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Studies on Calli Mediated Multiple Shoot Regeneration from *Rumex vesicarius* L.

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Authors' contributions

This work was carried out in collaboration between all authors. The conceptual idea of this article was given by author CR. Experimented by author LK. Authors MB and MSRK supported the work by providing material support. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Rumex vesicarius L is a valuable potent medicinal herb, which belongs to family Polygonaceae. It possesses antimicrobial, anti- inflammatory, antidiarrhoeal and antioxidant properties. An attempt to study the *In vitro* callus induction and regeneration of plantlets from calli of leaf and nodal segments as explants has been achieved. Initially mature seeds were excised from plants grown in the departmental garden of KL University. The sterilized seed explants were inoculated aseptically to the solid basal SH media without any growth regulators for seed germination. Effective plantlets observed after 1 week of culture inoculation under maintained controlled conditions. From these *In vitro* plantlets, leaf and nodal segments were taken as explants for this study. These explants were inoculated on SH medium supplemented with different concentrations of BA (0.5-5.0mg/l) and 2, 4-D (0.5-3.0mg/l) for callus induction and multiple shoot regeneration. 90% of callus induction was observed on media containing BA 4.0mg/l and efficient multiple shoot induction (96.6%) was observed on media containing BA 1.0mg/l from leaf explants. 90% of callus induction and 85% of multiple shoot induction observed on media containing BA 2.0mg/l from nodal explants. Roots were induced from *In vitro* shoots on SH medium supplemented with 1mg/l IBA after 1 week. Leaf explants were more regenerative with 96.6% response compared to nodal explants 85%. Finally these *In*

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vitro regenerated plantlets were hardened, acclimatized and successfully transferred to the field. This protocol will be useful for mass multiplication of plantlets and maintenance of germplasm throughout the year.

Keywords: Rumex vesicarius L; Schenk and Hildebrandt medium; In vitro; Callusinduction; regeneration; plantlets; seedlings; growth regulators.

1. INTRODUCTION

Rumex vesicarius L is an annual, pale green, glabrous herb branched from the root, it belongs to family Polygonaceae [1]. It is known by different vernacular names *viz* chukkakura (Telugu), bladder dock (English), chooka (Hindi), and amlavetasa (Sanskrit). It is widely cultivated as a green leafy vegetable in many parts of India. Flowers are hermaphrodite having both male and female organs and are pollinated by wind. It can grow in a moist moderately fertile well drained soil in sunny position [2]. This can be a wild edible plant, used like sorrel as a flavoring in salads or as a spinach [3]. Leaves are collected in spring time, eaten fresh or cooked and it shows an acid flavor [4]. The leaves contains astringent, aperient, diuretic and cooling properties, roasted seeds are used for the treatment of dysentery and the juice of the plant is cooling used in treating heat of the stomach, to allay toothache and by its astringent properties to check nausea [5]. This plant is also used to reduce biliary disorders and control cholesterol levels. Methanolic extract of *R. vesicarius* exhibits antimicrobial and antioxidant properties [6,7]. Dark green to brown and dark grey dyes can be obtained from the roots of many species in this genus, they do not need a mordant [8,9]. The principle constituents of *R. vesicarius* include phenols, alkaloids, flavanoids, triterpenoids, saponins, anthraquinones, reducing sugars, lipids and carbohydrates [8,10,11]. The dietary intake of bioactive phytochemicals like carotenodis, phenolic compounds, flavanoids and isothiocyanates will lead to the protection against chronic diseases [8,12,13]. *R. vesicarius* is a good source of minerals Na, K, Ca, Fe, and Mn in different organs at different stages of development from flowering to fruiting stages during spring, autumn and winter seasons [8,14,15]. This plant is an environmental weed, with the potential to have a significant impact on the natural flora and fauna in areas where it grows, seeds are polymorphic (light and dark of various shades) and of high potential viability. Seeds are enclosed within showy, papery fruiting valves at maturation. Extracted seeds are characterized by non-deep physiological dormancy. Light and dark seeds usually require an after-ripening period of several months, but thereafter germinate at any time of the year in a light–dark rhythm. Light seeds also show excellent germination in constant darkness; they are non-dormant. Dark seeds show conditional dormancy, where germination is inhibited in darkness, but not in a light–dark rhythm. Scarification experiments indicate that the conditional dormancy of dark seeds is related to the pericarp. The pericarp may restrict oxygen consumption by the embryo, contain chemical inhibitors and impede radical protrusion. A range of environmental variables is likely to affect the specific germination requirements of particular seed types. Environmental conditions may induce secondary dormancy, in both light and dark seeds [8,16]. Other view showed that, the seeds of this plant are regarded as dormant, so seed germination is complex process in *R. vesicarius*. Dormancy broken by treatment with GA_3 after two weeks, it will result in low seed germination percentage (20%) under laboratory and dark conditions (23°C) [17]. Continuous use of this plant for medicinal purpose threatens its population. Rapid *In vitro* clonal propagation by using *Ex vitro* nodal explants have been reported in *R. vesicarius* [18]. In *R. vesicarius In vitro* propagation and flowering from *Ex vitro* nodal explants has also been reported [19]. Hence this is an attempt to develop an effective reproducible and simple protocol for calli mediated multiple shoot

regeneration by using leaf and nodal explants to make it available throughout the year for pharmaceutical usage and also for germplasm conservation.

2. MATERIALS AND METHODS

2.1 Culture Medium and Conditions

For the initial experiments, mature seeds were excised from plants grown in the Botanical garden at KL University campus, Vaddeswaram, Andhra Pradesh, India. After selection of healthy seeds, seeds are washed with tap water for 3-4 times, followed by chemical sterilization with 0.1% (w/v) $HgCl_2$ for 2-3 minutes and then seeds were washed in sterile distilled water to remove traces of $HgCl_2$. Finally these seeds were flame sterilized with whatman filter paper and supplemented on the surface of the nutrient culture medium SH without growth regulators. Effective plantlets developed from these seeds within one week. After two weeks, leaves and nodal segments were taken as explants for callus induction. We found that compare to *Ex vitro* the *In vitro* leaf and nodal explants were found to be appropriate as it was responding well under *In vitro* conditions.

The leaf and nodal explants (0.6cm) were excised from *In vitro* grown plants for callus induction. These explants as ideal for this experiment were utilized for further studies on the effect of growth hormones BA, 2, 4- D and IBA. All these explants were placed horizontally, with dorsal side in contact with the medium. The culture medium used for the explants selected was Schenk and Hildebrandt medium [20] supplemented with 0.8% (w/v) agar, 3% (w/v) sucrose and enriched with varying concentrations of 2, 4 -D, BA & IBA were used to determine the optimum growth regulator levels. pH was adjusted to the range of 5.6 with 1 N KOH or 1N HCl before molten media was dispensed into culture tubes and bottles (Borosil, India) and these media were autoclaved at 121ºC with 15 p.s.i pressure for 20 min. The cultures were maintained at 25±2ºC under a 16-h photoperiod of 50 µmol m−2 s−1 irradiance provided by cool white fluorescent tubes.

2.2 Induction of Callus and Regeneration of Multiple Shoots

Basal SH medium supplemented with different concentrations of Benzyl amino purine (0.5,1.0, 2.0,3.0,4.0and 5.0 mg/l) and 2,4-D(0.5,1.0,2.0 and 3.0 mg/l) were tested for callus induction and regeneration of shoots from leaf and nodal explants. Callus was induced from both explants when cultured on SH medium supplemented with BAP and 2, 4-D.

All cultures with callus were sub cultured after two weeks on a fresh SH medium supplemented with BAP and 2, 4-D for four weeks to induce *In vitro* regeneration of shoot. The response of each explants with regard to the induction of shoots, the length of shoot and the percentage of response recorded after six weeks of culture.

2.3 *In vitro* Rooting

In an aseptic chamber *In vitro* regenerated shoots were separated gently from the culture bottles and transferred to another culture tubes containing SH medium supplemented with IBA (0.5,1.0 and 2.0mg/l). The response of each explants with respect to the number of induced roots and roots length per shoot were recorded after three weeks of culture.

2.4 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(n)). 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 these acclimatized plants were shifted to field conditions, 90% 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.

2.5 Data Collection and Statistical Analysis

The experiments were repeated thrice for each and every respective concentration followed by a completely randomized design. Ten explants per replicate were used for each treatment. Data for the percentage of response per explants with different concentrations of cytokinins and auxins with basal SH medium (% of callus induction ,morphology of callus, shoot regeneration from callus, shoot length, number of roots and root length) were recorded after six weeks of culture. Thus obtained data were analyzed by one- way ANOVA technique. The Mean values recorded from the experimental data were compared using Turkeys' HSD test at P=0.05 with SPSS ver.13.0. The results are expressed as Mean±SE of three experiments.

3. RESULTS AND DISCUSSION

To overcome seed dormancy initially seeds were soaked in sterile distilled water overnight. After soaking, the seeds washed with running tap water, followed by sterilization with 0.1% $HgCl_2$, double distilled water and finally sterize with Whatman filter paper and supplemented on the surface of the nutrient culture medium SH without growth regulators. 80% of *In vitro* germination observed from seeds within one week. After two weeks, leaves and nodal segments of these plantlets were taken as explants for callus induction (Fig. 1(a, b&g)). A similar work was carried out in seeds of *Rumex vesicarius* after *In vitro* germination gave rich plantlets with many valuable phytochemicals under investigation (such as: saponins, alkaloids, flavanoids, tannins, steroids, carbohydrates, glycosides etc.,) [8].

3.1 Induction of Callus and Multiple Shoots Regeneration from Leaf Explants

Leaf explants showed callus induction initially at the cut ends of explants and subsequently from the entire surface of each explants after 2 weeks. Further subcultures of this little mass of callus were rapidly multiplied into large mass of greenish yellow friable callus and brown friable callus on SH medium supplemented with various concentrations of BA (0.5,1.0,2.0,3.0,4.0 and 5.0mg/l) and 2,4-D (0.5,1.0,2.0 and 3.0mg/l).In both cases callus induction was observed after one week of inoculation. Percentage of callus induction, morphology of callus, percentage of multiple shoots, number of shoots, average length of shoots from *In vitro* derived calli were recorded after six weeks of culture inoculation using different concentrations of different growth regulators. The explants inoculated onto SH medium supplemented with various concentrations of BA, greenish yellow friable callus was

observed in 2 weeks (Fig. 1(c)) and multiple shoots regenerated from this calli after three weeks of culture incubation. Leaf explants inoculated onto SH medium supplemented with 0.5mg/l BA showed 50% of callus induction and 75% of multiple shoot response. At 1.0 mg/l BA 60% of callus induction and 96.6%of multiple shoot response were observed. When the concentration of BA was increased to 2.0mg/l 75% of callus induction and 90% of shooting response was observed. 80% of callus induction and 80% of shooting response were observed when explants inoculated on SH medium with BA 3.0mg/l. At 4.0mg/l 90% of callus induction and 63.3% of shooting response was observed. 70% of callus induction and 50% of shooting response was observed at BA 5.0mg/l.

If leaf explants inoculated onto SH medium with 2,4-D, only brown friable calli was observed within two weeks of inoculation. SH medium with 0.5mg/l 2,4-D showed 50% of callus induction. At 1.0mg/l 2,4-D 60% of callus response were observed. When concentration of 2,4-D was increased to 2.0 mg/lt 90% of callus induction was observed. 70% of callus induction will be at 3.0 mg/l 2,4-D. 90% of callus induction was observed at 4.0 mg/l of BA and 2.0 mg/l of 2,4-D and maximum shooting response (96.6%) was observed from *In vitro* derived leaf calli at BA1.0mg/l (Fig. 1(d,e&f).

3.2 Callus induction and multiple shoots regeneration from nodal explants

The effect of different concentrations of BA and 2, 4-D under investigation on nodal segments for multiple shoots response was investigated. Initially, callus was induced at the basal end of nodal explants and subsequently from the entire surface of segments in 3weeks. Further subculture of these little masses of calli were rapidly multiplied into large mass of compact greenish and white brown compact calli on SH media supplemented with various concentrations of BA (0.5, 1, 2, 3, 4 and 5 mg/l) and 2,4-D (0.5, 1, 2 and 3mg/l). In both cases callus induction was observed after 1 week of inoculation (Fig. 1(h)). At 0.5 mg/mi of BA, 40% of callus induction was obtained and 50% of shooting response was induced from nodal explants. When concentration of BA was increased to 1.0 mg/ml, 60% of callus induction and 70% of shooting response were observed. 90% of callus induction and 85% of shooting response were observed in full strength SH medium supplemented with BA at 2mg/l (Fig. 1(i,j)). At 3 mg/l of BA, 80% of callus induction and 60% of shooting response were observed. , 70% of callus induction and 50% of shooting response were observed when explants inoculated on SH media containing 4 mg/l of BA. At 5.0mg/l of BA, 50% of callus induction and 40% of shooting response were observed from nodal explants.

If nodal explants inoculated onto SH medium with 2,4-D , only brown compact calli will be observed within two weeks of inoculation . SH medium with 0.5mg/l of 2,4-D showed 60% of callus induction. At 1.0mg/l of 2,4-D, 70% of callus response were observed. When concentration of 2,4-D was increased to 2.0mg/l, 90% of callus induction was observed. 60% of callus induction was obtained at 3.0 mg/l of 2,4-D. 90% of callus induction was observed at 2.0mg/l of BA and 2.0 mg/l of 2,4-D and 85% of shooting response was observed from nodal callus at 2.0mg/l of BA (Table 2).

The growth of callus was slow and took nearly 20 days for complete proliferation into a rapid mass of callus from nodal explants (Fig. k). 96.6% of multiple shoots response was obtained from leaf explants and 85% of this response was obtained from nodal explants. However, indirect shoot organogenesis has been achieved from both leaf and nodal explants derived callus culture. Leaf explants were responded better when compared to nodal explants for the induction of shoots after six weeks of culture inoculation. Maximum shoot growth (19.20±1.13) and shoot elongation (7.65±0.34cm) were achieved on SH medium supplemented with BA (1.0mg/l) from leaf explants derived calli (Table 1 and Fig. 1(f)). SH medium supplemented

with BA (2.0 mg/l) elicited the maximum shoot regeneration (11.40±0.94) and shoot elongation (5.12±0.37cm) from nodal explants derived calli, from these elongated plantlets after 20 days *In vitro* flowering observed (Fig. 1(l)). Effect of auxin and cytokinin on multiple shoot production and *In vitro* flowering in R.vesicarius reported by [18,19].

Calli mediated regeneration in *Rumex vesicarius* through leaf and nodal explants.

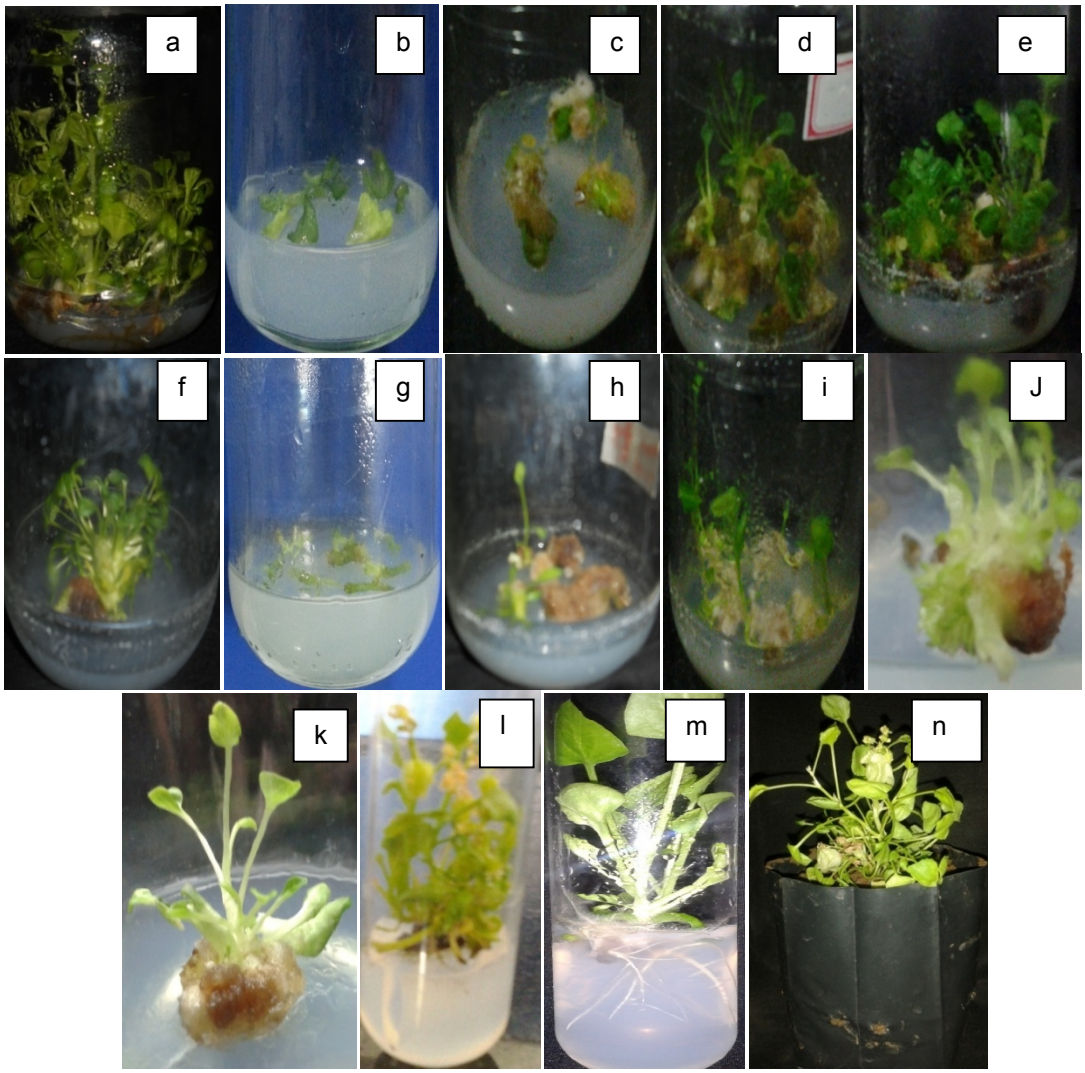

Fig. 1. Calli mediated regeneration in *Rumex vesicarius* through leaf and nodal explants

(a). In vitro plantlets from seed explants after two weeks.(b). Leaf explants for callus induction from in vitro regenerated plantlets. (c). Initiation of callus from leaf explants. (d). Shoots derived from leaf callus. (e). Induction of multiple shoots from leaf callus. (f).Elongation of shoots from leaf callus. (g). Nodal explants for callus induction from In vitro regenerated plantlets (h).Initiation of callus from nodal explants. (i). Induction of shoots from nodal callus. (j). Multiple shoots regenerated from nodal callus. (k).Elongation of shoots from nodal callus. (l).Initiation of flowers from elongated shoots in vitro. (m). Roots initiated from elongated shoots. (n). Acclimatization of the plantlet

Table 1. Influence of BA and 2,4-D individually for callus induction and multiple shoot induction from leaf explants of *Rumex vesicarius* on SH medium after six weeks

Sample number	Concentration of growth regulators (mg/ml)	% of callus induction	Morphology of callus	% of multiple shoots	No. Of shoots from leaf calli (mean±se)	Average length of shoots from leaf calli (mean±se)
1	BA(0.50)	50.00	GREENISH YELLOW FRIABLE CALLUS	75.00	12.85^{A}±1.12	7.14^{B}±0.62
2	BA(1.00)	60.00	GREENISH YELLOW FRIABLE CALLUS	96.60	19.20^{C} ±1.13	7.65^{C}±0.34
3	BA(2.00)	75.00	GREENISH YELLOW FRIABLE CALLUS	90.00	18.22^{B}±1.21	7.11^{B} ±0.43
4	BA(3.00)	80.00	GREENISH YELLOW FRIABLE CALLUS	80.00	17.80^{B} ±1.47	7.04^{B}±0.54
5	BA(4.00)	90.00	WELL DEVELOPED GREENISH YELLOW CALLUS	63.30	13.57^{A}±1.36	6.94^{A}±0.63
6	BA(5.00)	70.00	GREENISH YELLOW FRIABLE CALLUS	50.00	9.60^{A} ±0.91	5.33^{A}±0.51
7	2,4-D(0.50)	50.00	BROWN FRIABLE CALLUS	-	-	-
8	2,4-D(1.00)	60.00	BROWN FRIABLE CALLUS	-	-	-
9	2,4-D(2.00)	90.00	BROWN FRIABLE CALLUS WITH HAIRY ROOTS	-	-	-
10	2,4-D(3.00)	70.00	BROWN FRIABLE CALLUS	-	-	-

Table 2. Influence of BA and 2,4-D individually for callus induction and multiple shoot regeneration from nodal explants of *Rumex vesicarius* on SH medium after six weeks

Sample number	Concentration of growth regulators (mg/ml)	% of callus induction	Morphology of callus	% of multiple shoots	No. Of shoots from nodal calli (mean±se)	Average length of shoots from nodal calli (mean±se)
1	BA(0.50)	40.00	GREENISH WHITE COMPACT CALLUS	50.00	5.27^{A}±0.50	3.74^{A}±0.34
2	BA(1.00)	60.00	GREENISH WHITE COMPACT CALLUS	70.00	6.48^{B} ±0.57	4.00^{B}±0.36
3	BA(2.00)	90.00	GREENISH WHITE COMPACT CALLUS	85.00	11.40^{C}±0.94	5.12^{C}±0.37
4	BA(3.00)	80.00	GREENISH WHITE COMPACT CALLUS	60.00	5.22^{A} ±.51	4.50^{A}±0.43
5	BA(4.00)	70.00	GREENISH WHITE COMPACT CALLUS	50.00	4.80^{A}±0.43	3.86^{A}±0.37
6	BA(5.00)	50.00	GREENISH WHITE COMPACT CALLUS	40.00	4.16^{A} ±0.38	3.08^{A}±0.29
7	2,4-D(0.50)	60.00	BROWN COMPACT CALLUS	-	-	-
8	2,4-D(1.00)	70.00	BROWN COMPACT CALLUS	-	-	-
9	2,4-D(2.00)	90.00	BROWN COMPACT CALLUS	-	-	-
10	2,4-D(3.00)	60.00	BROWN COMPACT CALLUS	-	-	-

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

In the present study, leaf callus is friable, while nodal callus is compact. Additionally, leaf callus showed better response than nodal callus in induction of shoots and its elongation. Similar observations have also been observed in *Momordica cymbalaria* using nodal, intermodal and leaf explants [21]. Additionally rich calli with many valuable phytochemicals such as: Saponins, alkaloids, flavanoids, tannins, steroids, carbohydrates, glycosides etc., are under investigation using leaves and node segments as explants in this experiment. All the concentrations of 2,4-D under investigation have not any observed shooting responses using either leaf callus or nodal callus (Tables 1 and 2).

3.3 *In vitro* Rooting from Regenerated Shoots

In vitro grown shoots of *R. vesicarius* L. were separated and transferred to SH medium containing different concentrations of IBA individually for the induction of roots. *In vitro* rooting (100%) was observed from shoots derived from leaf calli on SH medium supplemented with 1.0mg/l of IBA. Where as SH media with 0.5 mg/l of IBA showed 60% of rooting response. Where as shoots shifted on to SH medium with 2.0mg/l of IBA showed only 80% of the response. It is found that, SH medium supplemented with 1.0mg/l of IBA was the best for the induction of *In vitro* roots from shoots. The maximum number of roots per shoot (8.83±0.20) with a maximum length (13.07±0.433cm) was obtained from *In vitro* shoots derived from leaf calli (Table 3 and Fig. 1(m)).

In this study, it was observed that, the thick and long roots were developed from *In vitro* grown shoots on SH medium supplemented with IBA (1.0mg/l).

Table 3. Influence of different concentrations of IBA on root induction from *In vitro* regenerated shoots

Sample number	Concentration of growth regulators (mg/ml)	% of rooting response from leaf derived calli	No. of roots/ leaf derived calli (mean±se)	Average length of roots from leaf derived calli (mean±se)
1	IBA(0.5)	60.00	4.17^{A}±0.40	7.88±0.76
2	IBA(1.0)	100.00	8.83^{C}±0.20	3.07^{C}±0.43
3	IBA(2.0)	80.00	7.19^{B}±0.63	10.00^{B}±0.87

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

4. CONCLUSION

Indirect organogenesis as described in this report is more desirable because this mode of regeneration gives better chance of producing genetically uniform plant. Till today there is no report on indirect organogenesis in *R. vesicarius*. This protocol used to make this plant available throughout the year for the production of secondary metabolites for pharmaceutical and economic usages, germplasm conservation and also for the commercial cultivation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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