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In vitro Plant Regeneration of *Rauvolfia tetraphylla* L.: A Threatened Medicinal Plant

Rabbi Hoque, Rita Sarah Borna, M. Imdadul Hoque and R. H. Sarker*

Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

Key words: *Rauvolfia tetraphylla*, *In vitro* seed germination, Regeneration, Seedling development

Abstract

Poor seed germination is the main obstacle for seed propagation of *Rauwolfia tetraphylla* L. in nature. The high viability (83.1%) of seeds in nature and the presence of viable embryo indicate that viability is not the only reason behind poor seed germination of this valuable medicinal plant. However, to overcome the problem of poor seed propagation, an efficient protocol has been developed for *R. tetraphylla.* Among the various treatments the maximum rate of *ex vitro* seed germination (13.33%) was found when the seeds were treated with 100 ppm of GA_3. Response of *in vitro* germination was found to vary under different conditions. Not only that the rate was much higher compared to *ex vitro* germination. The rate of seed germination was found to be 78% in MS without PGR (plant growth regulator) supplements, while it was 70 and 78% in cotton bed under light and dark, respectively. It was 80% in incubator at 37°C. In *R. tetraphylla* the hard seed coat is regarded as one of the barriers for germination and it can be easily eliminated by removing the hard seed coat. *In vitro* raised plantlets were reared in nature following proper acclimatization where they produced flowers as well as seeds. Apart from the said investigation, a method for *in vitro* shoot formation was also developed. Best response (90.7%) towards *in vitro* shoot regeneration was obtained from nodal segment when they were cultured on MS supplemented with 2.2 mg/l BA and 0.1 mg/l NAA. It took about 10-12 days to initiate shoots. About 9.9 ± 0.87 shoots were obtained per explants and their length was recorded as 2.28 ± 0.21 cm after six weeks. Various concentrations of IBA and NAA were used for *in vitro* root induction, but the *in vitro* raised shoots did not produce roots.

Introduction

Rauvolfia tetraphylla L. belonging to Apocynaceae is a small evergreen woody shrub and commonly known as 'Be Still Tree', 'Devil Pepper', and 'Four-leaf Devil Pepper'. Locally it is called 'BaroChadar'. It is native to Central America and Northern South America.

*Author for correspondence: <rhsarker2000@yahoo.co.uk>.

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Introduced and naturalized in many countries including Bangladesh, India, Myanmar, Nepal, Pakistan and Sri Lanka (Ahmed et al. 2008). This medicinal plant used in traditional medicine is at present rarely available in Bangladesh.

Rauvolfia tetraphylla has a great therapeutic potential and possesses significant pharmacological activities. It contains a number of alkaloids, namely ajmaline, ajmalicine, reserpine, serpentine and tetraphyllincine (Faisal et al. 2005). Specially root tissue of this plant contains various monoterpene indole alkaloids and reserpine is reported to be present in more than 50% of total alkaloids (Anitha and Kumari 2013). Apart from this, *Rauvolfia tetraphylla* is used in the treatment of cardiovascular, hypertension and various psychiatric diseases (Faisal and Anis 2002).

Rauvolfia tetraphylla can be cultivated through seeds, but it has poor rate of seed germination, slow growing and its development is dependent on its age and season. Beside this it has a certain seed dormancy period (Dey and Dee 2011). Therefore, conventional plant propagation through seeds is not enough to meet the demand of this plant required for the purpose of producing the desired quantity of alkaloids.

Destruction of habitat and over-exploitation for the purpose of medicinal uses invites threats to this plant. Being shrubby in nature and restricted distribution makes this plant highly vulnerable to human exploitation. Moreover, pharmaceutical companies largely depend upon material procured from naturally occurring plants that causes the depletion of this threatened plant species at an alarming rate. The indiscriminate collection and limited cultivation have made this plant unavailable normally and it has been listed as an endangered plant in India (Swarup and Arora 2000).According to Hussain and Jha (2014), this plant is threatened because of extensive utilization for its wide-ranging medicinal application. In another report, this plant has been described as critically endangered due to its extensive indiscriminate collection from wild, poor seed germination and lack of adequate commercial plantation (Anon 2003). The status of conservation of *R. tetraphylla* in Bangladesh has been regarded as "Not Evaluated (NE)" but appeared to be rare and no measure has been taken for the conservation of this important plant (Ahmed et al. 2008). With such natural and ecological constraints, overcoming the problem associated with the supply and need of this valuable medicinal plant is known to be a serious issue. Thus, it is necessary to adopt proper strategy for the propagation as well as conservation of *R. tetraphylla* in Bangladesh.

To develop appropriate conservation strategies, seed germination studies of this medicinal plant, has been considered to be useful (Kandari et al. 2007). Moreover, *in vitro* propagation through is another possible way to conserve this plant species by which many genetically uniform plants can be propagated. A few reports are available for *in vitro* propagation of *R. serpentina*, (Khan et al. 2018, Susila et al. 2013, Alatar et al. 2012). On the other hand, very–limited information is available on *in vitro* propagation and cultivation of *R. tetraphylla* (Faisal et al. 2012, Harisaranraj et al. 2009, Faisal et al. 2005).

Considering the importance of its use in medicine a study was conducted for *in vitro* propagation of *R. tetraphylla*. In the present study, authors report on the capability of germination of naturally available seeds also an efficient protocol for *in vitro* seed germination and regeneration of *R. tetraphylla*.

Materials and Methods

Fully mature seeds were collected from the ripened fruits of *Ravoulfia tetraphylla* L. plants growing in the Botanical Garden of the Department of Botany, University of Dhaka and were used for germination and seedling development. Various explants, such as, nodal segments, internodes and leaves used for *in vitro* regeneration were collected freshly from the plants growing in the Botanical Garden. For testing the viability of seeds, 0.1% 2, 3, 5-tri-phenyltetrazolium chloride solution (TTC) was used. Sun-dry seeds were soaked in distilled water for 15 min and then cut vertically into two equal parts (Fig. 1). After that they were placed in a Petri dish containing TTC solution and were incubated at 30°C for 2 - 3 hrs in a dark chamber. After incubation, TTC was decanted off. Stained cut seeds were then rinsed several times with distilled water until the water become clear and the observations were recorded under stereo microscope (Nikon SMZ1000). Photographs were taken using a camera (Nikon Digital Sight DS-Fi2) attached to the microscope.

The seed coat of *Rafoulfia tetraphylla* is hard and as such it is apparently difficult to germinate them. Several treatments were applied to examine the capability of germination of seed germination. For *ex vitro* germination seeds were treated with either GA_3 and H_2SO_4 or hot water. Four different concentrations of GA_3 (50, 100, 150 and 200 ppm) and four different timings (5, 10, 15 and 20 min) of H_2SO_4 were tested for seed germination. All experiments were conducted with fully mature seeds with their control for all sets of experiments. For GA_3 and hot water treatment, seeds were incubated for 16 hrs in the respective solution/hot water at room temperature. Following different treatments, with specific incubation period seeds were sown in the earthen pots containing soil and were maintained in the net house under a natural condition. Twenty seeds with three replications were used for each treatment. The observations for seed germination were recorded regularly up to 30 days.

Apart from this, experiments were conducted for *in vitro* seed germination and seedling development. For this, seeds were surface sterilized with 2 - 3 drops of Tween-20 for 5 - 10 min with constant shaking and then rinsed briefly with sterilized distilled water. Seeds were kept in 70% ethanol for 5 - 10 min followed by washing thoroughly with sterilized distilled water for 2 - 3 times. The seeds were then treated with 0.1% $HgCl_2$ solution by shaking gently for 10 - 15 min. To remove $HgCl_2$, the materials were then washed 3 - 5 times with distilled water. The entire sterilization of seeds was carried out in a laminar air flow cabinet. After that the seeds were taken in a sterilized glass bottle containing autoclaved distilled water. The sealed bottles containing seeds were

incubated at 4ºC in a refrigerator for 7 - 10 days. Sterilized seeds were taken in the laminar air flow cabinet and one edge of the seed was cut to facilitate germination. For *in vitro* seed germination, 3 techniques were applied using the pre-cut seeds: (i) cut seeds were inoculated on agar solidified MS without PGRs (plant growth regulators) containing only 3% sucrose; (ii) incubation of seeds on water soaked sterilized cotton bed in a glass bottle in light and dark chambers to find out their effect on germination. The cultures of seeds on MS and cotton beds were maintained at 25 ± 2ºC under 16hrs light and 8hrs dark condition. Each of the culture vessels containing five seeds were inoculated, and (iii) the rate of germination was also tested at 37ºC in an root system were taken out from the culture vessels and were transplanted to small pots containing sterilized soil. They were then acclimatized following standard protocols and establish them in natural environment.

For *in vitro* regeneration freshly collected explants from *R. tetraphylla* were used. Explants were prepared by washing thoroughly under running tap water for 30 min to clean the dust and surface contaminants. They were then surface sterilized with detergent for 2 - 3 min and then with 2 - 3 drops of Tween-20 in water for 3 - 5 min with constant shaking. This was followed by washing with distilled water several times to remove the sterilizing agents completely. Further surface sterilization of explants was carried out in the laminar flow by rinsing with 70% ethanol for 30 - 60 seconds and washed with autoclaved distilled water 2 - 3 times. The materials were then suspended into 0.1% $HgCl_2$ solution with constant gentle shaking for 5 - 10 min. Finally, $HgCl_2$ solution was removed by washing them thoroughly 3 - 5 times with sterilized distilled water. After sterilization small segments (5 - 8 mm) of explants were isolated and cultured on agar solidified MS for initiation, elongation and multiplication of shoots. For regeneration of shoots MS was supplemented with various concentrations and combinations of different PGRs, such as, BA BAP, NAA, TDZ and Kn. As and when required sub-cultured were done in a fresh medium at an interval of 21 - 28 days for multiple shoot regeneration.

For the induction of roots 2.0 - 2.5 cm long regenerated shoots were isolated and were cultured on either MS or half the strength of MS supplemented with different concentrations and combinations of IBA, IAA and NAA. Gelrite (Duchefa, Netherlands) was used as solidifying agent for rooting. All the *in vitro* culture vessels were maintained at 25 ± 2ºC under 16hrs light and 8hrs dark condition with a photosynthetic light intensity of 3200 lux.

Results and Discussions

The viability of seeds of *Ravoulfia tetraphylla* L. available in nature was examined as the seeds rarely germinate in nature. More so the seeds are small, oblong in shape with uneven surface (Fig. 1c). Seed length and width were 7.22 and 3.04 mm, respectively. Seed coat is very hard which indicated a strong dormancy for germination in nature. The viability of seeds was tested using 0.1% 2, 3, 5-TTC (Fig. 1). Endosperm and embryo were

observed under stereo microscope after TCC treatment. It was found that larger seeds contain uniformly red coloured embryo, whereas smaller seeds were non-embryonic.

Endosperm from both smaller and larger seeds was light pink in colour. Larger seeds were healthy with both endosperm and embryo. The viability of *R. tetraphylla* seeds was observed to be 83.1% (Table 1). These results were in conformity with the findings of Hussain and Jha (2014). Embryo of *R. tetraphylla* was present in the middle of blunt side of the seed. Embryo is regarded as the essential structure within a seed and detection of the presence and position of embryo within the seed is very important (Kozlowski and Pallardy 1997). Viability of these seeds can be determined by locating the position of embryo with the seed.

Table 1. Viability of *Rauvolfia tetraphylla* seeds as determined by 0.1% TTC solution.

Lot no.	No. of seed tested	No. of viable seeds	% of viable seeds	Viability (%)
1	100	83	83.0	83.1
2	92	76	82.6	
3	86	72	83.7	

The seed coat of *R. tetraphylla* is naturally very hard, and this is believed to be associated with low rate of germination of seeds. Eventually the availability of this plant is very limited in the natural population. One of the objectives of this study was to develop a suitable protocol for seed germination to facilitate the breaking of seed dormancy. Both *ex-* and *in vitro* methods for seed germination were tried and several treatments were applied to achieve a better rate of germination as well as development of plantlets so that the protocol can be utilized in raising required number of plants of this valuable and threatened species.

A total of nine different treatments were applied for *ex vitro* seed germination. The highest percentage of germination of seeds of *R. tetraphylla* was 13.33 in 100 ppm GA_3. In this treatment germination of seeds initiated after 21 days of sowing (data not shown). The germination percentages in case of 50, 150, and 200 ppm of GA_3 were 5.0, 6.67 and 3.7, respectably. It was also noticed that the increasing concentration of GA_3 decreased the rate of germination. No seed germinated in H_2SO_4 and hot water treatments. In all cases in control condition seeds did not germinate. GA_3 was the most effective PGR which exhibited good response in increasing the rate of germination. A good number of plants were developed using the seeds following GA_3 treatment. This finding was almost identical to those reported by Baskin and Baskin (1991).

Seed germination was found to be enhanced by soaking the seeds of *Osmorhiza claytonia* in GA_3. However, it is reported that GA_3 is effective in breaking the non-deep physiological dormancy, but it does not overcome the deep physiological dormancy (Baskin and Baskin 2004). H_2SO_4 treatments did not initiate germination of *R. tetraphylla* which may be due to the damage caused to the seed or specifically damage to the embryo

through long time exposure in acid. Wang et al. (2007) reported that due to incubation in H_2SO_4 caused damage to the seed as well its germination. Moreover, seeds did not germinate in hot water treatment, as such high temperature also appeared to be not effective in this case.

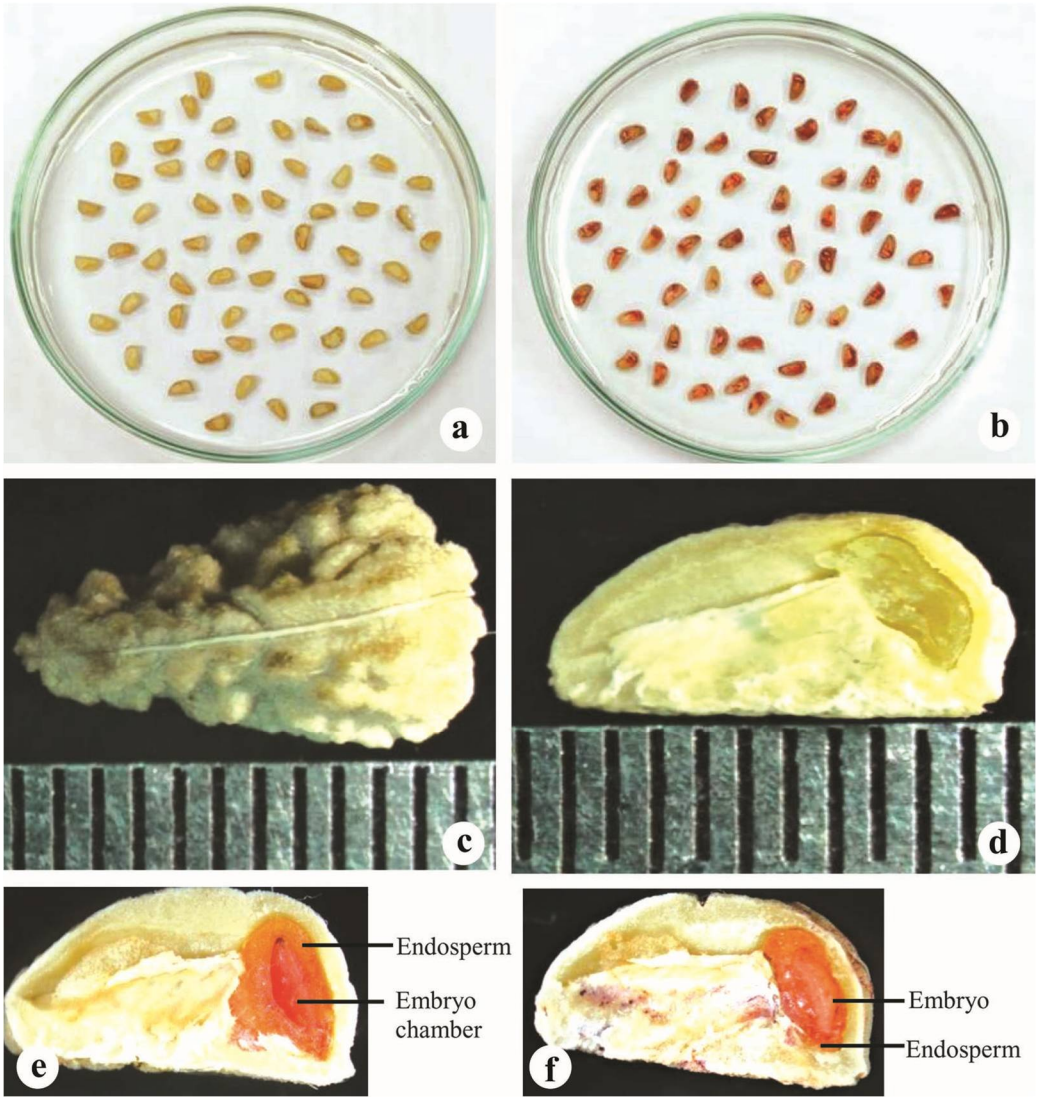

Fig. 1. Seed viability test. a. Vertically cut seeds before dipping into TTC solution. b. Embryos became red in color after incubation in TTC solution. c. Seed under stereo microscope showing the uneven surface of seed coat. 12x. d. Magnified view of vertically cut seed under stereomicroscope. 12x. e. Seed showing endosperm and embryo chamber but without the embryo under stereomicroscope 10x. f. Seed showing red color embryo under stereomicroscope. 10x.

In vitro techniques were applied to develop a suitable protocol for seed germination. To enhance the rate of germination one edge of each sterilized seed was cut as a result part of the hard seed coat was removed. Uncut seeds were treated as control. Before cutting the seeds, cold treatment was given to them for 7 - 10 days at 4°C. The cold treated pre-cut seeds were then exposed to three different conditions, namely MS medium, sterilized cotton bed and incubation at 37°C for optimization of *in vitro* germination.

It was observed that 78.00% of the seeds germinated on agar solidified MS with 3% sucrose but without any PGR supplements. Similar results were obtained by Utami and Hariyanto (2019) in *Phalaenopsis amboinensis* J.J. Sm (orchid), Islam et al. (2018) in *Eustoma* and Darso and Feyissa (2015) in *Cordeauxia edulis*. No report was available for germination of seeds of *R. tetraphylla* in MS. Seeds started germination after 10 days of inoculation. However, uncut seeds showed no germination mainly due to the presence of hard seed coat. Darso and Feyissa (2015) reported 73.3% germination of *Cordeauxia edulis* seeds on MS when their seed coat was removed. In contrast, they did not observe any germination of seeds when seed coat was present. It was suggested that hard seed coat may hamper water uptake, resulting in little or no germination in low moisture conditions. Seeds were also incubated on water soaked sterilized cotton bed in a glass bottle under light and dark conditions to test their effects on germination. On cotton beds, approximately 70.00% and 78.00% of seeds were germinated in light and dark condition, respectively. In both the conditions germination initiated in 3 - 5 days of culture. The cultures of seeds on MS and cotton beds were maintained at 25 ± 2°C under 16 hrs light and 8 hrs dark condition. On cotton bed germination of pre-cut seeds was as good as on MS. Germination started after 3 - 5 days of inoculation. Anitha and Kumari (2013) conducted similar experiment in *R. tetraphylla*, but they used GA_3 before splitting the seeds and inoculated in moist cotton. They obtained 90% germination following this technique. Various stages of *in vitro* seed germination and development of plantlets have been presented in Fig. 2. Germination of pre-cut seeds was also tested at 37°C in an incubator with proper replications where 80% of seeds were found to germinate. Thus, temperature played an important role in seed germination (Godoi and Takaki 2004) and at 37°C the enzymatic activity was supposed to be very high and that produced high rate of germination. Maraghni et al. (2010) showed seed germination of *Ziziphus lotus* at 35°C. Control seeds did not germinate on cotton bed.

In this experiment the seeds were also exposed to a specific low temperature. Such pretreatment of seeds under cold condition may have triggered in breaking the seed dormancy. Cold treatment is reported to promote production of GA_3 and can contribute in breaking the seed dormancy (Roberts 1988). *In vitro* grown plantlets with enough strong root system were taken out from the culture vessels and were transplanted to small pots containing sterilized soil. They were then acclimatized following the standard protocols and established them in the natural environment. The survival rate of the

transplanted plants was found to be approximately 90% (data not shown). The plants flowered within 4 - 5 months and set seeds normally.

From the results of seed germination, it was observed that rate of *ex vitro* seed germination was very low compared to *in vitro* seed germination. Nevertheless, by cutting one edge the seeds can germinate easily in different *in vitro* conditions. Therefore, it is concluded that hard seed coat is certainly a barrier to seed germination in *R. tetraphylla*. Overcoming this barrier seeds can easily be germinated if hard seed coat be removed at least partially. Beside this embryo of *R. tetraphylla* was also small and covered by thick endosperm, there was a possibility that embryo cannot take sufficient water to germinate.

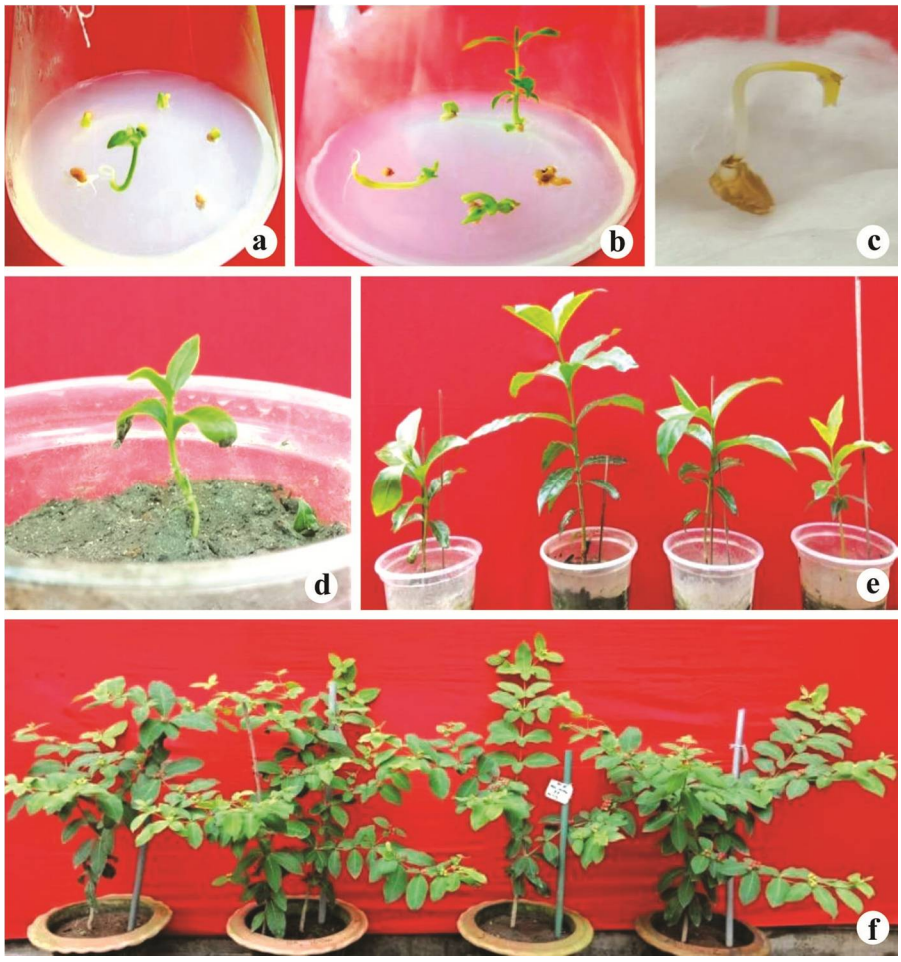

Fig. 2. *In vitro* seed germination and development of plantlets: a. Inoculation of seeds on MS. b. Plants growing on MS–after 1 month. c. Germination of seeds on cotton bed. d. Transplanted plantlet in soil after two months of culture. e. Four-month-old developing plantlets derived from *in vitro* raised seedlings. f. Fully developed plants of *R. tetraphylla*.

In another investigation experiments were conducted to find out the suitable explant and media combination for *in vitro* regeneration and multiplication of shoots of *R. tetraphylla*. Nodal segment, shoot tip, leaf and inter node were used as explants for shoot regeneration. Different concentrations and combinations of BA, NAA, BAP, Kn and TDZ were used in MS to determine the optimum PGR composition for initiation and development of multiple shoots.

The results obtained in the study demonstrated that MS with different concentrations and combinations of BA with NAA was effective towards regenerating multiple shoots directly from nodal segments of *R. tetraphylla*. Best response towards *in vitro* shoot regeneration was obtained when nodal segments (90.77%) were cultured on MS supplemented with 2.2 mg/l BA and 0.1 mg/l NAA through direct organogenesis **(**Fig. 3a**).** It took about 10 - 12 days to initiate shoots from nodal explant. About 9.9 ± 0.87 shoots were obtained per explant and length of shoot was recorded to be 2.28 ± 0.21 cm after six weeks of culture (Table 2). Such developing shoots can be multiplied through sub-culturing in the same medium (Fig. 3d**).** No response was found from leaf and internode explants but shoot tip explants showed little response towered shoot regeneration.

Table 2. Effect of BA and NAA on shoot proliferation of *R. tetraphylla* from nodal segments.

MS +BA + NAA (mg/l)		% of shoot regeneration	No. of shoot/ explant (mean ± SE)	Shoot length (cm)mean ± SE	Days to shoot initiation
1.0	0.1	39.13	2.7 ± 0.25	0.93 ± 0.12	20
1.5	0.1	55.55	2.9 ± 0.34	1.34 ± 0.57	15
2.0	0.1	63.33	3.5 ± 0.1	1.2 ± 0.34	15
2.2	**0.1**	**90.77**	**9.9 ± 0.87**	**2.28 ± 0.21**	**10 - 12**
2.5	0.1	73.33	6.8 ± 0.53	1.68 ± 0.25	12 - 15
3.0	0.1	70.00	6.1 ± 0.4	1.72 ± 0.01	15
3.5	0.1	66.67	7.2 ± 0.47	2.1 ± 0.37	15
4.0	0.1	43.33	4.8 ± 0.4	1.12 ± 0.25	15 - 20

This result is in conformity with that reported by Faisal et al. (2012) for high frequency shoot initiation. Effect of BA on various shoot bud inductions have been reported in several cases using a range of explants (Faisal et al. 2007, Benmahioul et al. 2012, Shahzad et al. 2011).The synergistic effect of BA in combination with an auxin for shoot multiplication was also reported in other medicinal plants, such as *Mucuna pruriens* (Faisal et al. 2006), *Metabriggsia ovalifolia* (Ma et al. 2011) and *Rauvolfia serpen*tina (Alatar et al. 2012).

For shoot regeneration, four different combinations of BAP (0.4 - 0.5 mg/l) and Kn (0.1 - 0.2 mg/l) were also used with MS to see their effects on regeneration of shoots of *R. tetraphylla*. The best response was observed on MS containing 0.5 mg/l BAP and 0.1 mg/l Kn (Fig. 3b). About 66.67% shoot regeneration with 2.18 shoots/explant were recorded on the same media combination. Elongation of shoots was observed within six to eight weeks of culture. Occasionally *in vitro* flower bud formation was observed in such cultures containing 0.5 mg/l BAP and 0.1 mg/l Kn **(**Fig. 3f). It was observed that shoot regeneration decreased by increasing concentration of BAP. The results with these PGR supplements were similar to that of Sarma et al. (1999). Bhalsing and Deshpande (2015) found BAP and Kn combination suitable for complete plant regeneration.

Apart from this, various concentrations of TDZ (0.1 - 2.0 mg/l) in MS were used for the induction of shoots. The best response was observed on MS containing 1.5 mg/l TDZ. About 62.5% of nodal segments exhibited regeneration with 3.18 shoots/explant after 5-8 weeks (Fig. 3c). But the increasing concentration of TDZ (2.0 mg/l) was found to decrease the shoot regeneration capability. Faisal et al. (2005) also used TDZ in MS for shoot regeneration of *R. tetraphylla* from nodal explants. They found 90% shoot regeneration frequency with 18.00 shoots per explant when cultured on MS medium supplemented with 5µM TDZ.

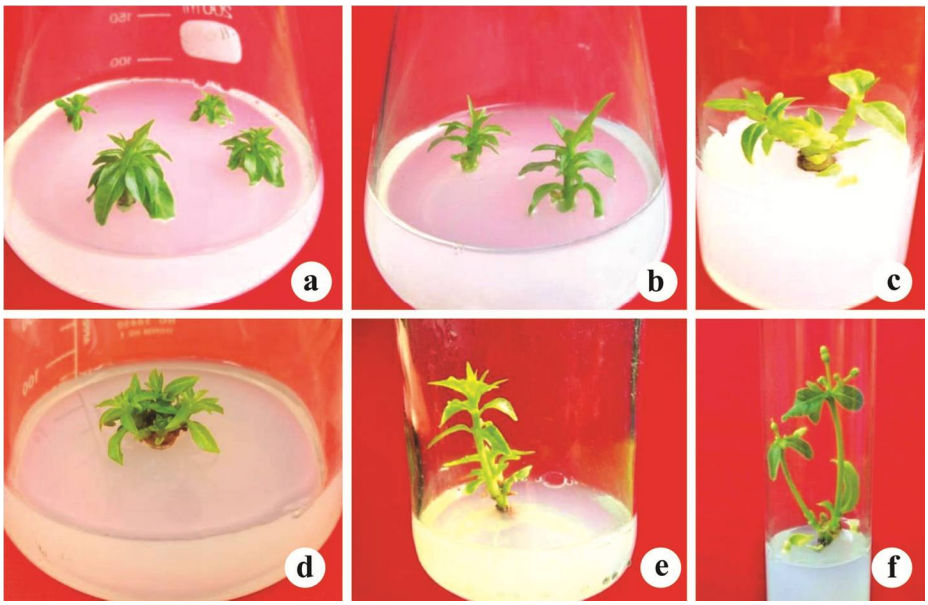

Fig. 3. Development of shoots of *R. tetraphylla* from nodal segment explants: a. MS media supplemented with 2.2 mg/l BA and 0.1 mg/l NAA after 4 weeks of culture. b. MS + 0.5 mg/l BAP and 0.1 mg/l Kn after 5 weeks of culture. c. MS + 1.5 mg/l TDZ after 6 weeks of culture. d. Multiplication of shoots following subculture on same media as shown in Fig. 3a after 8 weeks of culture. e. Same as Fig. 3d but showing the elongation of isolated shoot. (f) *In vitro* flower bud formation on the shoots in the cultures containing 0.5 mg/l BAP and 0.1 mg/l Kn.

To induce root from *in vitro* regenerated shoots, 2 - 3 cm long individual shoots were excised and cultured on MS as well as half strength of MS with or without different concentrations of IBA and NAA. But none of the cultures showed induction of roots. Faisal et al. (2005) observed *in vitro* rooting of *R. tetraphylla* in IBA containing medium. Sarma et al. (1999) found root induction in medium containing NAA. Rohela et al. (2019) also found root induction of roots in media supplemented with IAA and IBA.

It was observed that the hard seed coat of this *Ravoulfia* species was responsible and acted as the barrier of seed germination. Protocols for *ex vitro* as well as *in vitro* seed germination have been developed for *R. tetraphylla*. Seed germination barrier can easily be overcome by removing the hard seed coat. *In vitro* raised plants can be established in natural environment. Thus, the propagation of this valuable and threatened medicinal plant can be maintained and be enhanced.

Although a good method for *in vitro* shoot regeneration was developed but a method for complete development of plantlet was hampered due to the lack of *in vitro* rooting from the *in vitro* derived shoots. In the future studies more emphasis should be given for the establishment of a reproduceable protocol for *in vitro* regeneration of *Ravoulfia tetraphylla*.

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