Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 9, 2015

Original Article

IN VITRO MULTIPLE SHOOT REGENERATION STUDIES IN *CYPERUS ROTUNDUS* L.

LAVANYA KAKARLA, CHAKRAVARTHY RAMA, MAHENDRAN BOTLAGUNTA*

Department of Biotechnology, K L E F (Koneru Lakshmaiah Education Foundation) University, Vaddeswaram, Guntur 522502, Andhra Pradesh, India
Email: bmnchowdary@gmail.com

Received: 17 Jun 2015 Revised and Accepted: 11 Jul 2015

ABSTRACT

Objective: *Cyperus rotundus* L (Cyperaceae) is one of the highly valuable, potent, multipurpose medicinal herb used in traditional medicine for treatment of several human alignments. To meet the growing herbal needs of pharma industry, we made an attempt on *in vitro* regeneration of *C. rotundus* by using rhizome explants.

Methods: Rhizome explants were inoculated on both solid and liquid Murashige and Skoog [MS] media supplemented with various concentrations of Kinetin [Kn], 2,4-Dichloro phenoxy acetic acid [2,4-D], Naphthalene acetic acid [NAA], Indole-3-butyric acid [IBA] and activated charcoal for *in vitro* regeneration of multiple shoots and root induction.

Results: Efficient multiple shoots induction (88.9%) was observed on MS liquid media containing 3.0 mg/l 2,4-D+3.0 mg/l NAA+0.5 mg/l Kn after two weeks of explant inoculation with an average number of shoots 9.56±0.35 per explant. Further, we noticed effective root induction (82.3%) from these *in vitro* grown shoots on root induction media containing 1.0 mg/l IBA with 500 mg/l activated charcoal.

Conclusion: Overall, it suggests that this rapid and reliable protocol will be useful for mass multiplication of plantlets and maintenance of germ plasm throughout the year without seasonal constraints.

Keywords: *Cyperus rotundus* L, MS (Murashige and Skoog) medium, Rhizome explants, *In vitro* regeneration, 2,4-Dichloro phenoxy acetic acid.

INTRODUCTION

The World Health Organization (WHO) estimates that approximately 80 % of the world's populations depends primarily on traditional medicines as sources for health care [1]. Plants and plant products are reported to exhibit a wide range of biological activity profiles which include nootropics, psychoactive agents, dependence attenuators, anticonvulsants, sedatives, analgesics, autonomic, anti-inflammatory agents, antipyretics, neurotransmission modulators, anticoagulants, cardio protectants and bone healing agents etc [2]. This has been possible largely due to their incredible and diverse array of the carbon skeletons and functional groups. In order to conserve these natural resources biotechnology offers plant tissue culture is one of the techniques used in the field of plant breeding and conservation of many endangered plants. Tissue culture technology has proved to be a versatile tool for propagating elite clones and the screening for useful variants [3]**.** To the meet the growing herbal needs of pharma industries and for protecting the genetic erosion there is a need for mass propagation of these plantlets. In the present study, we are choosen one of the highly valuable medicinal plant *Cyperus rotundus* L.

Cyperus rotundus L is a cosmopolitan herbaceous weed, is found in moist temperate to wet tropical regions of the world and it belongs to Cyperaceae family [4]. The family comprises about 104 genera and more than 5000 species worldwide, although estimates of numbers vary greatly due to differing taxonomic concepts of individual researchers. The largest genus is *Carex* with about 2000 species worldwide, followed by *Cyperus* with about 550 species [5]. *C. rotundus* is a grass like weed, about 80-90 cm tall, with an extensive underground network of basal bulbs, fibrous roots, thin wiry rhizomes and tubers born in chains of 2-6 or more on rhizomes, with tubers spaced 3-4 mm apart.

The leaves are mostly basal, dark green, with a prominent midrib and an abrupt taper at the top. The purplish to red-brown inflorescence is borne on a stem that is triangular in cross section and usually taller than the foliage [6]. The tuberous roots are collected, dried and used in traditional medicine [7]. The nut grass is used in hair and skin care products, it stimulates sebaceous glands near hair roots. The tubers are credited with astringent, diaphoretic, diuretic, dessicant, cordial and stomachache properties [8]. A decoction of the tuber is used for washing hair, treating gonorrhea and syphilis. It is also given in diarrhea and for general weakness. This plant contains a battery of antioxidant biomolecules and has been considered as edible plant source for Ayurveda, nutraceutical and pharmaceutical applications.

The demand for *Cyperus rotundus* rhizomes and its unregulated harvesting has lead to near extinction of the species. *In vitro* micropropagation is useful for continuous supply of raw material required for herbal industry without seasonal constraints [9]. In the present investigation, induction of multiple shoots and roots from *Cyperus rotundus* rhizome explants is reported, which could be used as an alternative source to *Cyperus rotundus* rhizomes maintenance and conservation of germ plasm.

MATERIALS AND METHODS

Plant material

C. rotundus plants were collected from the botanical garden at K L E F University campus, Vaddeswaram, Andhra Pradesh, India. These plants were taxonomically identified by Dr. A. Prasada Rao, senior taxonomist in K L University, Vijayawada, Andhra Pradesh, India. Voucher specimen has been deposited at K L University Botanical garden (voucher specimen number KLU-1251) for further use.

Explant collection and sterilization

Healthy young rhizomes 2-3 cm were excised from plants grown in the botanical garden. After selection of rhizomes as explants are washed in tap water for 30 min to remove soil and other unwanted contamination. They were kept in double distilled water for 3-4hr to eliminate phenolic substances leach out of explants. This was followed by rinsing with a solution of 1% sodium hypochlorite (v/v) for 5 min, washing under running tap water for 15 min followed by surface sterilization with 0.1% (w/v) $HgCl_2$ for 2-3 minutes and finally explants were washed in autoclaved distilled water for 5-6 times to remove traces of $HgCl_2$. Later collect sterile whatmann filter paper and flame sterilize in the laminar air flow cabinet. Dry the explants on a filter paper and cut the terminal ends of explants. Finally these explants were inoculated on to the Murashige and

Skoog [MS] basal medium supplemented with different combinations and concentrations of phyto hormones for shoot bud induction.

Culture medium and conditions

The culture medium used for the explant selection is Murashige and Skoog [MS] medium [10] solid and liquid media's were used throughout the experiment was supplemented with and without addition of 0.8% (w/v) agar, 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8 using 0.1N NaOH (or) 0.1N HCl before autoclaving at 121ºC for 15 min. The cultures were incubated at 25±2 °C, 60-65% relative humidity and a 16/8 hr (light/dark) photoperiod with a light intensity of 50 $\mu mol^{-2}S^{-1}$ supplied by cool white fluorescent light. Thermolabile constituents like vitamins and phytohormones were filter sterilized through 0.2µm. membranes before addition to the sterilized medium. For shoot bud initiation rhizomes of *C. rotundus* were inoculated on MS media supplemented with different concentrations of kinetin [Kn] (0.25-2.0 mg/l), 2, 4-Dichloro phenoxy acetic acid [2,4-D] (1.0-4.0 mg/l) and Naphthalene acetic acid [NAA] (1.0–4.0 mg/l) either independently (or) in combination with other growth hormones for two to three weeks. After shoot bud initiation and elongation the excised shoots were transferred to root induction media supplemented with different concentrations of Indole-3-butyric acid [IBA]. All these results were tabulated after three to four weeks of inoculation.

Statistical analysis

The experiments were performed using completely, randomized experimental design was applied. In this study, every experiment ten explants was used, all experiments were repeated three times. Data (Percentage of shoot induction and multiple shooting, average no of shoots and their length, rooting, average number of roots and their length) were analyzed by one-way ANOVA technique. The Mean values recorded from the experimental data were compared using Tukeys' HSD test at P = 0.05 with SPSS ver.13.0. The results are expressed as mean±SE of three experiments.

RESULTS

C. rotundus rhizome explants were cultured on MS medium without growth regulator gave no regeneration response. The explants inoculated on MS medium supplemented with various concentrations of Kn (0.25, 0.50, 0.75, 1.0, 1.5 and 2.0 mg/l). The number of shoots and shoot induction frequency was recorded after two weeks of culture inoculation using different growth parameters.

The explants inoculated on to MS medium with 0.25 mg/l showed 40% of shooting response was observed with two shoots from each explant. At 0.50 mg/l showed 80% of response with five shoots per explant. 72% of shooting induction response observed in 0.75 mg/l with four shoots per explant. At 1.0 mg/l concentration 60% of response with three shoots. 30% of response with two shoots per explant was observed at 1.5 mg/l.

When concentration of Kn was increased to 2.0 mg/l 10% of shoot induction was observed with the single shoot formation. Among different selected concentrations of Kn alone, 0.5 mg/l Kn showed 80% of shoot induction, so further experiments we performed with this concentration of Kn [table 1, fig. 1].

Table 1: Shoot induction from rhizome explants of *C. rotundus* in as a function of different concentrations of kinetin in MS basal medium

S. No.	Basal medium supplemented with Kinetin (mg/ml)	Frequency of shoot induction (%)	No. of shoots per explant
1	0.25	40	Two
2	0.50	80	Five
3	0.75	72	Four
4	1.00	60	Three
5	1.50	30	Two
6	2.00	10	Single

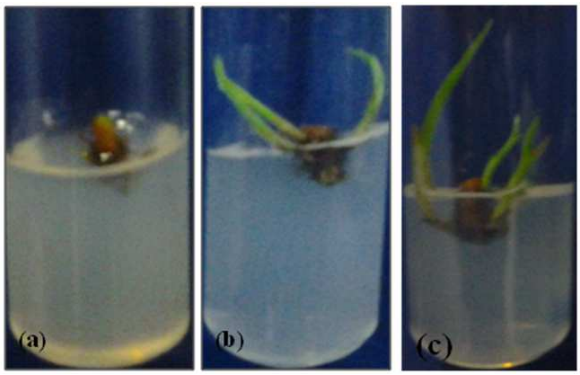

Fig. 1: (a) Explant inoculated on full strength MS Media. (b) Initiation of shoots from rhizome explants on media. (c) Elongation of shoots on MS media containing Kn 0.5 mg/l

Multiple shooting

C. rotundus plants were efficiently regenerated from rhizome explants from field grown young plants on liquid MS medium supplemented with 0.5 mg/l Kn, 1.0–4.0 mg/l 2,4-D and 1.0–4.0 mg/l NAA for multiple shoot induction. The multiple shoot induction with respect to the test concentrations of growth hormones were presented in (table 2). When MS liquid medium supplemented with 0.5 mg/l Kn and various concentrations of 2,4-D. At 1.0 mg/l 2,4-D with 0.5 mg/l Kn showed 42.3% multiple shoot induction was observed. 53.4% of shoot induction was observed with 2.0 mg/l 2,4-D with Kn. When medium supplemented with 3.0 mg/l 2,4-D 74.3 % shoot inductions was observed. 62.5 % shoot induction were noticed on medium supplemented with 4.0 mg/l 2,4-D combination with Kn. Among these tested concentrations of Kn combination with 2,4-D higher multiple shoots were noticed on medium containing 3.0 mg/l 2,4-D with 0.5 mg/l Kn with average number of shoots were 4.16±0.16 with shoot length 2.79±0.18 (Fig.2(a)).

Fig. 2: (a) Multiple shoot induction from rhizome explants on media containing 3.0 mg/l 2,4-D+0.5 mg/l Kn. (b) Multiple shoot induction in liquid media containing 3.0 mg/l 2,4-D+3.0 mg/l NAA+0.5 mg/l Kn after three weeks of inoculation

Further we supplemented 3.0 mg/l 2,4-D+0.5 mg/l Kn with various concentrations of NAA 1.0–4.0 mg/l). When MS liquid media with 3.0 mg/l 2, 4-D+1.0 mg/l NAA+0.5 mg/l Kn showed 51.3% multiple

shoot induction. At 3.0 mg/l 2, 4-D+2.0 mg/l NAA+0.5 mg/l Kn 55.6% response was observed. When NAA concentration was increased to 3.0 mg/l along with constant concentrations of 2,4-D and Kn 88.9% response was observed. 64.5% of multiple shooting was observed, when explants inoculated on to media containing 4.0 mg/l NAA along with 2,4-D and Kn. Among all these hormonal concentrations alone and in combinations 3.0 mg/l NAA+3.0 mg/l 2,4-D+0.5 mg/l Kn showed 88.9% multiple shoot induction with an average number of shoots 9.56±0.35 with shoot length 4.78±0.36 (Fig.2(b)). Finally these shoots were incubated more than three weeks of duration under strictly controlled conditions for shoot elongation medium containing 3.0 mg/l NAA+3.0 mg/l 2,4-D+0.5 mg/l Kn.

Rooting of shoots

Elongated shoots (3-5 cm) were excised and placed on full strength MS medium supplemented with various concentrations of IBA and 500 mg/l activated charcoal for rhizogenesis. When MS medium with IBA 1.0 mg/l showed 82.3% of root development in the shooted explants within 10-12 days. Whereas, MS medium with IBA 0.50 mg/l showed after 20 days of time, but the percentage of rooting response was lower 62.4%. Whereas shoots shifted onto MS medium with IBA 1.50 mg/l showed 74.5%. 58.9% of rooting response was observed with IBA 2.0 mg/l. While increasing the concentration of IBA above or below 1.0 mg/l the percentage of rooting was lower. Shoots formed roots 82.3% on media containing 1.0 mg/l IBA in this medium the number of roots was highest 5.23±0.18 and their length 2.74±0.18, it is evident from (table 3, Fig.3 (a, b &c)). Hence, MS medium with Kn 0.75 mg/l, 3.0 mg/l 2,4-D, 3.0 mg/l NAA and 1.0 mg/l IBA were found to be ideal concentrations for complete multiple shoot and root induction in *C. rotundus* L.

Acclimatization and field establishment

Well-developed rooted plantlets were gently removed from the test tubes and thoroughly washed with sterile water to remove adhered agar and traces of medium to avoid contamination, plantlets were transferred to plastic pots containing autoclaved and annealed soil with nutrient rich vermiculite (1:1) (fig. 3(d)). In the first week of transplantation the plantlets were encapsulated with polyethylene sheet rinsed with 70% ethanol to provide high humidity, allow sufficient light and to curb the affect of contaminants. The polyethylene sheet was removed periodically and progressively whenever the leaves appeared to be wet. The polyethylene sheet was withdrawn completely after three weeks of hardening. After 3 weeks, the plants were transferred to larger pots filled with soil and organic manure for further growth. Finally the acclimatized plants were shifted to field conditions, 81.13% of them having survived. The growth characteristics of plants raised *in vitro* did not show any significant morphological variations from those of the natural habitat.

Table 2: Multiple Shoot induction from rhizome explants of *C. rotundus*

S. No.	Growth regulator concentration in (mg/l)	Multiple shooting percentages (%)	Average no. of shoots/explant	Average shoot length/explant
1	2,4-D 1.0+Kn 0.5	42.3	$1.76^{a}\pm0.25$	$0.84^{a}\pm0.21$
2	2,4-D 2.0+Kn 0.5	53.4	$2.58^{b}\pm0.14$	$1.18^{b}\pm0.16$
3	2,4-D 3.0+Kn 0.5	74.3	$4.16^{c}\pm0.16$	$2.79^{c}\pm0.18$
4	2,4-D 4.0+Kn 0.5	62.5	$3.68^{b}\pm0.17$	$1.64^{b}\pm0.15$
5	2,4-D 3.0+NAA 1.0+Kn 0.5	51.3	$2.34^{a}\pm0.32$	$1.14^{a}\pm0.18$
6	2,4-D 3.0+NAA 2.0+Kn 0.5	55.6	$2.58^{a}\pm0.24$	$1.21^{a}\pm0.24$
7	**2,4-D 3.0+NAA 3.0+Kn 0.5**	**88.9**	**$9.56^{c}\pm0.35$**	**$4.78^{c}\pm0.36$**
8	2,4-D 3.0+NAA 4.0+Kn 0.5	64.5	$3.68^{b}\pm0.21$	$1.86^{b}\pm0.14$

Values are expressed as mean±SE (n=10 in replicate). Mean followed by same letters do not differ significantly at $p \geq 0.05$ by Tukey's HSD test

Table 3: Root induction from rhizome explants of *C. rotundus*

S. No.	Growth regulator concentration in (mg/l)	Multiple shooting percentages (%)	Average no. of roots/explant	Average root length/explant
1	IBA 0.5	62.4	$3.34^{a}\pm0.26$	$1.86^{a}\pm0.14$
2	IBA 1.0	82.3	$5.23^{c}\pm0.18$	$2.74^{c}\pm0.18$
3	IBA 1.5	74.5	$4.16^{b}\pm0.14$	$2.16^{b}\pm0.12$
4	IBA 2.0	58.9	$3.68^{a}\pm0.26$	$1.42^{a}\pm0.13$

Values are expressed as mean±SE (n=10 in replicate). Mean followed by same letters do not differ significantly at $p \geq 0.05$ by Tukey's HSD test.

Fig. 3: (a) Initiation of roots from excised shoots cultured *in vitro* solid MS medium supplemented with 1.0 mg/l IBA+500 mg/l activated charcoal. (b&c) Elongation of shoots and roots after four weeks in rooting media containing 1.0 mg/l IBA with charcoal. (d) Successful pot culture of *in vitro* grown plantlet *Cyperus rotundus* L

DISCUSSION

The effect of BA alone or in combination with Indole-3-acetic acid [IAA] for induction of multiple shoots and roots in *Cyperus rotundus* [11]. In *Cyperus alternifolius* multiple shoots induced from axillary buds [12]. Inflorescence and tuber buds of *Cyperus pangorei* used as explants for *in vitro* regeneration studies [13]. In *Cyperus aromaticus* callus derived from roots of *in vitro* grown plantlets [14]. But in our experiments, efficient multiple shoot induction was observed on liquid MS medium at 3.0 mg/l 2,4-D+3.0 mg/l NAA+0.5 mg/l Kn with an average number of shoots 9.56±0.35 and their shoot length 4.78±0.36 per explant. In addition to this rooting observed on solid MS medium with 1.0 mg/l IBA with 500 mg/l activated charcoal. Similarly, multiple shoots were induced on SH medium with Kn 1.50 mg/l+Adenosine [ADS] 1.0 mg/l+500 mg/l activated charcoal+5 % coconut water [CW] was found to be ideal concentrations for multiple shoot and root induction in *Cyperus scariosus* [9]. In this study, a combination of 2,4-D, NAA and Kn on liquid MS medium was found to be more effective to induce multiple shoot proliferation in *Cyperus rotundus*.

CONCLUSION

An efficient *in vitro* protocol for induction of multiple shoots, roots and tuber of *Cyperus rotundus* were reported here from single rhizome explant. This technique is necessary for mass propagation of uniform plants for pharmaceutically active compounds extraction, germ plasm conservation and breeding programmes for high yield production of therapeutically important compounds.

ACKNOWLEDGEMENT

We are very much thankful to the management, HOD, Principal for their kind support. This work was supported by a grant from the Women Scientist Award (DST, Government of India) to Lavanya Kakarla.

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Rajkumar V, Guha G, Kumar RA. Isolation and bioactivity evaluation of two metabolites from the methanolic extract of *Oroxylum indicum* stem bark. Asian Pac J Trop Biomed 2012;2 Suppl 1:7-11.
2. Dahanukar S, Kulkarni R, Rege N. Pharmacology of medicinal plants and natural products. Indian J Pharmacol 2000;32 Suppl:81-118.
3. Akin-Idowu PE, Ibitoye DO, Ademoyegun OT. Tissue culture as a plant production technique for horticultural crops. Afr J Biotechnol 2009;16:3782-8.
4. Goetghebeur P. Cyperaceae. In: K Kubitzki. The families and genera of vascular plants: Alismataneae and Commelinanae (except Gramineae). Springer-Verlag, Berlin; 1998. p. 141-90.
5. Archer C. Plant Life; 1998. p. 18, 14.
6. Marwat SK, Khan MA. Biodiversity of sedges in dera ismail khan district nwfp Pakistan. Sarhad J Agric 2008;2:293-304.
7. Chopra RN, Nayar SL, Chopra IC. Glossary of indian medicinal plants, Publications & Information Directorate: New Delhi, India; 1956.
8. Singh N, Kulshrestha VK, Gupta MB, Bhargava KP. "A pharmacological study of *Cyperus rotundus*." Indian J Med Res 1970;58:103–9.
9. Lavanya K, Chakravarthy R, Krishna MSR. *In vitro* flower induction and multiple shoot regeneration studies in *Cyperus Scariosus* R. Br from axillary bud explants. Int J Pharm Biol Sci 2014;1:697–705.
10. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco cultures. Physical Plant 1962;15:473-97.
11. Thepsithar C, Euawong N, Jonghomkajorn N. *In vitro* propagation of purple nutsedge (*Cyperus rotundus* L.) for useful chemical extraction. Int J Biol Food Veterinary Agric Eng 2013;8:454-8.
12. Mohamed-Yasseen Y, Splittstoesser WE. "Multiplication of *Cyperus alternifolius* from axillary buds *in vitro*: instructive laboratory exercise." Plant Growth Regul Soc Am 1992;20:83-9.
13. Benazir JF, Mathithumilan B, Ravichandran P, Jochebed V, Suganthi R, Manimekalai V. "*In vitro* regeneration of nut sedge (*Cyperus pangorei* Rottb)." J Plant Biochem Biotechnol 2009;18:209-15.
14. Lai KC, Pey SL, Mang LC, Pey LB. "Establishment of *Cyperus aromaticus* cell suspension cultures for the production of Juvenile hormone III". *In Vitro* Cell Dev Biol: Plant 2010;1:8-12.