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In vitro Growth and Shoot Production of Seeds of *Crassocephalum rubens* (Juss. Ex Jacq.) S. Moore (Asteraceae)

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Abstract: *Crassocephalum rubens* is a wild, economic and medicinal plant that is now threatened by genetic erosion in Nigerian ecosystem. The scarcity of the plant is due to indiscriminate collection from the wild and the difficulty in germinating the seeds. This study provides protocols for *in-vitro propagation* of *C. rubens* to supplement natural propagation, contribute to conserve the plant genetic resources and ensure its sustainable use as food and medicine in Nigeria. Freshly collected seeds were cultured on MS - basal media supplemented with growth regulators and water of immature *Cocos nucifera* (fruit) as source of vitamins. The growth and shoot production of *C. rubens* were evaluated using standard parameters: viability; number of shoot; shoot length; number of root; root length; number of leaf primordial; and % callus formation. Data were statistically analysed. *C. rubens* showed different growth patterns on the six media used for the tissue culture experiment. The control medium (CMC) supported shoot production (1.00), shoot elongation (15.67 mm), leaf formation (2.00) but it gave the least viability of 30% and did not support the callus formation. Medium CM5 was the best in the propagation of *C. rubens* with 70% viability; 2.33 shoots; 47.33 mm shoot length; 36.33 mm root length and 65% callus formation. The established protocols for the *in-vitro* propagation of *C. rubens* in the present study will enhance its sustainable use and conservation in Nigeria. Researchers can raise plantlets for laboratory experiments using the reported protocols. Furthermore *in-vitro* cultured plantlets can be supplied to vegetable farmers to support its sustainability as an economic plant in Nigeria.

Key words:*Crassocephalum rubens* · *In-vitro* culture · Coconut water · MS basal media · Growth regulators · Nigeria

INTRODUCTION

Crassocephalum rubens (Juss. Exjacq. S. Moore) belongs to plant family asteraceae. It is an erect herb of about 1 m high. Leaves arranged spirally, sessile; stipules absent; blade of lower leaves elliptical, oblanceolate or obovate, 4.5-16 cm×2-5 cm, either not lobed. Inflorescence a head, up to 18 heads arranged in a terminal corymb. The flowers are bisexual, equal; corolla tubular and 8-10 mm long, violet, mauve or purple. Fruit a ribbed achene, up to 2.5 mm long, crowned by white pappus hairs 8-12 mm long. It is distributed in lowlands and mountain situations from Guinea and Mali to Cameroon. It is found throughout tropical Africa including the Indian Ocean islands, where it is probably introduced. It is also reported from Lesotho, South Africa and Yemen [1].

The mucilaginous leaves of *Crassocephalum rubens* are slightly laxative and are given to women after childbirth for the treatment of stomach ache and liver complaints. An infusion of the leaves is taken against colds and leaf - poultices are applied to burns. Leaf sap is applied to sore eyes and to filarial (threadlike nematode worms) parasites from the eyes. The leaves crushed in water are rubbed into the ear for earache. The leaves are used for soups and sauces in the southwestern part of Nigeria and other humid zones of West and Central Africa. In Uganda the leaves are dried, chopped and cooked with peas or beans. In East Africa it is used as an antidote against any form of poisoning. The root is used as medicine for tumors and cancers. The sap is used for the treatment of cutaneous and sub cutaneous parasitic infections [2].

Fresh leaves of *C. rubens* contain per 100 g edible portion: 79.9 g Water, 269 kJ Energy (64 kcal), 3.2 g Protein, 0.7 g Fat, 14.0 g Carbohydrate, 1.0 g Fiber, 260 mg Calcium and 52 mg Phosphorus. Traces of alkaloids have been recorded in stems and leaves and an abundance of tannins in the roots [3]. Iwalewa *et al.* [4] reported the

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antioxidant and cyto-protective potentials of *C. rubens*. The volatile extracts from fresh leaves showed antibacterial activity against *Staphylococcus aureus*, *Streptococcus faecalis*, *Escherichia coli*, *Salmonella typhi* and *Candida albicans* with the Minimal Inhibitory Concentration (MIC) value of 0.54 - 4.38 mg/ml [5]. Ola-Adams and Onyeachusim [6] listed *C. rubens* among edible wild Nigerian plants that are threatened with genetic erosion. Genetic erosion could be due to abandonment of traditional agroecosystem by the indigenous population, socio-economic and cultural change and replacement of mixed cropping by monocropping, population migration and lack of scientific interest in the wild plants [7, 8]. The factor threatening the survival of *Crassocephalum rubens* in Nigeria is the fact that the storage method and germination pattern of this plant is not understood [9].

Keeping in view the economic importance and the threatened status of *C. rubens* in Nigeria, the present study was carried out to establish protocols for the *in-vitro* propagation of *Crassocephalum rubens*, explore the potential of coconut as a source of vitamin and substitute to the synthetic growth regulators in tissue culture media and contribute to the conservation of plant genetic resources in Nigeria.

MATERIALS AND METHODS

Collection and Identification of Plant Sample: Plants and seeds of *Crassocephalum rubens* were collected very early in the morning from five different locations in Ibadan, Nigeria: National Horticultural Research Institute of Nigeria, two vegetable farms, University of Ibadan premises and the field genebank of the National Centre for Genetic Resources and Biotechnology (NACGRAB). Species identification was done at the University of Ibadan Herbarium (UIH), University of Ibadan. As the seeds are dispersed by explosive mechanism, they were collected by tying transparent polythene bags to the receptacle of the flowers. The collected seeds were stored in dry glass bottles for experiments.

***In-vitro* Cultures:** The experiment was done in the tissue culture Laboratory of NAGRAB. The constituents of media used in this study are presented in Table 1. The seeds of *C. rubens* were cultured on MS-basal media supplemented with growth regulators and immature coconut water as shown in Table 1. The pH of each medium was adjusted to 5.7 with 1M NaOH or 1M HCl prior to the addition of 0.7% agar (Difco, USA). Media and instruments were autoclaved for 15 - 30 min at 121°C (1 atm). The seeds were surface-sterilized with 70% ethanol for 5 min.; 10% sodium hypochloride for 20 min.; and 5% sodium hypochloride for 10 min. Aseptic inoculation was carried out in a laminar flow hood using standard methods [10]. After inoculation, the glass tubes were sealed with paraffin wax and labeled accordingly. The cultures were incubated at 27±1°C with a photoperiod of 16 h in the growth room for a period of 30 - 90 days. Each treatment was replicated thrice. Plantlets with multiple shoots were frequently subcultured on rooting media.

Assessment of Growth of *In-vitro* Plantlets: Growth was evaluated weekly. The growth parameters like viability, shoot length, root length, number of leaf primordial, root number, shoot number, percentage callus formation and number of buds were observed, measured and recorded.

Statistical Analysis of Data: Analysis of variance was estimated at $P < 0.05$ level of significance and the means were compared using the Duncan multiple range test.

RESULTS AND DISCUSSION

The growth and shoot production of *C. rubens* on MS basal media supplemented with growth regulators and coconut water are as shown in Table 2. The highest viability of 80% obtained from medium CM1 (1/4 MS + 30% coconut water) could be attributed to the high constituent of coconut water in the medium and it could be inferred that the coconut water enhanced viability of the explants in cultures. The least viability of 30% was recorded on the control medium (CMC). Media CM3 and CM4 contained 10% and 25% coconut water respectively. Each of these media significantly supported shoot production with 3.17 shoots indicating that the coconut water as an organic supplement in cultures supported shoot proliferation. Furthermore, medium CM4 gave the highest number of leaf primordials with 10.67 leaflets. It also supported multiple shooting and callus formation (Plate 1). Medium CM2 gave 55.00% viability but did not produce any shoot. A combination of BAP and coconut water in the medium (CM2) adversely affected cell division and growth of *C. rubens* in culture.

Media CM5 significantly enhanced shoot elongation, root formation and elongation compared to other media used for the experiments. Callus formation was enhanced in all media except the control medium (CMC). Medium CM4 best supported callus formation with 80%. Overall, medium CM5 was the best for the *in-vitro* propagation of

Table 1: The media constituents used for the *in-vitro* development of *C. rubens*

Media code	1/4 MS	BAP (mg/l)	NAA (mg/l)	KIN (mg/l)	Coconut water % (v/v)	Sucrose (mg/l)
CM1	+	-	-	-	30.00	30.00
CM2	+	0.70	-	-	10.00	30.00
CM3	+	-	-	-	10.00	30.00
CM4	+	-	-	-	25.00	30.00
CM5	+	-	0.10	0.70	20.00	30.00
CMC (Control)	+	-	-	-	-	30.00

Legends: + = Present; - = Absent; MS = Murashige and Skoog salt base [11]; NAA = 1-napthalene acetic acid; BAP = Benzyl aminopurine; KIN = Kinetin.

Table 2: The growth and shoot production of *Crassocephalum rubens* in MS basal media

Media Code	(%) Viability	Number of shoot	Shoot Length (mm)	Number of Root	Root Length (mm)	No. of f Lea Primordial	% Callus Formation
CM1	80.00	*2.00±0.00^{b}	29.33±18.16^{abc}	0.00±0.00^{c}	0.00±0.00^{f}	9.33±4.46^{ab}	60.00
CM2	55.00	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{f}	0.00±0.00^{d}	70.00
CM3	60.00	3.17±0.75^{a}	14.00±7.46^{dc}	0.00±0.00^{c}	0.00±0.00^{f}	8.33±3.62^{abc}	60.00
CM4	50.00	3.17±0.75^{a}	14.17±7.91^{dc}	0.00±0.00^{c}	0.00±0.00^{f}	10.67±6.77^{a}	80.00
CM5	70.00	2.33±0.52^{b}	47.33±18.6^{a}	1.83±0.41^{a}	36.33±13.3^{a}	6.00±2.28^{bdc}	65.00
CMC	30.00	1.00±0.00^{b}	15.67±5.01^{bc}	0.00±0.00^{c}	0.00±0.00^{f}	2.00±0.00^{cd}	0.00

Legends: Evaluation was made after 60 days in culture. *Mean of 3 readings ± standard deviation. *Different letters in the same column indicate significant differences ($p < 0.05$). CM1 (1/4 MS + 30 %coconut water); CM2 (1/4 MS + 0.70 mg/L BAP + 10 %coconut water); CM3 (1/4 MS + 10 %coconut water); CM4 (1/4 MS + 25 %coconut water); CM5 (1/4 MS + 0.10 mg/L NAA + 0.70 mg/ L KIN + 20 %coconut water); CMC (1/4 MS only).

Plate 1: Multiple shoots and compact callus of Crassocephalum rubens on medium CM4 (1/4MS supplemented with 25% coconut water) after 60 days in culture.

Plate 2: Shoot production and Callus formation of Crassocephalum rubens on medium CM5 (1/4 MS + 0.10 NAA + 0.70 KIN + 20% Coconut water) after 30 days in culture.

C. rubens because it significantly enhanced viability, shoot elongation, root formation, root elongation and formation of callus (Table 2 and Plate 2).

Auxins and cytokinins generally induce cell division; differentiation and elongation of shoot; and rooting [12, 13]. The growth recorded on medium CM5 must have been due to NAA and KIN constituents in the medium. The findings of this study are in accordance with the previous studies on the roles of auxins and cytokinins in the *in-vitro* propagation of medicinal plants. Jose and Satheeshkumar [14] studied the *in-vitro* culture of *Ophiorrhiza mungo* (mongoose plant) and reported that its rooting was favored in MS basal medium supplemented with IBA (12.3 µM) + NAA (1.07µM). The best shoot proliferation of *Portulaca grandiflora* was established on MS + 4.0 mg/L BAP and the rooting of its microshoots was best on ½ MS + 0.75 mg/L NAA medium [15]. Ahmed and Anis [16] recorded optimum regeneration of *Cucumis sativus* on MS medium containing 1.0 µM of BA and the significant rooting of the *in-vitro* microshoots of the plant on ½ MS + 1.0 µM NAA. Studies on the *in-vitro regeneration* from callus culture of *Clematis gouriana* by Rajan and Krishna [17] stated that the shooting and rooting of the plant on MS medium supplemented with 0.5 mg/L IBA. Also in conformity with the present results, Sarika and Meenakbhi [18] reported the highest frequency of root induction of *Jatropha curcas* L. on MS medium supplemented with 3.0 mg/L IBA.

The established protocols for the *in-vitro* propagation of *C. rubens* could form the basis for the *in-vitro* production of secondary metabolites for this plant. Mulabagal *et al*. [19] reported the production of secondary metabolites from medicinal plants. Another significance of the present study is that *in-vitro* plantlets of *C. rubens* could be raised for economic purpose and distributed to vegetable farmers in Nigeria.

CONCLUSION

This study established protocols for the *in-vitro* propagation of *C. rebens* and reported the benefit of coconut water in its *in-vitro* cultures. Further work on conservation of *C. rubens* should include its cryopreservation and creation of its *in-vitro* genebank to fully ensure its sustainable use in Nigeria. Also, selection of desirable characteristics from wild varieties of *C. rubens* seems possible via tissue culture and biotechnology to enrich its germplasm conservation.

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