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MEDICINAL PLANTS

Organogenesis, direct somatic embryogenesis, and shoot proliferation of *Rheum spiciforme* Royle: an endemic and vulnerable medicinal herb from Indian Trans Himalayas

Mohd Ishfaq Khan[1,2] · Anwar Shahzad[2] · Irfan Bashir Ganie[2] · Shahzad A. Pandith[1]

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Abstract

Rheum spiciforme Royle is a high value medicinal herb restricted to NW Himalayas. The medicinal properties of *Rheum* include anti-oxidant, anti-microbial, antitumor, anti-inflammatory, anti-fungal, anti-atherosclerotic, anti-proliferative, hepatoprotective, and immuno-enhancing. The species is threatened and endemic which demands its conservation. In this context, we have developed a premiere efficient *in vitro* regeneration system for this herb. The seed germination displayed phenomenal increase when transferred from soil (13.6 ± 3.1%) to half-strength Murashige and Skoog (MS) medium (92.6 ± 1.3%) fortified with 0.005 mM gibberellic acid (GA_3) and 1 mM potassium nitrate (KNO_3) and calcium chloride ($CaCl_2$) each with mean germination time (MGT) of 8.5 ± 1.8 d. Among four types of explants used for callusing, leaf explants responded highest with 87.3 ± 1.4% at 2 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BA) each. Nodal-segment-derived brown calluses exhibited significantly high regeneration (96.3 ± 1.6 %) at 8.0 μM BA and kinetin (KIN) each with 4.0 μM GA_3. Leaf explants observed direct somatic embryogenesis which displayed maximum (91.0 ± 3.4%) germination at 25 μM BA, 1.0 μM NAA (naphthaleneacetic acid), 2.0 μM GA_3, 50.0 μM glutamine (GTM), and adenine sulfate (ADS) each. Multiple shoot induction with mean number of 10.1 ± 2.6 shoots and elongation 4.2 ± 0.4 cm was observed at 12.5 μM BA and 0.5 μM NAA along with 25 μM GTM and ADS each. The rooted seedlings developed in half-strength liquid MS with 2.5 μM NAA were hardened and subsequently transferred to the field. The developed protocol could be utilized for various attributes which include development of large-scale micropropagation system as a conservation measure, *Agrobacterium*-mediated genetic transformation studies, and industrial production of important bioactive chemical constituents.

Keywords Rhubarb · Micropropagation · Plant regeneration · Medicinal plant species

Introduction

Rheum Linn. (Polygonaceae) commonly known as rhubarb is a highly diversified genus consisting of about 60 extant species of perennial herbs. Different traditional systems of medicine are witness to the wide and efficacious use of rhubarb since antiquity to cure a wide range of ailments related to the circulatory, digestive, endocrine, respiratory, and skeletal systems. The remedying properties of *Rheum* are ascribed to a set of diverse bioactive secondary metabolite constituents, particularly anthraquinones and stilbenoids, besides the dietary flavonoids known for their putative health benefits (Zargar *et al.* 2011; Jeelani *et al.* 2017; Pandith *et al.* 2018). *R. spiciforme* Royle Syn. *R. scaberrimum* Lingelsheim ex Limpricht (web link - 2) is a perennial stemless herb with purgative root used as medicine. The species bears white green, unbranched spike like panicle (panicle spiciform) inflorescence and produces tri-winged pinkish red seeds (FOC Vol. 5 pp 349; FOC—Flora of China). *R. spiciforme* is a vulnerable (Kala 2005) medicinal herb that grows at an altitude of about 3047 to 4,876 m above mean sea level (asl) (Kumar *et al.* 2011). Besides being used as food/vegetable (leaves), the roots of *R. spiciforme* are also

✉ Shahzad A. Pandith
drshahzad@uok.edu.in

[1] Department of Botany, University of Kashmir, Hazratbal, Srinagar, Jammu, Kashmir 190006, India

[2] Plant Biotechnology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh 202002, India

used as coloring agent by locals in the areas of its occurrence (Dorjey *et al*. 2012).

In comparison to the long-going conventional approaches of multiplication of a particular species, *in vitro* micropropagation offers a clear advantage for the propagation and multiplication at large scale (Acemi 2020). Roggemans and Claes carried out the preliminary study on *in vitro* propagation of the rhubarb (*R. rhaponticum*) and noticed initiation of the axillary buds from shoot tips grown on MS medium fortified with IBA (indole-3-butyric acid) and BAP (6-benzylaminopurine) (Roggemans and Claes 1979). This was followed by additional investigations, though inadequate, which were focused on other species of this perennial herb, and include *R. palmatum* (Ishimaru *et al*. 1990; Cui *et al*. 2008), *R. tanguticum* (Xu *et al*. 2004), *R. ribes* (Sepehr and Ghorbanli 2005), *R. rhabarbarum* (Lepse 2007; Rayirath *et al*. 2011), *R. officinale* (Ji-yong 2010), *R. webbianum* (Rashid *et al*. 2014), *R. moorcroftianum* (Maithani 2015), and *R. coreanum* (Mun and Mun 2016). Indeed, a detailed report on the *in vitro* multiplication of one of the important species of Rhubarb, *R. australe*, is available in our recent communication (Pandith *et al*. 2018). Nonetheless, and owing to the pharmacological utility of Rhubarb species, these reports do not account much toward the *in vitro* regeneration system of this important medicinal herb. But, as a prelude, it provides a platform for advanced empirical studies imperative for a better and valuable output.

Pandith *et al*. (2020) has recently reviewed the utility of the technique of *in vitro* multiplication of some rhubarb species. However, to date, and to the best of our knowledge, there are no authentic reports on the detailed *in vitro* propagation of *R. spiciforme*, somatic embryogenesis, in particular. Therefore, and in light of the threatened and endemic nature of *R. spiciforme*, the aim of present study was to develop reproducible *in vitro* systems to produce regenerated plants through improved seed germination, indirect organogenesis through callus, and direct somatic embryogenesis from leaf explants. We speculated that explants are affected by PGRs (plant growth regulators) and the medium composition. Therefore, the regeneration capacity of four types of explants with effect of six PGRs on seed germination, callusing, shoot multiplication, and direct somatic embryogenesis were investigated. Also, the effects of different additives like $CaCl_2$, KNO_3, glutamine and adenine sulfate were evaluated. The protocol could be utilized in large-scale propagation, germplasm conservation, genetic transformation, and industrial production of important metabolites of therapeutic significance of this important medicinal herb.

Materials and Methods

Seed collection and sterilization The mature seeds of *R. spiciforme* were collected from Lachulangla, Leh, Ladakh, India (33° 04′ 53.49″ N/77° 38′ 08.32″ E; altitude 4,750 m asl) on 30th of July 2019 (Fig. 1*A*). The plant specimen was also deposited at the University of Kashmir Herbarium (KASH) receiving the voucher specimen number 2745-(KASH). The seeds were shade dried and stored in netted packets until use. Before transfer to culture medium, the seeds were surface sterilized according to Kauth *et al*. (2006) with certain modifications. Briefly, the seeds were thoroughly washed under tap water for about 2 h and treated with carbendazim 50% WP (Bavistin, Crystal Crop Protection, New Delhi, India) for 30 to 40 min to be washed again for another 30 min to remove any traces of Bavistin. Following this, they were treated with 1% (*v/v*) Tween 20 detergent (Sigma Aldrich, Saint Louis, MO) for 30 min and rinsed two times with distilled water. The seeds were then kept for imbibition in autoclaved water at room temperature (25 ± 2°C) in dark for 24 h. Meanwhile, all other materials required for culture were washed with laboline detergent, oven dried, and surface sterilized with 70% alcohol and autoclaved at 121°C and 15 psi pressure for 18 min. Finally, the seeds were treated with 0.1% of $HgCl_2$ for 5 min followed by 5 to 6 rinses with autoclaved double distilled water prior to inoculation.

Seed germination Following sterilization, the seeds were transferred to culture medium under aseptic conditions in laminar hood. The seed germination process was evaluated on Murashige and Skoog (MS; Murashige and Skoog 1962) full-strength medium (HiMedia, Mumbai, India) and MS-half strength medium supplemented with 30 g L^{-1} sucrose (HiMedia) and 0.8% (*w/v*) agar–agar (Sigma Aldrich). Both the medium types were augmented with different concentrations and combinations of gibberellic acid (GA_3), calcium chloride ($CaCl_2$), and potassium nitrate (KNO_3) which were purchased from Duchefa Biochemie (Haarlem, The Netherlands). The pH of the medium was set to 5.8 ± 0.2, heated until complete dissolution, poured into glass culture vials (25 × 200 mm) and autoclaved at 121°C temperature, 15 lb pressure for 18 min. The sterilized seeds were inoculated in glass culture vials at 25 ± 2°C with 55% humidity under 12-h photoperiod (50 to 60 mol m^{-2} s^{-1}) in a plant growth chamber. One seed was inoculated per vial, and 20 vials were used for each treatment. The observations were made with respect to each non-contaminated seed (experimental unit). Another set of sterilized seeds (20 seeds in each block with 5 replications) was sown in coco peat instead of the MS medium. Further, seeds (20 seeds in each block with 5 replications) were subjected to observe the germination percentage *ex vitro* using garden soil (soil/sand; 3:1 ratio) in pots inside greenhouse at 25°C. The seed germination was monitored on daily basis and data recorded. The mean germination time (MGT) was calculated according to Darrudi *et al*. (2014) using the equation:

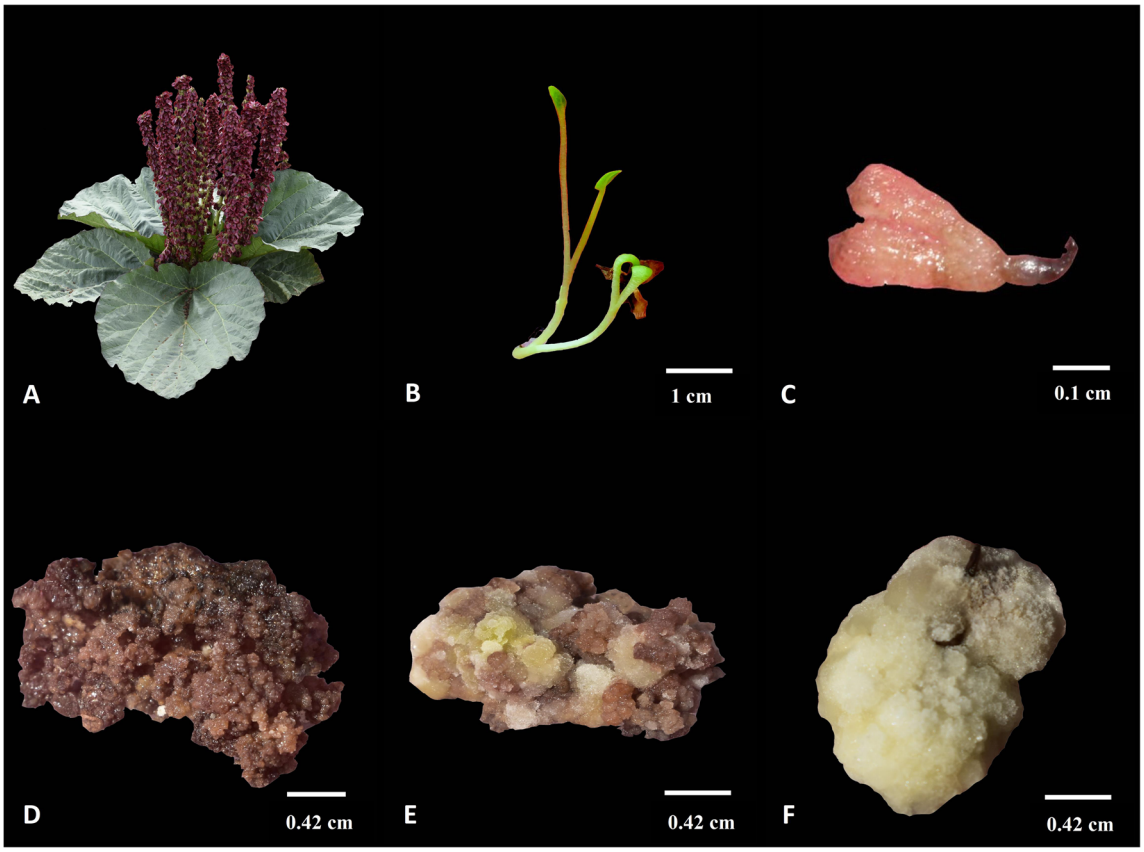

Figure. 1 Seed germination and callusing in different explants of *Rheum spiciforme* Royle. (*A*) Plant in natural habitat; (*B*) seed germination at 0.005 mM gibberellic acid (GA_3), 1 mM potassium nitrate, and 1 mM calcium chloride, (*C*) initiation of callusing from embryonic axis on medium with 8 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 8 μM thidiazuron (TDZ); (*D*) regenerative brown callus on medium containing 6 μM 2,4-D and 6 μM 6-benzylaminopurine (BA); (*E*) non-regenerative pale callus on medium containing 4 μM 2,4-D and 4 μM BA; and (*F*) non-regenerative white callus on medium containing 2 μM 2,4-D and 2 μM BA.

$$MGT = \frac{\sum(n \times D)}{N}$$

where n is the number of germinated seeds between recording intervals, D number of d from the beginning of the test, and N the total number of germinated seeds in the treatment at the end of experiment.

The percentage of seed germination was calculated using the equation:

$$\%\text{seed germination} = (\text{n}/\text{N}) \times 100$$

where n is the total number of germinated seeds and N the number of seeds used at the beginning of the experiment.

Callusing For callogenesis, node, leaf, root, and embryo segments were used as explants. Node, leaf, and root segments of about 1 cm were excised from the aseptically grown 6-wk-old *in vitro* seedlings, 4.9 ± 0.2 cm in height. In case of embryo, the surface sterilized seeds described in the previous section were used. As the seeds were swollen by imbibition, the embryos were excised with the help of scalpel and cut at both sides (from radicle and cotyledon side) to use the main embryonic axis (0.3 cm in length) for callusing. The segments of node, leaf, and root and embryonic axis were cultured in glass culture vials with 20 vials per treatment in MS medium supplemented with 0.0 to 10.00 μM 2,4-D (2,4-dichlorophenoxyacetic acid), BAP, KIN (kinetin) and TDZ (thidiazuron) in all suitable combinations for callogenesis. The callus derived after 7 wk was transferred on medium augmented with 2.0 to 10.00 μM BAP, KIN, and GA_3 for organogenesis. These additives were procured from Duchefa Biochemie. The percentage regeneration was calculated with respect to callus derived from different explant sources. Here, the regenerative callus is defined as the callus found capable of developing somatic embryos. The parameters like color, response percentage, and growth were evaluated. Moreover, response percentage of explants was calculated by counting the number of explants responded divided by total explants used and multiplied by 100. Furthermore, the growth of callus was evaluated in terms of volume (cm^3) of callus formed after 36 d of inoculation. The growth was expressed as –, +, ++, +++, and ++++ which indicates no, slight (less than 2 cm^3), moderate (2

to 4 cm^3), intense (4 to 6 cm^3), and very intense (more than 6 cm^3) callogenesis (growth), respectively.

Somatic embryogenesis For somatic embryogenesis, like callusing, single intact axillary leaves were used from the aseptically grown 6-wk-old *in vitro* seedlings, 4.9 ± 0.2 cm in height. The leaves along with petioles in both upright and horizontal (keeping abaxial side up in some explants and adaxial side in others) positions were cultured in glass culture vials with 20 vials per treatment containing MS medium fortified with 5.0 to 30.00 μM BAP, 0.0 to 01.00 μM NAA (naphthaleneacetic acid), 2.0 to 8.00 μM 2,4-D, 2.0 to 8.00 μM GA3, 25.0 to 100.00 μM GTM (glutamine), and 25.0 to 100.00 μM ADS (adenine sulfate) in all suitable combinations. The optimal condition for direct somatic embryogenesis in culture media at 25 ± 2°C with 55% humidity in dark for 1 wk was achieved by keeping vials inside a container covered completely by aluminum foil inside a plant growth chamber. Parameters including location of somatic embryo formation, number of somatic embryos formed, and percentage regeneration of these somatic embryos into plantlets were evaluated against each treatment.

Multiple shoot induction Nodal segments were excised from the aseptically grown 6-wk-old *in vitro* seedlings, 4.8 ± 0.5 cm in height. The single segments of about 1 cm were cultured in glass culture vials with 20 vials per treatment in MS medium supplemented with 0.0 to 30.00 μM BAP, 0.0 to 01.00 μM NAA, 1.0 to 15.00 μM KIN, 5.0 to 100.00 μM GTM, and 1.0 to 100.00 μM ADS in all possible combinations for shoot multiplication and evaluation of the effects of different combinations on shoot morphology. The *in vitro* regenerated shoots were multiplied and maintained by repeated transfer of mother explants and sub-culturing of *in vitro* raised shoots and callus. The mean number of shoots per explant, mean length of shoot, color of shoots and vigor of lamina, base callusing, percentage response and relative growth of callus in volume, location, number, and percentage regeneration of somatic embryos were recorded. Moreover, for shoot multiplication, callusing, and induction of somatic embryogenesis, the culture medium was initially supplemented with basic PGRs, and after recording the first set of results, the concentration of these PGRs was kept constant to evaluate the effect of others, and the trend was followed in each case.

Adventitious root induction and acclimatization The plantlets generated following the above-mentioned procedures were transferred for rooting to culture vials containing half-liquid Murashige and Skoog (LMS) medium and filter-bridge augmented with IAA (indole-3-acetic acid), IBA, and NAA at 0.5 to 2.5 μM concentrations. The one-half LMS medium devoid of any PGR was taken as control. Rooted seedlings from the culture vials were removed and transferred to thermocol cups having sanitized Soilrite™ (75% peat moss and 25% horticulture mark expanded perlite) (Keltech Energies Ltd., Bangalore, India). During hardening the plants were also sprinkled with quarter-strength MS inorganic solution and covered with polyethylene bags. The covered plantlets were kept in culture room at 25°C, and polyethylene bags were removed after 2 wk. The healthy plants were hardened finally in garden soil after keeping the pots in greenhouse for 4 wk and relocated to natural conditions subsequently.

Statistical analyses Each experiment was replicated three times with 20 samples in each, and the results were recorded after 6 wk. The results are expressed as the mean ± standard error. The treatments presented and compared in each table were evaluated in experiments carried out at same time, and the statistical analyses were carried out separately for each experiment. The data collected was analyzed in SPSS software (Version 25, SPSS Inc. Chicago, IL). The significance, analysis of variance, and significant differences among the treatments were determined based on one-way ANOVA and assessed following Tukey's and LSD Posthoc tests at 95% confidence limits ($P < 0.05$).

Results

Seeds exhibited better germination on half-strength MS medium supplemented with gibberellic acid, calcium chloride, and potassium nitrate Seeds of *R. spiciforme* showed a fairly significant increase in volume within the first 24 h when imbibed at room temperature (25 ± 2°C) in the dark. The percentage of seed germination in *R. spiciforme* was better in half-strength MS medium in comparison to full-strength MS medium. In MS-full medium, the percentage seed germination was the highest (71.5 ± 1.4%) with mean germination time (MGT) of 14.0 ± 1.7 d when supplemented with 0.005 mM GA_3, 1 mM $CaCl_2$, and KNO_3 each. On the other hand, in half-strength MS medium, the addition of 0.005 mM GA_3 significantly increased the rate of germination from 25.0 ± 0.6 to 75.2 ± 1.2 % with MGT of 10.6 ± 2.0 d (Table 1). So, while keeping the concentration of GA_3 constant at 0.005 mM, addition of 1.0 mM $CaCl_2$ was seen to increase the rate of seed germination to 91.4 ± 2.2 % and lowered the MGT to 9.1 ± 1.7 d. Moreover, the seed germination increased to 92.6 ± 1.3 % when 1 mM KNO_3 is supplemented along with 0.005 mM GA_3 and 1 mM $CaCl_2$ with MGT of 8.5 ± 1.8 d (Fig. 1*B*). The seeds were also grown in garden soil and coco-peat, and percentage germination of seeds in soil is very low (13.6 ± 3.1) compared to the coco-peat (58.5 ± 2.3 %) (Table 1).

Table 1 Effect of gibberellic acid (GA_3), calcium chloride ($CaCl_2$), and potassium nitrate (KNO_3) fortified Murashige and Skoog (MS) medium on seed germination of *Rheum spiciforme* Royle.

Serial number	GA_3 (mM)	$CaCl_2$ (mM)	KNO_3 (mM)	Mean germination time	Percentage germination
	MS half				
1	0.000	0.0	0.0	12.68 ± 1.20^{a}	25.00 ± 0.58^{a}
2	0.001	-	-	9.08 ± 1.50^{b}	37.42 ± 0.87^{b}
3	0.0025	-	-	16.55 ± 1.44^{c}	21.07 ± 0.64^{c}
4	0.005	-	-	10.67 ± 2.03^{j}	75.22 ± 1.18^{d}
5	0.01	-	-	14.08 ± 1.16^{d}	21.34 ± 0.88^{c}
6	0.005	1	-	9.07 ± 1.73^{b}	91.40 ± 2.21^{e}
7	0.005	2.5	-	12.21 ± 0.61^{k}	44.84 ± 0.72^{mp}
8	0.005	5	-	14.62 ± 1.45^{l}	35.90 ± 0.90^{bf}
9	0.005	10.0	-	9.17 ± 1.17^{be}	31.95 ± 0.98^{g}
10	0.005	-	1	8.55 ± 0.87^{f}	85.85 ± 0.93^{h}
11	0.005	-	2.5	35.0 ± 0.00^{i}	00.00 ± 0.00^{i}
12	0.005	-	5	10.86 ± 2.31^{m}	15.99 ± 0.58^{j}
13	0.005	-	10.0	35.0 ± 0.00^{i}	00.00 ± 0.00^{i}
14	0.005	1	1	8.52 ± 1.80^{f}	92.65 ± 1.33^{e}
15	0.005	2.5	2.5	9.98 ± 2.31^{n}	57.54 ± 1.44^{kl}
16	0.005	5	5	8.88 ± 1.16^{o}	74.41 ± 1.23^{d}
17	0.005	10.0	10.0	35.0 ± 0.00^{i}	00.00 ± 0.00^{i}
	MS full				
18	0.000	0.0	0.0	13.75 ± 0.90^{g}	21.21 ± 0.91^{c}
19	0.001	-	-	16.52 ± 1.44^{c}	54.51 ± 0.29^{l}
20	0.0025	-	-	14.89 ± 1.50^{p}	47.29 ± 1.46^{em}
21	0.005	-	-	35.0 ± 0.00^{i}	00.00 ± 0.00^{i}
22	0.01	-	-	9.23 ± 1.47^{e}	13.11 ± 0.86^{jo}
23	0.005	1	-	17.01 ± 1.73^{u}	33.66 ± 0.80^{fg}
24	0.005	2.5	-	12.56 ± 2.02^{a}	13.67 ± 0.88^{jo}
25	0.005	5	-	15.41 ± 0.59^{h}	42.88 ± 1.49^{ep}
26	0.005	10.0	-	18.15 ± 1.16^{q}	8.01 ± 0.27^{q}
27	0.005	-	1	13.62 ± 1.45^{g}	21.84 ± 2.05^{ac}
28	0.005	-	2.5	16.67 ± 0.88^{c}	56.60 ± 1.83^{kl}
29	0.005	-	5	11.95 ± 1.28^{r}	18.88 ± 1.61^{ej}
30	0.005	-	10.0	15.98 ± 1.26^{s}	53.56 ± 0.87^{l}
31	0.005	1	1	14.01 ± 1.73^{d}	71.54 ± 1.44^{k}
32	0.005	2.5	2.5	35.0 ± 0.00^{i}	00.00 ± 0.00^{i}
33	0.005	5	5	15.53 ± 1.81^{h}	8.53 ± 2.26^{r}
34	0.005	10.0	10.0	35.0 ± 0.00^{i}	00.00 ± 0.00^{i}
35	Coco peat			11.81 ± 3.2^{t}	58.51 ± 2.36^{eh}
36	Garden soil			16.27 ± 1.32^{c}	13.64 ± 3.18^{jo}

The data was recorded up to 6 wk. The data represents mean value ± SE (standard error) and was found statistically operative and significant by Tukey's test and LSD test at p = 0.05. The mean values with same *superscript(s)* within *columns* are not significantly different at p = 0.05 by the LSD test.

Callogenesis: 2,4-dichlorophenoxyacetic acid and 6-benzyl amino purine worked better on leaf and nodal explants; thidiazuron exhibited profound effect on the embryo In this study, four types of calluses were found in different explants with respect to different concentration and combination of PGRs (Table 2). Nevertheless, embryos responded with 67.9 ± 1.8% non-conspicuous callusing on medium containing 8 μM 2,4-D and 8 μM TDZ (Fig. 1*C*). The brown callus (Fig. 1*D*) was observed to undergo organogenesis. On the other hand, pale yellow- (Fig. 1*E*) and white- (Fig. 1*F*) colored callus cultures were very compact and feathery in appearance as compared to the loose and soft brown calluses. The red-

Table 2 Effect of different plant growth regulators (PGRs) on callusing from leaf, node, root, and embryo explants of *Rheum spiciforme* Royle.

PGR (μM)				Response percentage				Callus growth				Remarks
2,4 D	BA	KN	TDZ	Leaf (L)	Node (N)	Root (R)	Embryo (E)	Leaf	Node	Root	Embryo	
0.0	0.0	-	-	0.00±0.00^{g}	0.00±0.00^{h}	0.00±0.00^{f}	0.00±0.00^{m}	-	-	-	-	-
0.5	0.5	-	-	13.38±1.20^{a}	24.53±0.75^{b}	0.00±0.00^{f}	0.00±0.00^{m}	+	+	-	-	White in L and N
1.0	1.0	-	-	27.01±1.80^{h}	54.48±1.54^{e}	12.67±1.45^{a}	64.71±1.47a	+	++	+	+	White in L and N
2.0	2.0	-	-	87.38±1.47^{i}	74.17±2.21^{i}	19.95±1.85^{b}	35.82±0.88^{b}	+++	++	+	+	Brown in L,N/pale in R
4.0	4.0	-	-	56.16±1.91^{b}	81.72±1.20^{j}	62.79±1.12^{c}	59.93±2.09^{n}	++++	++++	++	++	Brown in L,N/pale in R/reddish in E
6.0	6.0	-	-	71.29±2.07^{c}	60.67±1.20^{c}	79.82±1.65^{g}	37.83±1.42^{bc}	+++	++	++++	+	Mostly whitish callusing
8.0	8.0	-	-	54.00±2.08^{b}	60.00±1.73^{c}	28.83±1.36^{d}	0.00±0.00^{m}	+	+++	+	-	Reddish in E
10.0	10.0	-	-	43.00±1.53^{j}	39.67±1.45^{d}	9.65±2.68^{a}	13.17±0.73^{d}	+	+	+	+	Reddish in E
0.5	-	0.5	-	0.00±0.00^{g}	0.00±0.00^{h}	0.00±0.00^{f}	0.00±0.00^{m}	-	-		-	-
1.0	-	1.0	-	18.33±1.86^{d}	24.67±2.60^{be}	28.62 ±1.69d	18.67±1.86^{f}	+	++	+	+	Died in 3rd wk in L,E,/black in R
2.0	-	2.0	-	0.00±0.00^{g}	21.00±0.58^{f}	49.33±4.26	43.00 ±1.73^{egk}	-	++	++	++	-
4.0	-	4.0	-	18.00±1.53^{d}	23.00 ±1.53b^{f}	41.67±3.18^{e}	24.67±2.60h	++	+++	++	+	Died in 3rd wk
6.0	-	6.0	-	57.33±1.45^{b}	63.67±2.33^{c}	0.00±0.00^{f}	81.00±1.15i	+++	++	-	+++	-
8.0	-	8.0	-	9.28±0.92^{e}	0.00±0.00^{h}	0.00±0.00^{f}	0.00±0.00^{m}	+	-	-	-	-
10.0	-	10.0	-	13.00±1.53^{af}	47.67±1.20^{k}	0.00±0.00^{f}	0.00±0.00^{m}	+	++	-	-	Black /swelling only
0.5	-	-	0.5	0.00±0.00^{g}	0.00±0.00^{h}	0.00±0.00^{f}	0.00±0.00^{m}	-	-	-	-	-
1.0	-	-	1.0	0.00±0.00^{g}	44.00±2.08^{l}	0.00±0.00^{f}	27.67±1.20hj		+	-	+	Black in E
2.0	-	-	2.0	22.67±1.45^{k}	16.00±0.58^{a}	0.00±0.00^{f}	46.00±1.15g	+	++	-	+	Black in E
4.0	-	-	4.0	0.00±0.00^{g}	0.00±0.00^{h}	0.00±0.00^{f}	51.00±0.58n	-	-	-	+++	Died in 3rd wk in E
6.0	-	-	6.0	0.00±0.00^{g}	0.00±0.00^{h}	14.00±1.53^{a}	40.00±1.53^{cek}	-	-	+	++	Died in 3rd wk E
8.0	-	-	8.0	0.00±0.00^{g}	0.00±0.00^{h}	37.48±2.48^{e}	67.95±1.78^{a}	-	-	+	++++	Brown in E
10.0	-	-	10.0	0.00±0.00^{g}	0.00±0.00^{h}	0.00±0.00^{f}	26.00±2.89^{h}	-	-	-	-	-
4.0	0.5	0.5	-	0.00±0.00^{g}	0.00±0.00^{h}	0.00±0.00^{f}	0.00±0.00^{m}	-	-	-	-	-
4.0	1.0	1.0	-	36.67±0.88^{l}	77.67±1.76^{m}	0.00±0.00^{f}	31.00±1.73^{j}	+	+++	-	+	White in L and N
4.0	2.0	2.0	-	72.83±1.17^{c}	59.84±1.02^{c}	18.33±1.86^{b}	80.17±0.73^{i}	++	++	++	++	-
4.0	4.0	4.0	-	16.67±0.88^{d}	22.00±0.58^{bf}	62.33±2.03^{c}	12.67±0.88^{d}	++	+	+++	+	Brown in L,N/pale in R
4.0	6.0	6.0	-	11.67 ±1.20^{aef}	17.67±0.88^{a}	39.67±2.96^{e}	0.00±0.00^{m}	+	+	+	-	White in L and N
4.0	8.0	8.0	-	0.00±0.00^{g}	39.33±1.76^{d}	0.00±0.00^{f}	20.00±1.73^{f}	-	+	-	+	-
4.0	10.0	10.0	-	0.00±0.00^{g}	0.00±0.00^{h}	0.00±0.00^{f}	0.00±0.00^{m}	-	-	-	-	-

The data was recorded up to 6 wk. The data represents mean value ± SE (standard error) and was found statistically operative and significant by Tukey's test and LSD test at p = 0.05. The mean values with same *superscript(s)* within *columns* are not significantly different at p = 0.05 by the LSD test. The signs −, +, + +, + + +, and ++++ indicate no, slight (less than 2 cm^3), moderate (2 to 4 cm^3), intense (4 to 6 cm^3), and very intense (more than 6 cm^3) callogenesis (growth), respectively. L, N, R, and E in remarks represent leaf, node, root, and embryo, respectively. Abbreviations: *2,4-D* 2,4-dichlorophenoxyacetic acid, *BA* 6-benzylaminopurine, *KIN* kinetin, and *TDZ* thidiazuron.

colored callus was also found to be non-viable. These observations can be correlated to Table 2. At lower concentrations of 2,4-D and BA (below 2 μM), white callus was formed in leaf and nodal explants with moderate growth. The percent callus response from leaf, node, and root explants increased with addition of 2,4 D and BA to the medium. Among four types of explants used for callusing, 87.4 ± 1.5% leaf explants responded at 2 μM 2,4-D and BA each; at 4 μM 2,4-D and BA, the percent response of explants decreased (56.2 ± 1.9%) with increased callogenesis. The callogenesis in node and root was best observed at 4 μM (81.7 ± 1.2%) and 6 μM (79.8 ± 1.6%) 2,4-D and BA each, respectively (Table 2). Although KIN in leaf and nodal explants slightly improved the percent response, however, callus died in third wk of culture. When KIN was supplemented along with 2,4-D and BA, it improved the formation of regenerating brown callus. The application of

TDZ however brought no significant effect on leaf, nodal, and root explants in this study. Pertinently, in embryo, TDZ was found to increase the percent response of explants along with the rate of callogenesis. Nevertheless, the callusing was not prominent in case of embryo explants.

Morphogenesis of regenerative callus: nodal segments displayed vibrant regeneration with intense rooting that shifted toward shooting with gibberellic acid The ultimate aim of callusing is to produce shoots for speedy propagation. In this study, callus transferred on medium fortified with equal concentration of BA and KIN only started the formation of shoot buds on leaf explant-derived callus (Fig. 2). Regeneration percentage of callus also varied with respect to explant used. The highest regeneration percentage of 81.3 ± 0.9 was observed in nodal segment-derived callus followed by calluses from leaf, embryo, and root with intense rooting at 8.0 μM BA and KIN each (Table 3). However, direct rooting from calluses was also observed which increased with increase in concentration of BA and KIN (Fig. 2*A*). To overcome this problem, GA_3 was supplemented along with BA and KIN. Addition of GA_3 improved bud maturation and regeneration percentage and decreased the emergence of rootlets (Fig. 2*B–D*). The highest percentage of regeneration (96.3 ± 1.6%) was observed in brown calluses derived from nodal segments followed by leaf (87.2 ± 1.5%) at 8.0 μM BA, KIN, and 4.0 μM GA_3 with slight rooting. In calluses derived from embryonic axis, lower (2 μM) concentration of GA_3 was found effective with percentage regeneration of 74.3 ± 0.7%. It was found that root-derived callus responded least to any supplement combination with highest regeneration rate of 43.2 ± 2.1% at 8.0 μM BA and KIN each and 6.0 μM GA3 (Table 3).

Axillary leaves lead to the direct induction of regenerative somatic embryos from both abaxial and adaxial sides of lamina and petiole The present investigation showed that BA at 15 to 25 μM concentration along with 1.0 μM NAA (Table 4) induces the formation of somatic embryos directly from leaf explants by culturing single intact axillary leaves on MS full medium. Formation of somatic embryos on adaxial side was observed after 2 wk. Initially, the leaf explants developed globular structures on adaxial side which later on grew into dark green shoots. When the concentration of BA was increased to 25 μM, emergence of mean 7.8 ± 0.9 somatic embryos were observed at juncture of lamina and petiole on adaxial side (jads) (Fig. 3*A–B*), juncture of lamina and petiole on abaxial side (jabs) (Fig. 3*C*), lamina on adaxial side (lad), and petiole (attached to lamina) (Fig. 3*D*) with 48.3 ± 1.7% regeneration. To increase the regeneration of somatic embryos into complete plantlets (Fig. 3*E–F*), along with 25 μM BA and 1.0 μM NAA, 2,4-D, GA_3, GTM and ADS were also added to the medium. Indeed, the addition 4 μM 2,4-D improved the rate of germination of somatic embryos up to 62.9 ± 2.9% which was further increased to 76.1 ± 2.3% by adding GA_3 at a concentration of 8 μM. It was observed that although mean number of somatic embryos per explant decreased to 6.2 ± 0.9, the percentage regeneration increased to the maximum

Table 3 Effect of different plant growth regulators (PGRs) on percentage regeneration of callus derived from leaf, node, root, and embryo explants of *Rheum spiciforme* Royle.

Treatment	PGR (μM)			Percentage regeneration of callus from different explants				Remarks
	BA	KIN	GA_3	Leaf (L)	Node (N)	Root (R)	Embryo (E)	
1	0.0	0.0	-	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	-
2	2.0	2.0	-	0.00±0.00^{a}	8.23±0.15^{b}	0.00±0.00^{a}	0.00±0.00^{a}	-
3	4.0	4.0	-	18.35±0.64^{b}	26.34±1.23^{c}	0.00±0.00^{a}	12.32±1.82^{b}	-
4	6.0	6.0	-	51.26±0.85^{c}	25.31±0.84^{c}	8.17±0.25^{b}	41.25±1.25 ^{c}	Moderate rooting
5	8.0	8.0	-	73.21±0.82^{d}	81.26 ±0.91^{ed}	28.26±1.21^{c}	62.34±1.02^{d}	Intense rooting
6	10.0	10.0	-	57.65±1.02^{c}	67.71±1.38^{f}	0.00±0.00^{a}	57.32±0.61^{d}	Intense rooting
7	8.0	8.0	2	76.25 ±0.78^{de}	79.35 ±0.23^{ed}	38.00 ±0.91^{d}	74.32±0.70^{e}	Slight rooting
8	8.0	8.0	4	87.25±1.53^{e}	96.32±1.65^{d}	36.26 ±1.00^{d}	63.07±0.13^{d}	Slight rooting
9	8.0	8.0	6	85.21±1.03^{e}	90.53±1.35^{d}	43.21 ±2.13^{d}	58.72±0.53^{d}	Moderate rooting

The data was recorded up to 6 wk. The data represents mean value ± SE (standard error) and was found statistically operative and significant by Tukey's test and LSD test at p = 0.05. The mean values with same *superscript(s)* within *columns* are not significantly different at p = 0.05 by the LSD test. Abbreviations: *BA* 6-benzylaminopurine, *KIN* Kinetin, and *GA_3* gibberellic acid.

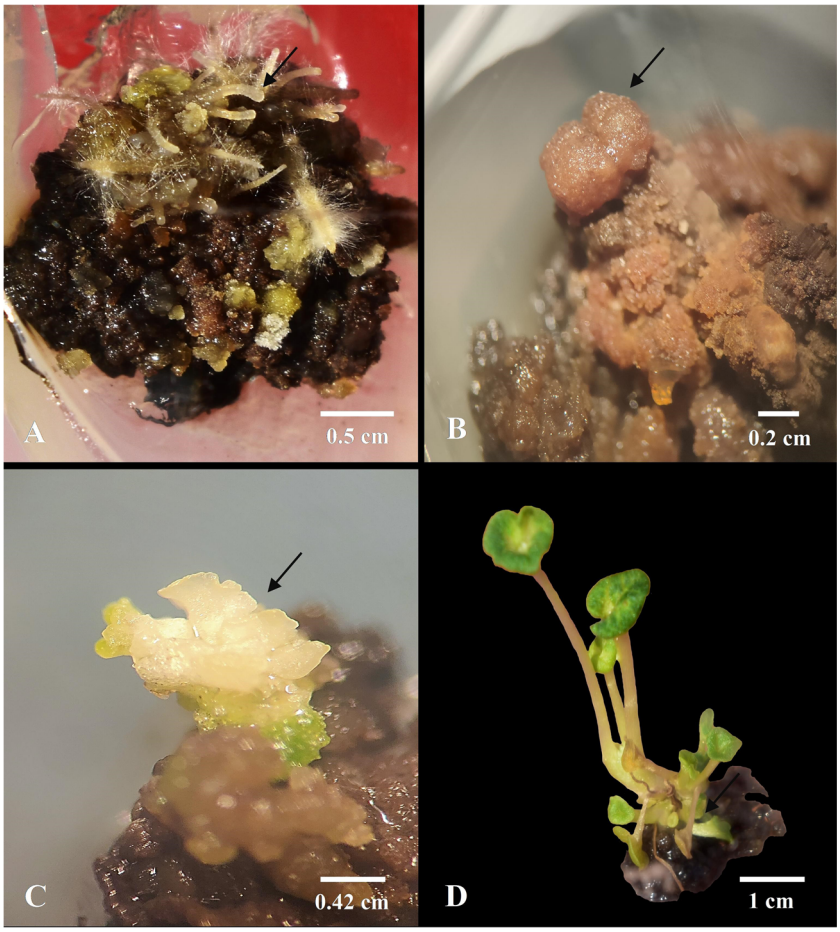

Figure. 2 Regeneration and organogenesis in callus from *Rheum spiciforme* Royle. (*A*) Root initiation on leaf derived callus at 8 μM 6-benzylaminopurine (BA) and 8 μM kinetin (KIN); (*B*) initiation of shoot bud formation in node-derived callus at 8 μM BA, 8 μM KIN, and 4 μM gibberellic acid (GA_3); (*C*) fully differentiated shoot from node-derived callus at 8 μM BA, 8 μM KIN, and 6 μM GA_3; and (*D*) regenerated plantlet from node-derived callus at 8 μM BA, 8 μM KIN, and 6 μM GA_3.

of 91.0 ± 3.5% at 25 μM BA, 1.0 μM NAA, 2.0 μM GA_3, and 50.0 μM GTM and ADS each. Also, it was found that maximum of 3.9 ± 1.0 plantlets were obtained from each explant fortified with 25 μM BA, 1.0 μM NAA, 2.0 μM GA_3, and 50.0 μM GTM and ADS each (Table 4).

A nitrogenous base (adenine sulfate) and an amino acid (glutamine) exhibited combinatorial effect in inducing extensive shoot multiplication in nodal segment-raised plants When single axillary shoots were grown on culture medium supplemented with 12.5 μM BA and 0.5 μM NAA, these multiplied extensively and produced mean 27.2 ± 2.9 shoots with mean shoot height of 3.4 ± 0.6 cm (Table 5). Nonetheless, it was observed that although shoot multiplication took place, shoots were found with underdeveloped lamina and pale-colored petioles (Fig. 4*A*). On the other hand, GTM and ADS supplemented at 25 μM concentration each (and not alone) along with 12.5 μM BA and 0.5 μM NAA produced mean 10.2 ± 2.6 shoots with mean 4.2 ± 0.4 cm shoot height, well developed green lamina with better overall vigor (Fig. 4*B–D*; Table 5). Therefore, for extensive shoot multiplication and maintenance of overall vigor, we suggest to grow single axillary explants on 12.5 μM BA and 0.5 μM NAA for 3 wk for multiplication and then transfer the culture on medium supplemented with 25 μM GTM and ADS each along with 12.5 μM BA and 0.5 μM NAA for 7 wk.

Naphthaleneacetic acid induced adventitious root system required for in vitro plants' acclimatization under natural conditions The plantlets initiated rooting in half-LMS supplemented with 2.5 μM NAA after 3 wk (Fig. 4*E*). The rooted plantlets were transferred to thermocol cups filled with sanitized Soilrite and hardened for 3 wk and subsequently transferred to pots in greenhouse (Fig. 4*F*).

Discussion

In vitro micropropagation/regeneration technique has the potential to provide an organized production system with uniform quality and yield. It has proven to be a useful asset for easy production of similar individuals of a particular genotype

Table 4 Effect of different plant growth regulators (PGRs) on direct somatic embryogenesis from leaf explants of *Rheum spiciforme* Royle.

Treatment	PGR (μM)						Somatic embryo formation/location	No. of somatic embryos formed	Percentage regeneration	Number of regenerated plants per explant
	BA	NAA	2,4-D	GA_3	GTM	ADS				
1	0.0	0.0			-	0.0	-	0.00 ± 0.00^{c}	0.00 ± 0.00^{g}	0.00 ± 0.00^{f}
2	5.0	1.0	-	-	-	-	-	0.00 ± 0.00^{c}	0.00 ± 0.00^{g}	0.00 ± 0.00^{f}
3	10.0	1.0	-	-	-	-	-	0.00 ± 0.00^{c}	0.00 ± 0.00^{g}	0.00 ± 0.00^{f}
4	15.0	1.0	-	-	-	-	+/Lad	2.10 ± 0.56^{abc}	18.02 ± 2.08^{a}	0.96 ± 0.11^{ad}
5	20.0	1.0	-	-	-	-	+/Lad	3.48 ± 0.48^{abd}	10.95 ± 3.22^{h}	0.71 ± 0.08^{a}
6	25.0	1.0	-	-	-	-	+/Jads, Jabs, Lad, Pet	7.83 ± 0.98^{g}	48.33 ± 1.76^{be}	2.01 ± 0.32^{b}
7	30.0	1.0	-	-	-	-	+/Lad	1.33 ± 0.88^{ac}	19.33 ± 3.93^{a}	0.46 ± 0.03^{c}
8	25.0	1.0	2.0	-	-	-	+/Lad	1.53 ± 0.29^{ac}	30.75 ± 2.16^{c}	0.52 ± 0.01^{c}
9	25.0	1.0	4.0	-	-	-	+/Jads, Lad, Pet	4.96 ± 0.43^{bdf}	62.89 ± 2.89^{d}	2.11 ± 0.23^{b}
10	25.0	1.0	8.0	-	-	-	-	0.00 ± 0.00^{c}	0.00 ± 0.00^{g}	0.00 ± 0.00^{f}
11	25.0	1.0	4.0	2.0	-	-	+-Lad	2.17 ± 0.20^{abc}	46.02 ± 2.08^{be}	1.02 ± 0.16^{d}
12	25.0	1.0	4.0	4.0	-	-	+/Lad	1.50 ± 0.44^{ac}	38.33 ± 2.03^{f}	0.84 ± 0.06^{a}
13	25.0	1.0	4.0	8.0	-	-	+/Lad	3.05 ± 1.16^{ab}	76.08 ± 2.31^{i}	1.22 ± 0.24^{d}
14	25.0	1.0	-	2.0	25.0	-	-	0.00 ± 0.00^{c}	0.00 ± 0.00^{g}	0.00 ± 0.00^{f}
15	25.0	1.0	-	2.0	50.0	-	+/Jads, Lad, Pet	3.05 ± 0.58^{ab}	32.83 ± 1.17^{cf}	1.36 ± 0.08^{d}
16	25.0	1.0	-	2.0	100.0	-	-	0.00 ± 0.00^{c}	0.00 ± 0.00^{g}	0.00 ± 0.00^{f}
17	25.0	1.0	-	2.0	50.0	25.0	+/Lad	2.86 ± 0.20^{a}	68.19 ± 4.05^{d}	1.74 ± 0.61^{b}
18	25.0	1.0	-	2.0	50.0	50.0	+/Jads, Jabs, Lad, Pet	6.17 ± 0.90^{df}	91.00 ± 3.46^{j}	3.97 ± 1.04^{e}
19	25.0	1.0	-	2.0	50.0	100.0	-	0.00 ± 0.00^{c}	0.00 ± 0.00^{g}	0.00 ± 0.00^{f}

The data was recorded up to 6 wk. The data represents mean value ± SE (standard error) and was found statistically operative and significant by Tukey's test and LSD test at p = 0.05. The mean values with same *superscript(s)* within *columns* are not significantly different at p = 0.05 by the LSD test. The "+" and "-" signs indicate presence and absence of the formation of somatic embryos, respectively. Jads, Jabs, Lad, and Pet indicate (position) juncture of lamina and petiole on adaxial side, juncture of lamina and petiole on abaxial side, lamina on adaxial side, and petiole (attached to lamina), respectively. Abbreviations: *BA* 6-benzylaminopurine, *NAA* naphthaleneacetic acid, *2,4-D* 2,4-dichlorophenoxyacetic acid, GA_3 gibberellic acid, *GTM* glutamine, and *ADS* adenine sulfate.

(Pandith *et al*. 2018). Tissue culture has been extensively employed for the *in vitro* propagation, regeneration, production of pharmaceutically important bioactive compounds, and germplasm conservation of rare and endangered medicinal herbs (Das and Rout 2002). Therefore, the present study was focused on production of the reproducible *in vitro* regeneration and multiplication system of an endemic and vulnerable medicinal herb *R. spiciforme*.

The seeds of *Rheum* have been shown to bear moderate levels of seed dormancy (Rutherford and Ali 1977) imposed by embryo and certain inherent inhibitors (Farzami and Ghorbanli 2011). Seed germination in *Rheum* is known to be species specific as well as habitat specific and exhibits susceptibility to even moderate changes in soil water condition (Bo Song 2013). In the present study, seed germination improved to 92.6 ± 1.3% on half-strength MS medium fortified with 1 mM KNO_3, 0.005 mM GA_3, and 1mM $CaCl_2$ with MGT of 8.5 ± 1.8 d. Similar results have been found in some other species of *Rheum* viz. *R. khorasanicum* (Darrudi *et al*. 2014). The GA_3 promotes seed germination by stimulating the growth potential of embryo and release of hydrolases that weaken the structures surrounding embryo (Kamiya *et al*. 2002). On the other hand, calcium improves seed germination by loosening the cell wall expanding highly hydrated gel networks (Lapasin and Pricl 1995), by providing structural integrity to cellular membranes (Burstrom 1968), and by regulating the activity of certain kinase and phosphatase enzymes that play important role in the signal transduction during seed germination process (Derek 1997; Harper *et al*. 2004). Although KNO_3 and other nitrogenous compounds elevate levels of seed viability and germination in many crop and tree species (Beligni and Lamattina 2000; Bethke *et al*. 2004; Gniazdowska *et al*. 2007; Gao *et al*. 2011; Gupta *et al*. 2011), the combined effect of $CaCl_2$ and KNO_3 has been found to be more pronounced than KNO_3 alone in *R. khorasanicum* (Darrudi *et al*. 2014). The nitrates possibly remove seed dormancy through pentose phosphate pathway and consume oxygen evolved during various oxidation processes during seed germination (Finch-Savage and Leubner-Metzger 2006). Moreover, KNO_3 and $CaCl_2$ are also known

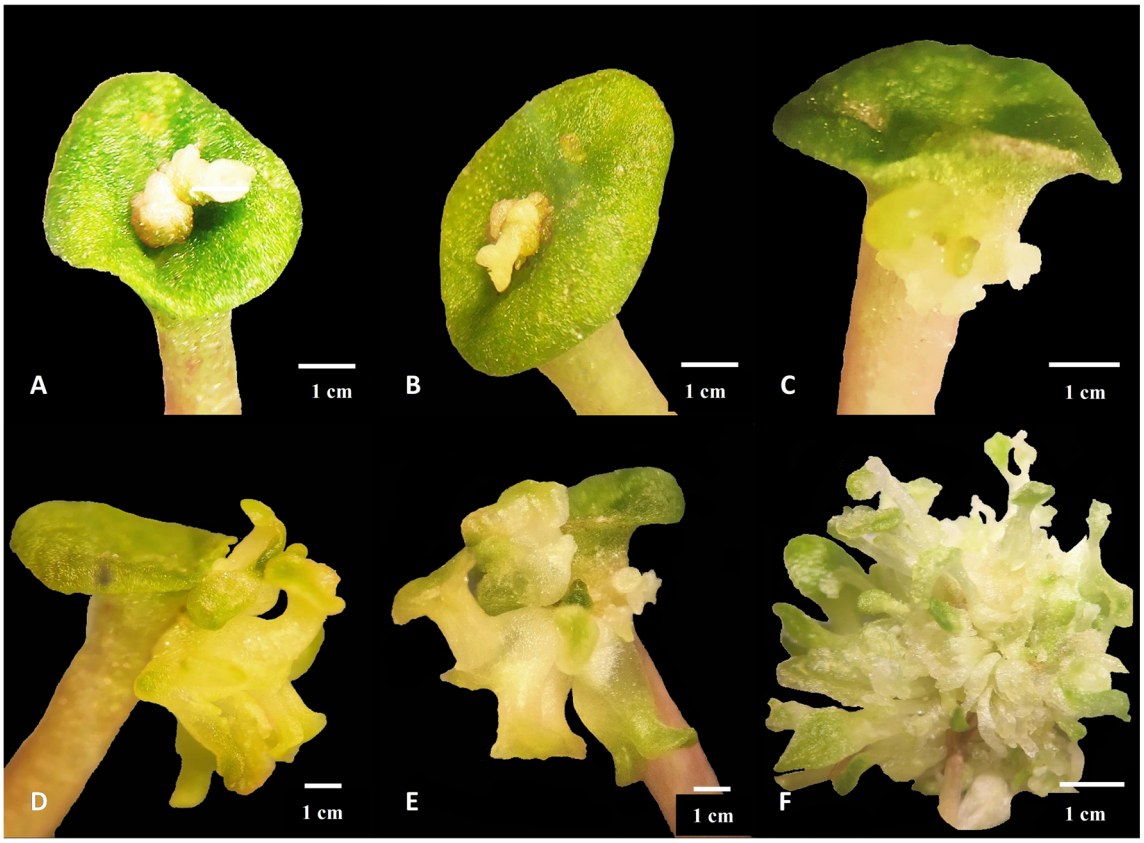

Figure. 3 Direct somatic embryogenesis in *Rheum spiciforme* Royle. (*A–B*) Somatic embryos formed on adaxial side of lamina at 25 μM 6-benzylaminopurine (BA) and 1 μM naphthaleneacetic acid (NAA); (*C*) somatic embryos formed on abaxial side of lamina at 25 μM BA and 1 μM NAA; (*D*) somatic embryos formed on petiole at 25 μM BA and 1 μM NAA; (*E*) fully grown somatic embryo at 25 μM BA, 1 μM NAA, 4 μM 2,4-dichlorophenoxyacetic acid (2,4-D), and 2 μM gibberellic acid (GA_3); and (*F*) regeneration of shoots from somatic embryos at 25 μM BA, 1 μM NAA, 2 μM GA_3, 50 μM glutamine (GTM), and 50 μM adenine sulfate (ADS).

to break dormancy and promote germination in *R. ribes* (Farzami and Ghorbanli 2011).

The induction and initiation of callusing is regulated by the relationship between cytokinin and auxin levels in the plant, with their contents being species and genotype specific (Gutiérrez *et al.* 2011). Auxins and cytokinins have been observed to show remarkable effects on growth, differentiation, and metabolism of cultured cells (Duangporn and Siripong 2009). Among auxins, 2,4-D is considered the best for initiation of callogenesis and is widely used in both monocot and eudicot callus cultures (Evans *et al.* 1981; Ho and Vasil 1983; Chee 1990; Mamun *et al.* 1996). The callus induction in most of the plant species follows classical equal cytokinin to auxin ratio (Skoog and Miller 1957). BA and KIN along with 2,4-D have been commonly used for callus induction (Chai and Sticklen 1998). In the present study, nodal and root explants responded best at 4 μM (81.7 ± 1.2) and 6 μM (79.8 ± 1.6%) 2,4-D and BA. Comparatively, in *R. moorcroftianum*, calluses were observed on MS basal medium containing 6.5 μM BA and 11.5 μM IAA (Maithani 2015). The formation and regeneration of different types of callus as a consequence of varying combination and concentration of PGRs is also observed in other species like *Coryphantha elephantidens* (Wakhlu and Bhau 2000), *Nerium odorum* (Rashmi and Trivedi 2014), and *Tussilago farfara* (Ren *et al.* 2017). Interestingly, in *R. spiciforme* embryo, TDZ was found to increase the percent response of embryo explants (67.9 ± 1.8%) along with very intense callusing at 8 μM 2,4-D and TDZ each. Indeed, TDZ, a synthetic phenylurea derivative, has shown to possess activities higher than adenine derivatives (Mok *et al.* 1982).

In different medicinal plants, like *Oroxylum indicum*, the two cytokinins BA and KIN have been used for *in vitro* regeneration and organogenesis where BA exhibited high percentage of shoot buds as compared to the KIN (Gokhale and Bansal 2009). BA is an important cytokinin and plays a key role in differentiation and formation of adventitious shoots (Fang *et al.* 2018; Tan *et al.* 2018). The efficiently metabolized phytohormone has been shown to stimulate the production of natural hormones in plant tissues leading to induction of organogenesis (Abbasi *et al.* 2013). The highest regeneration percentage observed in nodal segments with a proportionate percentage in the same explant derived brown calluses corroborates with similar results reported in *Oryza sativa* (Ray *et al.* 1996), *Arabidopsis thaliana* (Ikeda-Iwai *et al.*

Table 5 Effect of different plant growth regulators (PGRs) on shoot multiplication, mean height of shoots, and overall vigor of *Rheum spiciforme* Royle.

Treatment	PGR (μM)					Mean no. of shoots	Mean height of shoots (cm)	Base callusing	Remarks
	BA	NAA	KIN	GTM	ADS				
1	0.0	0.0	0.0	0.0	0.0	3.44±0.55^{a}	2.49±0.37^{a}	-	-
2	1.0	0.5	-	-	-	1.67±0.67^{a}	2.40±0.87^{a}	-	-
3	2.5	0.5	-	-	-	2.36±0.88^{a}	2.20±0.61^{a}	-	-
4	5.0	0.5	-	-	-	6.21±2.32^{ag}	2.47±0.58^{a}	-	Underdeveloped lamina
5	7.5	0.5	-	-	-	6.03±0.58^{ag}	2.93±1.16^{ad}	+	Underdeveloped lamina
6	10.0	0.5	-	-	-	11.98±1.73^{bf}	3.13±0.39^{ad}	-	Underdeveloped lamina
7	12.5	0.5	-	-	-	27.19±2.89^{c}	3.38±0.61^{acd}	+	Underdeveloped lamina
8	15.0	0.5	-	-	-	20.28±2.6^{de}	5.10±0.42^{bc}	-	Underdeveloped lamina
9	17.5	0.5	-	-	-	4.89±1.16^{ag}	1.89±0.23^{a}	++	Underdeveloped lamina
10	20.0	0.5	-	-	-	2.13±0.59^{a}	2.18±0.73^{a}	-	-
11	22.5	0.5	-	-	-	3.39±0.94^{a}	1.69±0.26^{a}	-	-
12	25.0	0.5	-	-	-	11.90±1.16^{bf}	2.36±0.45^{a}	-	-
13	27.5	0.5	-	-	-	7.39±2.02^{ag}	3.31±0.59^{ad}	-	-
14	30.0	0.5	-	-	-	5.90±1.16^{ag}	3.43±0.58^{acd}	-	-
15	1.0	1.0	-	-	-	1.00±0.00^{a}	2.31±0.46^{a}	-	-
16	2.5	1.0	-	-	-	1.00±0.00^{a}	2.30±0.69^{a}	-	Yellow lamina
17	5.0	1.0	-	-	-	3.99±1.01^{a}	2.64±0.39^{ad}	+	Yellow lamina
18	7.5	1.0	-	-	-	2.12±1.49^{a}	3.05±0.69^{ad}	-	Yellow lamina
19	10.0	1.0	-	-	-	12.15±1.48^{bf}	2.41±0.38^{a}	-	Yellow lamina
20	12.5	1.0	-	-	-	3.49±2.02^{a}	4.47±0.41^{bcd}	-	Yellow lamina
21	15.0	1.0	-	-	-	13.61±0.87^{bef}	3.53±0.52^{acd}	++	Yellow lamina
22	17.5	1.0	-	-	-	5.52±3.50^{ag}	4.15±0.66^{bcd}	-	Yellow lamina
23	20.0	1.0	-	-	-	2.50±1.25^{a}	2.25±0.29^{a}	-	Yellow lamina
24	22.5	1.0	-	-	-	8.99±3.46b^{fg}	2.41±0.43^{a}	+	Yellow lamina
25	25.0	1.0	-	-	-	12.75±1.46^{b}	3.08±0.58^{ad}	+	Yellow lamina
26	27.5	1.0	-	-	-	2.16±1.48^{a}	2.36±0.58^{a}	-	-
27	30.0	1.0	-	-	-	2.47±0.87^{a}	2.16±0.20^{a}	-	-
28	12.5	0.5	1.0	-	-	2.27±1.46^{a}	3.05±0.58^{ad}	-	-
29	12.5	0.5	2.5	-	-	8.67±1.20^{bg}	4.54±0.32^{bcd}	+	-
30	12.5	0.5	5.0	-	-	17.45±2.37^{de}	3.07±0.58^{ad}	-	-
31	12.5	0.5	7.5	-	-	12.80±1.17^{f}	3.66±0.18^{c}	-	-
32	12.5	0.5	10.0	-	-	11.28±0.64^{bfg}	2.35±0.35^{a}	-	-
33	12.5	0.5	12.5	-	-	4.68±1.20^{ag}	2.06±0.22^{a}	-	-
34	12.5	0.5	15.0	-	-	3.71±2.33^{a}	2.24±0.29^{a}	-	-
35	12.5	0.5	-	0.0	-	1.00±0.00^{a}	1.93±0.07^{a}	-	-
36	12.5	0.5	-	5.0	-	2.78±1.75^{a}	3.12±0.07^{ad}	+	-
37	12.5	0.5	-	15.0	-	3.35±0.33^{a}	4.47±0.29^{bcd}	-	-
38	12.5	0.5	-	25.0	-	10.53±1.32^{bfg}	2.73±0.23^{ad}	++	-
39	12.5	0.5	-	50.0	-	7.76±1.18^{g}	2.01±0.27^{a}	-	-
40	12.5	0.5		100.0		1.00±0.00^{a}	2.20±0.3^{a}	-	-
41	12.5	0.5	-	-	5.0	8.43±2.02^{bg}	2.80±0.35^{ad}	-	-
42	12.5	0.5	-	-	15.0	3.32±0.86^{a}	2.77±0.43^{ad}	-	-
43	12.5	0.5	-	-	25.0	14.89±1.16^{bef}	2.11±0.20^{a}	-	-
44	12.5	0.5	-	-	50.0	19.36±1.45^{de}	3.17±0.48^{ad}	-	-
45	12.5	0.5	-	-	100.0	1.00±0.00^{a}	2.67±0.12^{ad}	-	-
46	12.5	0.5	-	25.0	5.0	11.50±1.04^{bf}	3.50±0.29^{acd}	-	Well-developed lamina

Table 5 (continued)

Treatment	PGR (μM)					Mean no. of shoots	Mean height of shoots (cm)	Base callusing	Remarks
	BA	NAA	KIN	GTM	ADS				
47	12.5	0.5	-	25.0	15.0	7.69±0.65^{g}	2.44±0.30^{a}	+	Well-developed lamina
48	12.5	0.5	-	25.0	25.0	10.19±2.62^{bfg}	4.20±0.44^{bd}	-	Well-developed lamina
49	12.5	0.5	-	25.0	50.0	13.09±1.16^{b}	2.46±0.53^{a}	++	Well-developed lamina
50	12.5	0.5	-	25.0	100.0	1.00±0.00^{a}	1.52±0.24^{a}	-	Well-developed lamina

The data was recorded up to 6 wk. The data represents mean value ± SE (standard error) and was found statistically operative and significant by Tukey's test and LSD test at p = 0.05. The mean values with same *superscript(s)* within *columns* are not significantly different at p = 0.05 by the LSD test. The signs –, +, and ++ indicate no, slight, and moderate base callogenesis, respectively. Remarks represent the overall vigor in terms of lamina development on leaf color. Abbreviations: *BA* 6-benzylaminopurine, *NAA* naphthaleneacetic acid, *KIN* kinetin, *GTM* glutamine, and *ADS*= adenine sulfate.

2002), *Coffea arabica* (Quiroz-Figueroa *et al*. 2002), and *Triticum aestivum* (Munazir *et al*. 2010). The callus in most of the *in vitro* experiments consists of different cell types, and only some of them could be involved in organ regeneration (Feher 2019) as seen in tobacco inter-nodal explants where shoot regeneration competence was diminished in more mature or elongated cells (Gilissen *et al*. 1996). This might be the reason that out of four types of calluses formed, only brown callus was found regenerative. In studies with similar results, GA_3 has been shown to induce very positive effect on regeneration responses in otherwise very poor responsive calluses and improved induction and germination of shoot buds (Hunault and Maatar 1995; Viéitez Martín and Barciela 1990).

The process of direct somatic embryogenesis has been used in several medicinal plants such as *Gymnema sylvestre* (Kumar *et al*. 2002), *Tylophora indica* (Jayanthi and Mandal 2001), *Psoralea corylifolia* (Chand and Sahrawat 2002), *Gymnema sylvestre* (Kumar *et al*. 2002), and *Holostemma ada-kodien* (Martin 2003). Importantly, in genus *Rheum*, *R. emodi* has been shown to produce direct shoot buds from

Figure. 4 Shoot multiplication, rooting, and acclimatization of *Rheum spiciforme* Royle. (*A*) Shoot multiplication (pale yellow) at 12.5 μM 6-benzylaminopurine (BA) and 0.5 μM naphthaleneacetic acid (NAA); (*B*) multiple shoot formation at 12.5 μM BA and 0.1 μM NAA; (*C*) shoot elongation and multiplication at 12.5 μM BA, 0.5 μM NAA, and 25 μM glutamine (GTM); (*D*) shoot elongation and multiplication at 12.5 μM BA, 0.5 μM NAA, 25 μM GTM, and 25 μM adenine sulfate (ADS); (*E*) rooting at 2.5 μM NAA; and (*F*) hardening.

leaves in medium containing 10.0 mM BA and 5.0 mM IBA (Lal and Ahuja 1989). However, in this study, it was observed that higher levels of BA (25 μM) along with 1.0 μM NAA induce direct somatic embryos in *R. spiciforme*. These results were in consonance with other studies on *Fragaria* × *ananassa* (strawberry), *Polygonum rosea*, and *P. zeylanica* in the medium containing BA in combination with IBA (Liu and Sanford 1988; Das and Rout 2002). Cell clusters formed in the cultures led to the generation of somatic embryos. This clustering and isolation of cells has been shown to lead to reprogramming of genomic and cellular functions essential for the acquisition of embryogenic competence (Verdeil *et al*. 2001). Moreover, the final response following use of PGRs may be associated with two or more hormones or related additives wherein one may induce the synthesis of another (Taiz and Zeiger 1991). The low regeneration issue of the direct somatic embryos was resolved by addition of GA_3, GTM, and ADS as later two have been shown to improve the induction and regeneration of somatic embryos in other species like *P. rosea* and *P. zeylanica* (Das and Rout 2002; Ipekci and Gozukirmizi 2004). Additionally, one of the main nutrients in plant tissue culture media is nitrogen with which cytokinins interact. A positive interaction of cytokinins in the regulation of enzymes is associated with the assimilation of this element which leads to the activation of the nitrate reductase enzyme thereby promoting cell multiplication (Samuelson *et al*. 1995).

Extensive shoot multiplication is the ultimate aim of tissue culture technique. Shoots derived either from germination of seeds, organogenesis *in vitro*, or direct somatic embryogenesis need to be multiplied for either transplantation into natural conditions or to extract constituents of therapeutic significance (in case of medicinal plants). Although the contents of culture media involve various essential minerals, each plant species has its specific elemental requirements and responds differently to various additives and media formulations (George *et al*. 2008). In rhubarbs, like *R. rhaponticum*, shoot multiplication has been shown to take place on medium supplemented with different concentrations of BA and IBA (Roggemans and Claes 1979; Lal and Ahuja 1989; Lassus and Voipio 1994). BA plays a key role in the process of induction of shoots in *R. rhaponticum* and in other Polygonaceae members including *Coccoloba uvifera* (Wojtania and Gabryszewska 2000), *Rumex acetosella*, *R. acetosa* (Ćulafić *et al*. 1987), and *Polygonum aubertii* (Dabski and Kozak 1998). In the current study, 12.5 μM BA and 0.5 μM NAA in addition to 25 μM GTM and ADS were found optimal for shoot multiplication. Interestingly, our results are consistent with the concept that higher concentrations of cytokinin in combination with lower auxin concentration in the shoot induction medium favor shoot formation (Sugimoto *et al*. 2011). Moreover, KIN is also known to play a role in shoot multiplication; however, no significant improvement was found in this study in comparison to other studies on *Leptadenia reticulata* (Shekhawat *et al*. 2006). Pertinently, studies on *Sarcostemma brevistigma* and *Sapindus trifoliatus* have advocated the superiority of BA over KIN in shoot induction as well as its multiplication (Thomas and Shankar 2009; Bisht *et al*. 2012a, b) probably due to the ability of plant tissue to metabolize BA more readily as compared to other cytokinins used for shoot multiplication (Abbasi *et al*. 2013). Certainly, to improve the overall vigor, adenine supplemented in various forms, including ADS, is known to induce the proliferation of axillary shoots and promote the adventitious shoot formation in different types of explants (Van Stedan *et al*. 2008). Additionally, the effectiveness of organic nitrogen source for shoot multiplication in the form of GTM has also been reported by many authors (Green *et al*. 1990; Vasudevan *et al*. 2004). Indeed, in order to improve the process of shoot multiplication and maintain the overall vigor, ADS supplied in combination with GTM has been proven more effective (Siwach and Gill 2011).

According to Mosca *et al*. (2017) and Teardo *et al*. (2019), it is the osmotic aspect of medium which affects root formation and development, and exogenous PGRs do regulate the osmotic pressure. Also, NAA, a synthetic auxin, has been shown to play a crucial role during root formation and development (Martinez-de la Cruz *et al*. 2015; Cui *et al*. 2019). In this study, however, the success rate was recorded below 33%. There are limited reports on the acclimatization of rhubarbs' obtained from *in vitro* cultures (Lal and Ahuja 1989). The probable reasons are that rhubarb plants are very difficult to be stabilized phenotypically after *in vitro* treatments (Zhao *et al*. 2002). Moreover, according to Zhao *et al*. (2006), the application of PGRs and their different concentrations cause morphological deformations in rhubarb plants which result in their low resistance. The changes may be a result of environmental factors and an inappropriate selection of a given genotype for *in vitro* propagation (Zhao *et al*. 2005, 2007).

Conclusions and Outlook

In conclusion, owing to the restricted zone of *R. spiciforme* besides the natural and anthropogenic interventions which threaten its survival, the plant species deserve immediate and effective attention to formulate sustainable conservation measures. In this context, the present study describes an efficient and reproducible *in vitro* regeneration and multiplication protocol for the large-scale propagation of *R. spiciforme* utilizing three different pathways: organogenesis, embryogenesis, and shoot proliferation. This study showed that the processes of seed germination, callus regeneration, shoot multiplication, and induction of direct somatic embryogenesis are affected by PGRs and other additives like adenine sulphate and

glutamine. It was found that for extensive shoot multiplication and maintenance of overall vigor in this species, single axillary explants need to be cultured on medium containing 12.5 μM BA and 0.5 μM NAA for 3 wk for multiplication and then transferred on medium supplemented with 25 μM GTM and ADS each along with 12.5 μM BA and 0.5 μM NAA for 7 wk. The developed protocol with a sufficiently high success rate offers to be the starting point for development of other contemporary biotechnological avenues toward the conservation and resource management of this valued herb. In addition, this study can also be used in industrial production of biologically active secondary metabolites from *in vitro* derived tissues under controlled conditions for their commercial exploitation. In this study, although a complete *in vitro* regeneration system was established, further research is required to utilize the platform as a prelude to enhance the production of pharmaceutically important metabolites through genetic transformation techniques and bioreactor systems.

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Author contribution Conceived and designed the experiments: AS and SAP. Performed the experiments: MIK and AS. Analyzed the data: AS, MIK, and SAP. Contributed reagents/materials/analysis tools: SAP and AS. Wrote the paper: MIK, AS, and SAP.

Declarations

Conflict of interest The authors declare no competing interests.

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