In Vitro Rapid Multiplication Protocol for *Ex Situ* Conservation of the Rare, Endemic Medicinal Plant *Achyranthes coynei*

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Abstract

Achyranthes coynei is a rare, endemic species with potential medicinal properties. The present study aimed to develop an efficient *in vitro* rapid multiplication protocol for *A. coynei*. It was successfully propagated *in vitro* using apical shoot tips as explants. A combination of 6-benzylaminopurine (3.0 mg·L^{-1}), thidiazuron (0.5 mg·L^{-1}), and kinetin (0.5 mg·L^{-1}) in MS basal proved best for multiple shoot induction, yielding 4.8 shoots per explant after 60 days of culture. The shoots were rooted with a frequency of 80% after 30 days of culture on MS agar medium fortified with IBA/NAA (0.5 mg·L^{-1}) before being acclimatized in the greenhouse. The developed protocol will serve as a tool to restore the rare status of the plant and will append its sustainable utilization.

Key words

Achyranthes coynei · Amaranthaceae · *in vitro* · rapid multiplication · kinetin

Abbreviations

BAP:	6-benzylaminopurine
IBA:	indolebutyric acid
Kn:	kinetin
MS basal:	Murashige and Skoog basal medium
NAA:	a-naphthaleneacetic acid
TDZ:	thidiazuron

Achyranthes coynei Santapau (Amaranthaceae) is an endemic, potential medicinal plant from India [1]. It has been rated as rare, endangered [2] and reported from Gujarat, Maharashtra and the Karnataka states of India [1,3–5]. The plant is used in the treatment of various ailments by traditional practitioners of the region [1,6]. The plant has been scientifically studied for its antimicrobial and antioxidant activities, correlating its medicinal properties with the phytoconstituents present [6–11].

A. coynei is a perennial, profusely branching shrub growing uneven on road sides and on canal bunds along with *Achyranthes aspera* L. (**Fig. 1 a**) [1]. Fast erosion of medicinal plants has been reported from the study region [12,13]. Parameters such has deforestation, reduced rainfall, dam construction, grazing, fire wood collection, and expansion of cultivation land are hindering the natural habitat and population of medicinal plants including *A. coynei* [1,12,13]. The plant needs immediate attention to retain its medicinal properties displayed by the presence of richer triterpenoids over *A. aspera* [2,6,11]. The endemic and rare status of the plant by virtue of its assorted distribution makes it a vulnerable component of the community. And as quoted, endemic species confined to threatened ecosystems will certainly hit by extinction episodes and are most in need of rapid and effective conservation action [1,14]. Under these circumstances there is a need to facilitate an alternative strategy for its conservation and sustainable use. An *in vitro* system provides such an alternative tool. To the best of the authors knowledge, no work has been carried out on the *in vitro* multiplication of this rare, medicinal plant. Thus, the study was taken up to develop an efficient protocol for its rapid multiplication and *ex situ* conservation.

Germination studies (1 to 30 day) showed 70% seed germination, and the first seed germinated on 7th day. The germination was higher in seeds deprived of a persistent calyx (80%) compared to seeds bearing a calyx (60%).

Higher numbers of multiple shoots were observed after 4 weeks of inoculation on BAP (3.0 mg·L^{-1}), TDZ (0.5 mg·L^{-1}), and Kn (1.5 mg·L^{-1}) added individually to MS basal (**Table 1**). The shoot length was below 1 cm in BAP 3.0 mg·L^{-1} and TDZ 0.5 mg·L^{-1} (**Fig. 1 b, c**), whereas Kn 1.5 mg·L^{-1} (**Fig. 1 d**) individually showed a higher shoot length (1.67 ± 0.91 cm). Further increase in concentration of Kn (2.0 mg·L^{-1}) produced longer shoots (2.14 ± 1.45 cm), but with only one shoot per explant (**Fig. 1 e**). MS basal fortified with Kn resulted in a higher number of nodes per shoot (**Table 1** and **Fig. 1 d**), indicating its role in shoot elongation. The results were in accordance with that observed in *Pinus canariensis* C.Sm. (Pinaceae) by Pulido et al. [15], wherein kinetin influenced shoot elongation.

Different concentrations and combinations of cytokinins (BAP, TDZ, and Kn) were attempted to observe the synergistic effect on multiple shoot production. MS basal with BAP 3.0 mg·L^{-1} and TDZ 0.5 mg·L^{-1} produced higher average shoots with a shorter shoot length (**Fig. 1 f**), whereas a combination of BAP 3.0 mg·L^{-1}, TDZ 0.5 mg·L^{-1}, and Kn 1.5 mg·L^{-1} showed a lower shoot number (3.80 ± 2.06) with a 1.02 ± 0.96 cm shoot length (**Table 1**). The combination of BAP 3.0 mg·L^{-1}, TDZ 0.5 mg·L^{-1}, and Kn 2.0 mg·L^{-1} resulted in a multiple number of shoots per explant (~ 5) with more than a 2-cm shoot length and a higher number of nodes (2.36 ± 1.62) (**Fig. 1 g** and **Table 1**).

Four-week-old axillary shoots were separated and transferred individually into tubes containing MS medium without growth regulators and/or with various concentrations of auxins (**Table 2**) for rooting. A total of 30 shoots were used in the experiment and the results are presented in **Table 2**. NAA and IBA showed a good response in root induction at the 0.5 mg·L^{-1} concentration with the highest percent response and number of roots (**Table 2**). Callus formations were observed at the base of the explants in MS supplemented with NAA and were not considered for further multiplication or rooting of *A. coynei* plants (**Fig. 1 h**), as also observed in *Boscia senegalensis* Lam. (Capparaceae) by Daffalla and coworkers [16]. The callus formation may possibly be due to increased auxin accumulation. The highest numbers of roots per shoot were observed in NAA 0.5 mg/l, however, 76.66% of explants produced callus at the base (**Table 2** and **Fig. 1 h**). Optimally, the highest numbers of roots per shoot (4.20 ± 1.88) with a significant root length (2.92 ± 2.01 cm) were obtained when the medium was incorporated with 0.5 mg·L^{-1} IBA (**Fig. 1 i**). A higher concentration of IBA (1.0 mg·L^{-1}) significantly inhibited the percent response (30%) and reduced root branching (**Table**

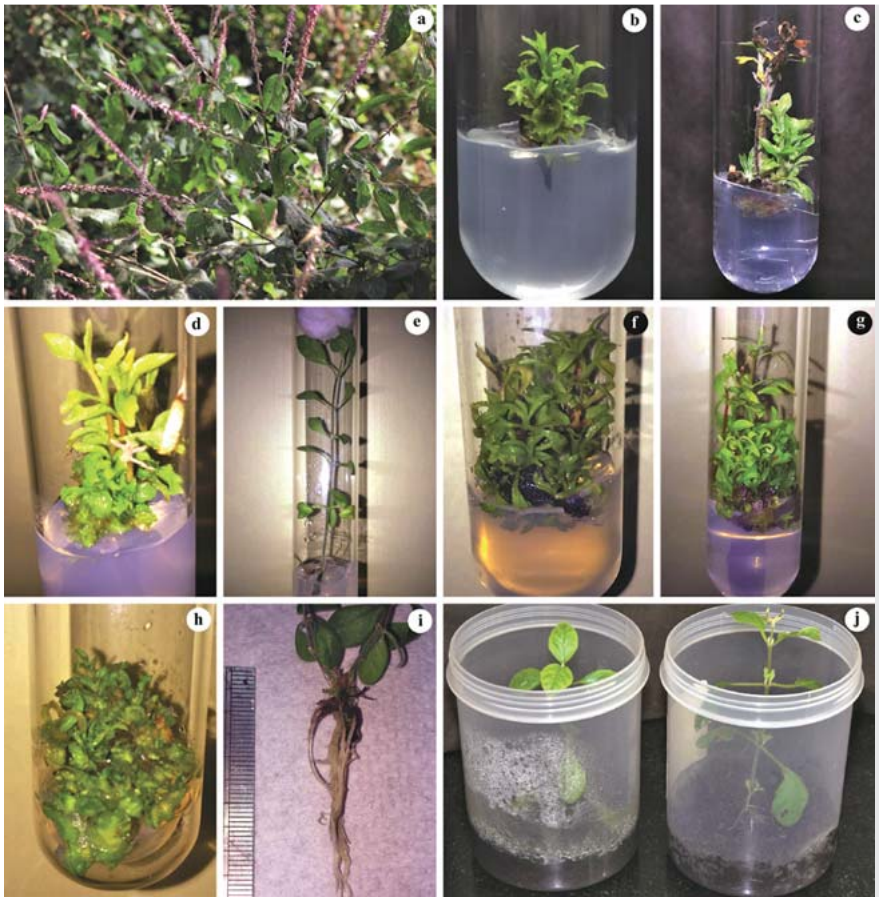

Fig. 1 **a** *Achyranthes coynei* Habit; **b** Multiple shoots with shorter multiple shoots on MS basal + BAP 3.0 mg · L^{-1}; **c** Multiple shoots with shorter multiple shoots on MS basal + TDZ 0.5 mg · L^{-1}; Improved shoot length on **d** MS basal + Kn 1.5 mg · L^{-1}, **e** MS basal + Kn 2.0 mg · L^{-1}; **f** Multiple shoot formation in MS basal + BAP 3.0 and TDZ 0.5 mg · L^{-1}; **g** Multiple shoot formation with longer shoots and more numbers of internodes on MS basal + BAP 3.0 + TDZ 0.5 + Kn 2.0 mg · L^{-1}; **h** Callus formation at the base of the explant on MS medium with NAA 0.5 mg · L^{-1}; **i** Rooting on MS basal + IBA 0.5 mg · L^{-1}; **j** *In vitro* grown, hardened *A. coynei* plants.

Table 1 Effect of various cytokinins on % shoot production, average shoot length, and shoot multiplication in *A. coynei*.

Cytokinins mg L^{-1}			% of Explants producing axillary shoots	Average number of shoots/explant	Average length of shoots/explant (cm)	Average number of nodes per shoot
BAP	TDZ	Kn				
0.0	0.0	0.0	00	0.00	0.00	0.00
0.5	–	–	70	1.90 ± 0.56**	0.59 ± 0.26	NR
1.0	–	–	90	2.24 ± 0.72**	0.56 ± 0.33	NR
1.5	–	–	80	1.75 ± 0.58**	0.48 ± 0.18	NR
2.0	–	–	80	1.50 ± 0.52**	0.67 ± 0.26	NR
3.0	–	–	80	2.28 ± 0.69**	0.79 ± 0.26	NR
–	0.5	–	80	2.05 ± 0.10**	0.89 ± 0.21	NR
–	1.0	–	40	1.75 ± 0.35**	0.88 ± 0.02	NR
–	1.5	–	70	1.85 ± 0.47**	0.89 ± 0.34	NR
–	2.0	–	70	1.92 ± 0.34**	1.41 ± 0.95	NR
–	3.0	–	40	1.55 ± 0.38**	0.81 ± 0.13	NR
–	–	0.5	70	1.00 ± 0.00**	1.55 ± 1.38	1.57 ± 1.98
–	–	1.0	50	1.00 ± 0.00**	1.04 ± 0.32	1.40 ± 0.89
–	–	1.5	70	1.25 ± 0.33**	1.67 ± 0.91	1.18 ± 1.48
–	–	2.0	70	1.00 ± 0.00**	2.14 ± 1.45	1.71 ± 1.70
–	–	3.0	80	1.00 ± 0.00**	1.56 ± 8.86	1.50 ± 1.41
3.0	0.5	–	90	4.50 ± 1.03**	0.88 ± 0.08	NR
3.0	0.5	1.5	90	3.80 ± 2.06**	1.02 ± 0.96	1.76 ± 0.98
3.0	0.5	2.0	100	4.80 ± 1.63**	2.06 ± 0.62	2.36 ± 0.74

The values represent the mean ± SE of three independent experiments. Ten shoots were raised for each experiment. ** significant at $p < 0.05$ according to Dunnett's multiple comparison test. NR: No response.

2). Interestingly, all of the concentrations of IBA improved shoot height and the number of internodes as also reported in *Embelia ribes* Burm.f. (Primulaceae) by Annapurna and Rathore [17].

Well-rooted 4-week-old plantlets with two or three pairs of leaves were washed with water to remove the agar (**Fig. 1 i**) and were transferred into pots (Ø 10 cm) containing a sterilized mixture of soil, sand, and peat (4 : 3 : 3 v/v/v). The plantlets were

Table 2 Effect of different auxin (NAA and IBA) concentrations on *in vitro* rooting in *A. coynei*.

Auxins mg·L^{-1}		% Response	Number of roots	Length (cm)	% Response	% of Explants producing callus*
NAA	IBA					
0.0	0.0	00	0.00	0.00	– – – –	00
0.1	–	40	3.25 ± 1.25**	2.03 ± 2.09	– – – –	56.66
0.5	–	80	4.25 ± 2.37**	3.13 ± 1.35	+ + – –	76.66
1.0	–	70	3.71 ± 3.07**	2.42 ± 1.72	+ – – –	93.33
–	0.1	40	4.00 ± 2.64**	0.96 ± 0.45	– – – –	NC
–	0.5	80	4.20 ± 1.88**	2.92 ± 2.01	+ + – –	NC
–	1.0	30	4.00 ± 0.40**	3.78 ± 1.00	+ – – –	NC

Data is given as the mean ± SE (n = 30). ** significant at $p < 0.05$ according to Dunnett's multiple comparison test; (+) positive and (−) negative response in the formation of root branches; *Callus formed at the base of explants; NC: No callus.

initially covered with polythene bags to ensure high humidity. The polythene covers were gradually removed during the acclimatization period (14 days). The potted plantlets were maintained inside a growth chamber at 26 ± 2 °C under a 16-h light regime (cool white fluorescent light; 40 µmol·m^{-2}s^{-1}), for 4 weeks (**Fig. 1 j**). The acclimatized plants were transferred to a greenhouse and survival rates were recorded. The *in vitro* grown transferred plants showed a survival rate of over 90%.

Conclusively, an efficient *in vitro* rapid multiplication protocol for *ex situ* conservation of the rare, potential medicinal plant *A. coynei* was developed. Optimum results were obtained by using 12- to 18-day-old germinated seedlings as explants on MS basal fortified with BA (3.0 mg·L^{-1}), TDZ (0.5 mg·L^{-1}), and Kn (0.5 mg·L^{-1}) and rooting on MS basal + IBA 0.5 mg·L^{-1}. A single explant can produce on an average 8 hardened plants within 18 weeks using this protocol. The developed protocol will be useful in the propagation of *A. coynei* and will also help to restore its status.

Materials and Methods

The seeds were obtained in 2013 from a single population near Madanbavi village in the Belagavi district. A herbarium of the identified plant specimen was authenticated and deposited at the Regional Medical Research Centre, ICMR, Belagavi (Voucher Number: RMRC 790).

Seed germination studies were carried out to understand the germination percentage of the seeds obtained from the wild using a standard technique [18]. Seeds were washed under running tap water (30 min), surface-sterilized using 1% (v/v) mercuric chloride solution for 10 min, rinsed thrice with sterile, distilled water, and placed in petri dishes for germination. Three replicates with 10 seeds per plate were used to determine the percent seed germination. Seeds of *A. coynei* bear a persistent dried calyx, so two separate groups with and without a calyx were used. The emergence of plumule and radicle was considered the indicator for seed germination.

Seeds were also germinated in plastic trays (21 × 16 cm) containing autoclaved coco-peat for *in vitro* studies. Three to five cm long shoot tips were derived from 12- to 18-day-old seedlings and were transferred aseptically to glass tubes containing MS medium supplemented with different combinations and concentrations of BAP, TDZ, and Kn (**Table 1**). The pH of the media was adjusted to 5.9 by 0.1 N sodium hydroxide solution. Media were autoclaved at 0.1 MPa, 121 °C, for 17 min. The cultures were kept in the growth chamber at 25 ± 2 °C under a 16-h light/8-h dark photoperiod (cool white fluorescent light; 30 µmol·m^{-2}s^{-1}).

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Conflict of Interest

There is no conflict of interest among all authors.

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