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IN VITRO FLOWER INDUCTION AND MULTIPLE SHOOT REGENERATION STUDIES IN *CYPERUS SCARIOSUS* R.Br FROM AXILLARY BUD EXPLANTS

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ABSTRACT

Cyperus scariosus R.Br is a valuable multipurpose medicinal herb, which belongs to family *Cyperaceae*. It possesses astringent, anti-inflammatory, diaphoretic, diuretic, cordial and stomach ache properties. An attempt has been made to study in *vitro* regeneration, floral bud induction, multiple shooting and multiple rooting has been achieved via healthy, moderate axillary buds as explants. The axillary bud explants were inoculated on Schenk and Hildebrandt medium with various concentrations of BA (0.5-2.0mg/lt) and Kn (0.5-2.0mg/lt) for *in vitro* regeneration. Effective, healthy shoots were observed from Kn 0.75mg/lt with activated charcoal 500mg/lt. These shoots were further subculture on Kn 0.75mg/lt with various concentrations of Adenosine (ADS) 0.5-2.0mg/lt and activated charcoal 500 mg/lt. Efficient flowering 80% was noted on SH media, when supplemented with Kn 0.75mg/lt+ADS 1.0mg/lt+activated charcoal 500 mg/lt. 98% of multiple shooting and multiple rooting was effective on full strength SH medium supplemented with Kn1.5mg/lt+ADS1.0mg/lt+ activated charcoal 500mg/lt+5% coconut water. *In vitro* regenerated plantlets were transferred onto vermiculite pots, which were further acclimatized in the field.

KEYWORDS: Schenk and Hildebrandt medium, *In vitro* flowering, Benzyl adenine and Furfuryl Kinetin.

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INTRODUCTION

Cyperus scariosus R.Br is commonly known as umbrella sedge[1] also known in English as cypriol. It belongs to family *Cyperaceae* and widely distributed in central India. It is a third largest family in monocotyledons[2]. *C.scariosus* grows rapidly and fills the soil with tangle of roots and rhizomes. It grows about a meter tall, arising from rhizomes and tubers. The stems are three sided and triangular in cross section and there is an umbrella like tuft of long narrow leaves at the top. The leaves are yellow to green in color with a distinct ridge. This plant is invasive and adversely affects many crops, including rice and many vegetables[3]. It is especially prevalent in southern India[4], where its essential oil is employed in perfume industry[5] and the nut grass is used in the formulation of hair and skin care products, it stimulates sebaceous glands near hair roots[6]. Dried tuberous roots of *C. scariosus* are used in traditional medicine[7]. The tubers are credited with astringent, diaphoretic[8], diuretic[9], desiccant, cordial and stomachache properties[10]. In traditional medicine, the rhizomes of the plant are used in the treatment of inflammation[11]. *C. scariosus* provides interesting secondary metabolites, of which terpenoids[12] xylopyranosides and essential oils are of special interest as they exhibit varied properties of pharmaceutical value[13]. Due to unrestricted large scale exploitation of this natural resource, the wild stock of this species has been markedly depleted. Plant tissue culture promises the continuous supply of raw material required for herbal industry without seasonal constraints. An efficient protocol is necessary for maintaining an *In vitro* line of this plant for further manipulations like mutations to improve secondary metabolite production. The present paper deals with the development of a reproducible protocol for micro propagation and *in vitro* flowering in *C.scariosus* through axillary bud explants.

MATERIALS AND METHODS

Plant material was collected from the Botanical garden at K L University campus, Vaddeswaram, Andhra Pradesh, India. For the initial experiments, young, immature, healthy moderate axillary buds 0.4cm-0.6cm and rhizomes 0.2-0.3cm were excised from plants grown in the botanical garden. After selection of axillary bud as an ideal explants are washed in tap water for 3-4 times, further rinsed with tween-20 for 5-10 minutes, later treated with 1%(w/v) bavastin for 15-20 minutes. Explants were sterilized 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 $HgCl_2$. Collect sterile what man 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 surface of SH medium with plant growth regulators.

Culture medium and conditions

The culture medium used for the explant selection is SH[14] (Schenk and Hildebrandt 1972) medium supplemented with 0.8% (w/v) agar, 3% (w/v) sucrose with various concentrations of BA and Kinetin were used to determine optimum growth regulator levels. The concentrations tested for BA were (0.50-2.00 mg/lt). While those for Kinetin was (0.50-2.00 mg/lt) with activated charcoal 500mg/lt. Each data nodule, shoot bud initiation were recorded after two weeks of culture initiation. Effective healthy shoots observed from Kn 0.75 mg/lt+ activated charcoal 500 mg/lt. The *in vitro* shoot buds when reached to a size of 3-4 cm after an incubation of two weeks in the culture room maintaining controlled conditions were further subculture on Kn 0.75 mg/lt with various concentrations of Adenosine (0.5-2.0 mg/lt) and Activated charcoal 500mg/lt. Efficient flowering 80% was noted on SH media, when supplemented with Kn 0.75mg/lt+Adenosine1.0mg/lt+ activated charcoal 500mg/lt. Each treatment was carried out for three times in 30 replicates. *In vitro* flowering response were recorded after two weeks of culture inoculation. When axillary bud explants were inoculated on full strength SH salts with various concentrations of Kn(0.75-2.0 mg/lt) +Adenosine 1.0 mg/lt+activated

charcoal 500 mg/lt and 5% coconut water .While effective multiple shooting and multiple rooting(98%) was observed at Kn 1.50 mg/lt + Adenosine 1.0 mg/ml+ Activated charcoal 500 mg/lt and 5% coconut water. The p^H of the medium was adjusted to 5.6 with 1 N NaOH or 1N HCl before molten media were dispensed into test tubes (Borosil, India) and the media were autoclaved at 121 ˚C at 15 p.s.i pressure for 15 min. The cultures were maintained at 25±2 ˚C under a 16-h photoperiod of 50μmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes.

RESULTS

Among the various media tried it was found that SH gave a higher percentage of response than MS medium after one week of inoculation. As the response was poor in MS medium, further studies were carried out with SH medium. C. *scarious* plants were efficiently regenerated from axillary buds. These were cultured on SH medium without growth regulator gave no regeneration response, therefore two types of cytokines (BA and Kn) were used for shoot regeneration from axillary buds . The explants were inoculated on to SH medium supplemented with various concentrations of BA were (0.50-2.00 mg/lt) and Kn (0.50-2.00 mg/lt) with activated charcoal 500 mg/lt. Shoot induction percentage were recorded after two weeks of culture inoculation. In both the cases shoot buds were observed after 8-10 days of inoculation. No intermediate callus formation was observed. Percentages of shooting response were recorded after two weeks of culture initiation using different growth parameters. Of the two cytokinins tested, Kn-treated explants achieved higher regeneration than those treated with BA. Among different selected concentrations of Kinetin, 0.75 mg/lt Kn treatment yielded higher regeneration. At Kn concentrations higher than 0.75 mg/lt, the percentage of response were lower. The shoots were incubated more than three weeks of duration under strictly controlled conditions for shoot elongation medium containing 0.75 mg/lt Kn+500 mg/lt activated charcoal (Figures-1 (A,B) and Table-1).

In vitro flowering

These shoots were further subculture on Kn0.75mg/lt with various concentrations of adenosine and 500mg/lt activated charcoal .The explants inoculated on to SH medium with Kn0.75mg/lt+ADS 0.5 mg/lt+charcoal 500mg/lt showed 46.6% of flowering response. At Kn0.75mg/lt+ADS 0.75mg/lt+charcoal500mg/lt 63.3% were observed. When concentration of ADS was increased to 1.0 mg/lt 80.0% of flowerings were observed with Kn0.75 mg/lt+ ADS1.0 mg/lt+charcoal500 mg/lt. 70.0% of flowering response were observe, when explants inoculated on SH medium with Kn0.75 mg/lt+ ADS1.25 mg/lt+charcoal500 mg/lt. At Kn0.75mg/lt+ ADS 1.50 mg/lt+charcoal500 mg/lt 53.3% of flowering response will be observed. 40.0% of flowering response will be observed at Kn0.75mg/lt+ADS1.75 mg/lt+charcoal500mg/lt. At Kn0.75mg/lt+ ADS 2.00mg/lt+charcoal 500mg /lt 26.6% of flowering response will be observed. Effective flowering was observed at ADS1.0 mg/lt with average number of flowers 1.916±0.2075, and percentage of flowering response will be 80.0%. Gradual reduction in number of flowers and percentage of flowering response was observed when treated with lower and higher concentrations of ADS 1.0mg/lt (Table 2, Figure-1 (C&D) and Graph-1).

Multiple shooting and multiple rooting

The axillary bud explants were placed on full strength SH medium supplemented with various concentrations of Kn+ADS1.0mg/lt+charcoal500mg/lt+5% coconut water (CW). Where SH medium with 0.75mg/lt Kn + ADS 1.0 mg/lt+activated charcoal 500 mg/lt + 5%CW 56.6% of multiple shoots and multiple roots were formed from single axillary bud explant. When concentration of Kn was increased to1.00mg/lt + ADS1.0mg/ml+ charcoal 500mg/lt + 5% CW 63.3% of response were observed. At 1.25 mg /lt Kn+ ADS 1.0 mg/ml+charcoal 500 mg/lt+5%CW 73.3% of multiple shoots and multiple roots were formed from axillary bud explants. A maximum of 98.0% response in terms of number of shoots, number of roots were obtained in full strength SH media supplemented with 1.50mg/lt Kn+ ADS 1.0

mg/ml+charcoal 500mg/lt+5% CW. At 1.75mg/lt Kn+ ADS 1.0mg/ml+charcoal 500mg/lt+5%CW 83.3% of multiple shoots & multiple roots were formed from a single axillary bud explants. 50.0% of multiple shoots and multiple roots response were observed when explants inoculated on SH media containing 2.0 mg /lt Kn+ ADS 1.0 mg/ml+charcoal 500 mg/lt+5% CW. Among different selected concentrations, Kn 1.50mg/lt + ADS 1.0mg/ml+ charcoal 500 mg/lt + 5% CW treatment yielded higher regeneration (98%) with average number of shoots 5.79±0.2695 and roots 7.10±0.2701 (Table-3, Figure-1(E,F,G) and Graph-2,). At Kn concentrations higher than1.50 mg /lt, the numbers of shoots and roots as well as the percentage of response were lower. The shoots were incubated more than three weeks of duration under strictly controlled conditions for shoot & root elongation medium containing Kn 1.50 mg/lt + ADS 1.0 mg/ml+ charcoal 500 mg/lt+5% CW. Whereas the shoots shifted onto SH medium with IBA 0.25 mg/lt showed only 40.0% of roots in three weeks of time. Whereas SH medium with IBA0.50 mg/lt showed very poor root development accounting for 23.3%. While increasing the concentration of IBA the percentage of rooting response will be lower. Hence SH medium with Kn1.50 mg/lt+ADS 1.0 mg/lt+charcoal500 mg/lt+5% CW was found to be ideal concentration for multiple shooting and multiple rooting regeneration in C.*scariosus*. The incidence of highly efficient root formation on auxin free medium may be due to the availability of higher quantity of endogenous auxin.

Statistical analysis

A completely randomized experimental design was applied. In this study every experiment ten explants were used, all experiments were repeated three times. Data (percentage of flowering, percentage of multiple shooting and rooting, shoot length, number of shoots response) 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.

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-1(H)). 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 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 1
Influence of different concentrations of BA and Kn on *In vitro* shoot induction in *Cyperus scariosus*

S.No	Medium + Growth regulator(mg/lt)	Shooting percentage (%)
1	SH+BA0.50	40
2	SH+BA0.75	60
3	SH+BA1.0	82
4	SH+BA1.50	70
5	SH+BA2.0	50
6	SH+Kn0.50	55
7	SH+Kn0.75+ Charcoal 500	95
8	SH+Kn1.0+ Charcoal 500	80
9	SH+Kn1.5+ Charcoal 500	70
10	SH+Kn2.0+ Charcoal 500	60

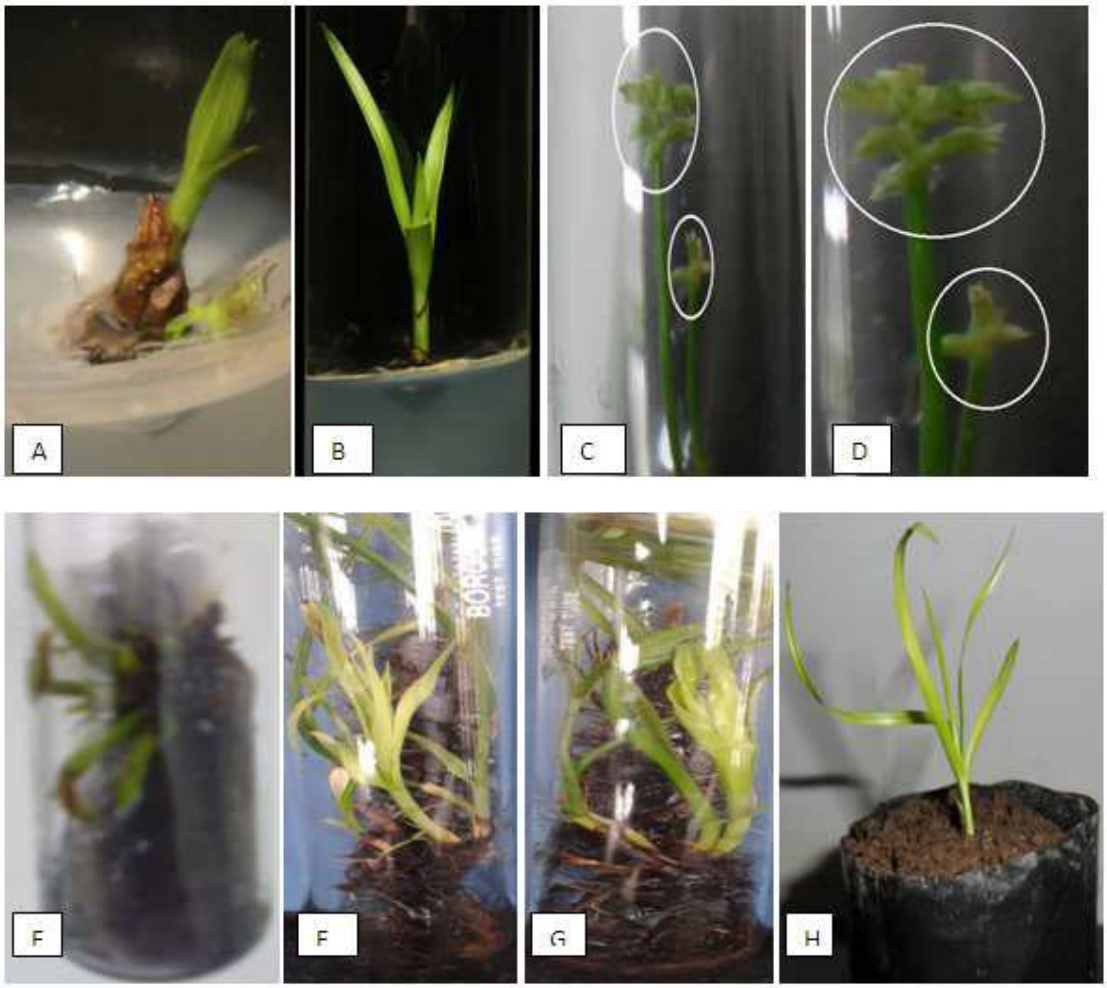
Table 2
Influence of Kn, Adenosine and activated charcoal on invitro floral bud induction in Cyperus scariosus. Values are expressed as mean± SE (n=10 in replicate).Mean followed by same letters do not differ significantly at p≥ 0.05 by Tukey's HSD test.

S.No	Growth regulator concentration in (mg/lt)	Flowering Percentage(%)	Average no. of flowers/explant
1	Kn0.75+ADS0.5+charcoal500	46.6	1.428^{a}±0.1465
2	Kn0.75+ADS0.75+charcoal500	63.3	1.631^{b}±0.1825
3	Kn0.75+ADS1.0+charcoal500	80.0	1.916^{c}±0.2075
4	Kn0.75+ADS1.25+charcoal500	70.0	1.809^{b}±0.1656
5	Kn0.75+ADS1.50+charcoal500	53.3	1.375^{a}±0.1433
6	Kn0.75+ADS1.75+charcoal500	40.0	1.25^{a}±0.1246
7	Kn0.75+ADS2.0+charcoal500	26.6	1.125^{a}±0.0977

Table 3
Influence of Kn, Adenosine, IBA, activated charcoal and coconut water on invitro multiple shooting and multiple rooting induction in Cyperus scariosus. Values are expressed as mean± SE (n=10 in replicate).Mean followed by same letters do not differ significantly at p≥ 0.05 by Tukey's HSD test.

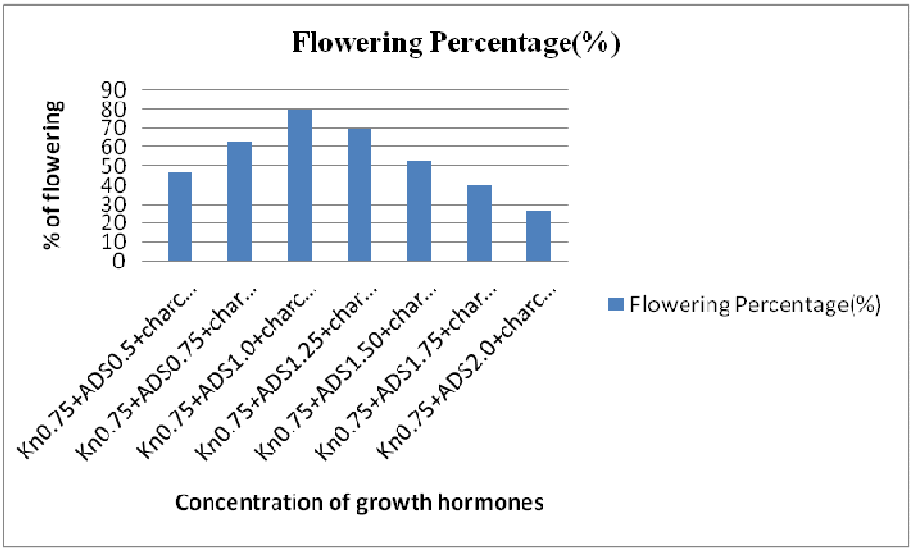
S.No	Growth regulator concentration in (mg/lt)	Multiple Shooting and rooting Percentage(%)	Average no. of Shoots/explant	Average no. of roots/explant
1	Kn0.75+ADS1.0+ charcoal500+5%CW	56.6	4.94^{a}±0.4683	5.52^{a}±0.5158
2	Kn1.0+ADS1.0+ charcoal500+5%CW	63.3	5.05^{a}±0.4756	6.105^{a}±0.5668
3	Kn1.25+ADS1.0+ charcoal500+5%CW	73.3	5.45^{b}±0.4549	6.45^{b}±0.5359
4	Kn1.50+ADS1.0+ charcoal500+5%CW	97.5	5.79^{c}±0.2695	7.10^{c}±0.2701
5	Kn1.75+ADS1.0+ charcoal500+5%CW	83.3	5.56^{b}±0.3942	6.96^{b}±0.4828
6	Kn2.00+ADS1.0+ charcoal500+5%CW	50.0	4.83^{a}±0.4538	5.33^{a}±0.499
7	IBA0.25+ charcoal500	Rooting40.0%	-	5.16^{a}±0.4721
8	IBA0.50+ charcoal500	Rooting23.3%	-	4.85^{a}±0.3858

Figure 1
In vitro flower induction and multiple shoot regeneration studies in _Cyperus scariosus_

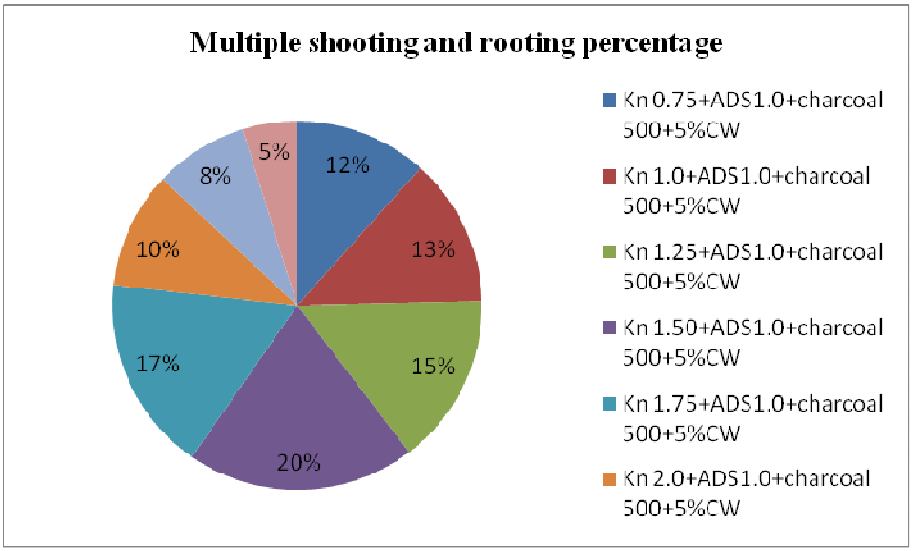


A. ***Initiation of shoot bud from axillary bud in SH+Kn0.75 mg/lt.***
B. ***Elongation of healthy shoot from axillary bud explants in SH+Kn0.75 mg/lt+ activated charcoal 500 mg/lt.***
C. ***Initiation of floral buds from elongated shoots in a medium containing SH+Kn0.75 mg/lt+ ADS 1.0 mg/lt+activated charcoal 500 mg/lt.***
D. ***Development of flowers after 25-30days.***
E. ***Initiation of multiple shoots in a media containing SH+Kn1.50 mg/lt+activated charcoal 500 mg/lt+5% CW from a single axillary bud explant.***
F. ***Elongation of multiple shoots and roots after two weeks of initiation.***
G. ***Complete plantlet showing multiple shoots and roots.***
H. ***Acclimatization of plantlet in vermiculite pot culture.***

Graph 1
Percentage of flowering response in *Cyperus scariosus*



Graph 2
Percentage of multiple shooting and multiple rooting in *Cyperus scariosus*



DISCUSSION

The effect of different cytokinines for induction of *In vitro* flowering in *Bambusa arundinaceae* reported by[15]. In *Cichorium intybus*[16] maximum number of flowers achieved on MS+B5 medium with 6.6µM BA, 2.85 µM IAA and 1.360 µM ADS. MS medium without plant growth regulators and 6 µM Kn induce flowers in Cucumber[17]. *In vitro* flowering from nodal explants of *Ceropegia bulbosa* will be cultured on B5 medium with different concentrations of BA and ADS with combinations of NAA[18]. But in our experiments efficient flowering was observed at ADS1.0 mg/lt with average number of flowers 1.916±0.2075 and percentage of flowering response will be 80.0%. Inclusion of ADS 25mg/lt in the culture medium improved the frequency of multiple shoot production[19]. Similarly multiple shoots were induced on MS medium supplemented with 4.44 µM BA or in combination with 2.85

µM IAA in *Cyperus rotundus*[20]. However roots were produced when callus transferred to low auxin or auxin free in *Cyperus rotundus*[21]. In our study SH medium with Kn1.50 mg/lt+ADS 1.0 mg/lt+charcoal500 mg/lt+5% CW was found to be ideal concentration for multiple shooting and multiple rooting regeneration in *C.scariosus.* In the present study a combination of Kn and ADS was found to be more effective to induce *in vitro* flowers formation. Addition of Kn along with ADS and 5% coconut water was important to induce the maximum multiple shoot proliferation.

CONCLUSION

An efficient I*n vitro* propagation and flower induction protocol from axillary bud explants of *Cyperus scariosus* has been achieved, which is a simple and effective protocol for mass multiplication, germplasm conservation and increasing the secondary metabolite production through genetic transformation studies.

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