research*, vol. 21, pp. 724-730, 2007.
[5] N. B. Singh, B. N. Pandey, A. Singh, "Allelopathic effects of *Cyperus rotundus* extract *in vitro* and *ex vitro* on banana." *Acta Physiol. Plant.* Vol. 31, pp. 633-638, 2009.
[6] O. Kaewmun, N. WathaNaapa, W. Ngow, and T. Pewnim, "Biochemical characteristics of *Cyperus iria* and *Cyperus rotundus. 31st Congress on Science and Technology of Thailand*, Suranaree University of Technology. 18-20 October, 2005.
[7] C. C. Weng, C. L. Keng, B. P. Lim, "Detection of insect juvenile hormone III and it's precursors from *in vitro* plantlets of *Cyperus rotundus."* *J. Plant Biol*., vol. 47, vol. 3, pp. 187-193, 2004.
[8] K. Samariya, and R. Sarin, "Isolation and identification of flavonoids from *Cyperus rotundus* Linn. *in vivo* and *in vitro*." *J. Drug Deliv. Therap.*, vol. 3, no. 2, pp. 109-113, 2013.
[9] J. B. Fisher, "Callus, cell suspensions, and organogenesis in tissue cultures of purple nutsedge (*Cyperus rotundus*)". *Bot. Gaz.*, vol. 138, no. 3, pp. 291-297, 1997
[10] Y. Mohamed-Yasseen, and W. E. Splittstoesser, "Multiplication of *Cyperus alternifolius* from axillary buds *in vitro*: instructive laboratory exercise." *Plant Growth Reg. Soc. Amer.*, vol. 20, pp. 83-89, 1992.
[11] J. F. Benazir, B. Mathithumilan, P. Ravichandran, V. Jochebed, R. Suganthi, and V. Manimekalai, "*In vitro* regeneration of nut sedge (*Cyperus pangorei* Rottb)." *J. Plant Biochem. Biotechnol*., vol. 18, pp. 209-215, 2009.
[12] K. C. Lai, S. L. Pey, L. C. Mang, and L. B. Pey, "Establishment of *Cyperus aromaticus* cell suspension cultures for the production of Juvenile hormone III." *In Vitro Cell. Dev. Biol. Plant,* vol. 46, no. 1, pp. 8-12, 2010.
[13] J. Webber, M. E. Johnston, A. H. Wearing, "High irradiance increases organogenesis in friable callus of *Caustis blakei* (Cyperaceae). *In Vitro Cell. Dev. Biol.-Plant*., vol. 39, no. 2, 139-141, 2003.
[14] S. M. D. Rogers, "Tissue culture and wetland establishment of the freshwater monocots *Carex, Juncus, Scirpus,* and *Typha.*" *In Vitro Cell. Dev. Biol.-Plant.* vol. 39, no. 1, pp. 1-5, 2003.
[15] T. Murashige, and F. Skoog, 1962. A revised medium for rapid growth and bio-assay with tobacco tissue culture. *Physiol. Plantarum*, vol. 15, pp. 473-497, 1962.
[16] D. B. Duncan, "Multiple range and multiple F test." *Biometrics*, vol. 11, pp. 1–42, 1955.