Efficient in vitro plant regeneration from leaf-derived callus and genetic fidelity assessment of an endemic medicinal plant Ranunculus wallichianus Wight & Arnn by using RAPD and ISSR markers

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Abstract

Ranunculus wallichianus is a medicinally important plant and an endemic species to the Western Ghats of South India. An efficient and reliable indirect regeneration protocol system for R. wallichianus was developed from leaf explants in the present investigation. Leaf explants were cultured on both full-strength and half-strength MS (Murashige & Skoog) medium supplemented with different concentrations (0.5 mg L⁻¹ to 3.0 mg L⁻¹) of 2,4-D and NAA. Among the different concentrations tested, the highest percentage of yellowish-green compact nodular callus formation was observed on a half-strength MS medium with 2.0 mg L⁻¹ of 2,4-D. Then, the in vitro raised organogenic callus was cultured on a half-strength MS medium containing various concentrations (0.5 mg L⁻¹ to 3.0 mg L⁻¹) of BA, KIN, and TDZ with 0.5 mg L⁻¹ NAA and 10% CW for in vitro shoot regeneration. The highest percentage of regeneration response (97%) and a maximum number of shoots formation (11.1 ± 0.13 shoots/culture with 9.2 ± 0.21 cm mean shoot length) were obtained from MS medium containing 2.5 mg L⁻¹ BA with 0.5 mg L⁻¹ NAA and 10% CW. The well elongated in vitro raised shoots were effectively rooted in a half-strength MS medium with 2.5 mg L⁻¹ IBA + 250 mg L⁻¹ activated charcoal. The well-rooted plantlets were successfully hardened and acclimatized with a survival rate of 94%. Clonal fidelity of in vitro raised plantlets was assessed by using DNA-based RAPD and ISSR molecular markers. A total of 56 and 47 monomorphic bands were obtained from RAPD and ISSR markers respectively. This present in vitro propagation protocol system could be effective for the conservation of R. wallichianus with their genetic purity and its further investigations.

Key message
The present study develops a protocol for mass multiplication of Ranunculus wallichianus through indirect organogenesis and evaluates the clonal fidelity of regenerants by using RAPD and ISSR markers.

Keywords Ranunculus wallichianus · MS medium · Coconut water · Organogenic callus · Molecular markers · Monomorphic band

Abbreviations

| Abbreviation | Symbol | Description |
|--------------|--------|-------------|
| BA           | 6-Benzyladenine |
| KIN          | Kinetin |
| TDZ          | Thidiazuron |
| CW           | Coconut Water |
| 2,4-D        | 2,4-dichlorophenoxyacetic acid |
| NAA          | 1-Naphthyl Acetic Acid |
| IAA          | Indole-3-Acetic Acid |
| IBA          | Indole-3-Butyric acid |
| PGR          | Plant Growth Regulator |
| DNA          | Deoxyribonucleic acid |
| RAPD         | Random Amplified polymorphic DNA |
| ISSR         | Inter Simple Sequence Repeats |

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Ranunculus wallichianus Wight & Arn (Wallich buttercup) is an endemic medicinal plant that belongs to the Ranunculaceae family (Mathew 1996; Kumar 2004). The genus Ranunculus has diverse pharmacological actions. Being an endemic with medicinal worth, R. wallichianus has been constantly collected from the wild, and conventional propagation through seeds was unsuccessful because of the meagre level of embryo formation. Naturally, the species R. wallichianus is propagating through suckers. However, the plant cannot be reproducing their population because of their habitat destruction and seasonal variations. Hence, reliable and reproducible studies need for the conservation of R. wallichianus. In vitro multiple shoot induction using a callus is a most effective tool for the establishment and regeneration of a large number of plantlets (Hossain et al. 2003; Anu et al. 2004; Hammerschlag et al. 2006). In vitro propagation of R. wallichianus through nodal segments was achieved in our previous study (Srinivasan et al. 2021). Consequently, the present investigation was to accomplish the indirect regeneration protocol for R. wallichianus from leaf explants.

Somaclonal variations may be occurred in in vitro propagated plantlets due to various kinds of abiotic factors during the in vitro process, particularly in plant regeneration through callus mediated propagation (Breiman et al. 1987). Therefore, evaluation of genetic uniformity of in vitro raised plantlets is compulsory. At the present time, quite a lot of DNA based molecular markers for example Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Inter-Simple Sequence Repeat (ISSR), and Amplified Fragment Length Polymorphism (AFLP), have been employed to evaluate the genetic stability of a regenerants (Singh et al. 2013; Ebrahimi et al. 2018; El-Mahdy et al. 2004; Hammerschlag et al. 2006). In vitro propagation of R. wallichianus through nodal segments was achieved in our previous study (Srinivasan et al. 2021). Consequently, the present investigation was to accomplish the indirect regeneration protocol for R. wallichianus from leaf explants.

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The leaf explants were collected from the Pampar Shola of Kodaikanal, India. Explants were surface sterilized by 1 % (v/v) Teepol solution and disinfected with 5 % Bavistin for 3 min each. Then, the explants were treated with 0.1 % (w/v) HgCl₂ (Hi-Media, India) for 3 min under a laminar airflow chamber and washed with autoclaved double distilled water for 5 min to eliminate the excess of HgCl₂. The leaf explants were cultured on both full-strength and half-strength MS (Murashige and Skoog 1962) medium containing 30 g L⁻¹ sucrose and 8 g L⁻¹ agar with 0.5 mg L⁻¹ to 3.0 mg L⁻¹ 2, 4-D, and NAA. The pH of the media was adjusted to 5.7 before autoclaving. The inoculated leaves were incubated at 25 ± 2 °C with a 16 h photoperiod under white cool fluorescent tube light with 50 µEm²s⁻¹ Photon Flux Density. For multiple shoot regeneration, the 45 d old well-proliferated callus was transferred to the half-strength MS medium containing various concentrations (0.5 mg L⁻¹ to 3.0 mg L⁻¹) of BA, KIN, TDZ with 0.5 mg L⁻¹ NAA, and 10 % CW. The subcultures were made fifteen days intervals. The number of regenerated shoots and shoot length were recorded after 30 days of culture.

The in vitro raised shoots (3.0–5.0 cm long) were excised and transferred to a half-strength MS medium containing 250 mg L⁻¹ activated charcoal with different concentrations (0.5 mg L⁻¹ to 3.0 mg L⁻¹) IBA for in vitro rooting. The percentages of rooting, mean number of roots per shoot, and mean root length was recorded after 2 week of transfer onto the rooting medium. Finally, the in vitro rooted plantlets were acclimatized. The plantlets were placed in the pots containing a mixture of Sterilized Red soil, Vermiculite, and Vermicompost in the ratio of 1:1:1. The sterilized Coconut husk and dried Mosses were spread on the soil surface of pots to retain the moisture condition. The potted plantlets were irrigated with a quarter-strength of MS liquid medium for a week and maintain under a cultivation chamber at 25 ± 2 °C under white fluorescent tube light (50 µEm²s⁻¹ PFD). At end of the fourth week, the well-adapted plantlets were transferred to pots containing normal soil and maintained under greenhouse, then gradually transferred to the field condition.

The 500 mg of leaf sample from each in vitro plantlet was used for the extraction of genomic DNA by Cetyltrimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle 1987). The quality and quantity of the DNA isolated were checked using Agarose (0.8 %) gel electrophoresis stained with Ethidium Bromide (0.5 µg µL⁻¹) and visualized in a UV transilluminator. Preliminary screening was carried out with 15 sets of each RAPD and ISSR primers. PCR amplification reactions were carried out in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) using 15 µL reaction volume containing 1.5 µL of 1.2x Dream taq Buffer, 2 µL Template DNA, 1 µL Primer (10 µM), 1.5 µL of dNTPs, 0.2 µL Dream Taq Polymerase, 8.8 µL of Double Distilled Water. PCR amplification reaction was performed with an initial denaturation of DNA at 95 °C for 5 min, followed by 45 s denaturation at 94 °C, 1 min annealing at 42 °C and 90 s extension at 72 °C followed by 35 repeated cycles and the final extension was 10 min at 72 °C. The amplified PCR products were resolved by 1.2 % agarose gel electrophoresis and the size of the amplicons was estimated by using a 2-log DNA ladder (NEB Inc., USA). The DNA banding patterns were visualized in a UV transilluminator (GeNei, India) and the image was captured under UV light using a Gel documentation system (Bio-Rad, USA).
All the experiments were repeated three times with 15 replicates, and the data were analyzed with the SPSS Software package (version 17.0; SPSS INC., Chicago, IL, USA). The percentage of response, mean number of shoots, and shoot length with the standard error were calculated after 6 week, while, the mean number of roots, and root length were calculated after 4 week. The sample means were measured by using one-way ANOVA followed by Duncan’s Multiple Range Test (DMRT) at a 5% probability level (P ≤ 0.05).

Callus induction was obtained from leaf explants on half-strength MS medium fortified with 1 mg L\(^{-1}\) to 3 mg L\(^{-1}\) 2,4-D and 1.5 mg L\(^{-1}\) to 3.0 mg L\(^{-1}\) NAA. The callus initiation was observed after 7 days of culture (Fig. 1a). Of these various concentrations tested, half-strength MS medium with 2,4-D 2 mg L\(^{-1}\) produce a maximum of 92% results with yellowish green compact nodular callus after 6 week (Supplementary Table 1; Fig. 1b). Numerous studies were reported on the highest occurrence of callus induction with 2,4-D (Natarajan and Konar 1970; Meynet and Duclos 1990a; Ball et al. 1993; Beruto et al. 1996; Zheng and Konzak 1999; Morini et al. 2000; Pellegrineschi et al. 2004; Xing et al. 2010; Osman et al. 2016; Miri 2020; Wahyuni
et al. 2020). In contrast, NAA produces optimum results with 67% of results at 2.5 mg L\(^{-1}\) with friable green callus (Supplementary Table 1). Sarkar and Banerjee (2020) developed a friable callus in Solanum erianthum on medium supplemented with NAA alone. While none of the explants was responded on full-strength MS medium. A comparison efficiency of hormones indicates that 2,4-D is positively superior to NAA since the response of explants was 92% in 2,4-D (Fig. 1a). As a result, 2,4-D is ideal for the induction of yellowish green compact nodular organogenic callus from leaf explants of *R. wallichianus*.

Forty-five day-old calli were transferred to a half-strength MS medium containing various concentrations (0.5 mg L\(^{-1}\) to 3.0 mg L\(^{-1}\)) of BA, KIN, and TDZ with 0.5 mg L\(^{-1}\) NAA and 10% CW were used for in vitro shoot regeneration (Table 1). In our previous report, half-strength MS media containing 3 mg L\(^{-1}\) TDZ with 10% CW produced more shoot induction than BA and KIN from nodal segments (Srinivasan et al. 2021). However, the present study shows the highest regeneration of shoots (97%) from calli obtained in a half-strength MS medium containing 2.5 mg L\(^{-1}\) BA with 0.5 mg L\(^{-1}\) NAA and 10% CW (Table 1; Fig. 1d, e and f). The number of shoots per callus was 11.1 ± 0.13 with 9.2 ± 0.21 cm shoot length (Table 1). Half strength MS with BA and NAA was more appropriate PGR for regeneration of adventitious shoots from calli of two *Ranunculus* species such as *R. sceleratus* (Dorion et al. 1975) and *R. asiaticus* (Meynet and Duclos 1990b). Klimek-Chodacka et al. (2020) recently stated the medium combined with BA and NAA (BN medium) was more effective for the proliferation of shoots from calli in *Nigella damascena* L. Alternatively, TDZ 1.5 mg L\(^{-1}\) and KIN 3 mg L\(^{-1}\) with 0.5 mg L\(^{-1}\) NAA and 10% CW produced 76 (7.8 ± 0.22 shoots/callus) and 69 (5.3 ± 0.24 shoots/callus) percentages of shoot regenerations respectively (Table 1). The optimum average of shoot length in TDZ and KIN was 8.3 ± 0.18 cm and 7.4 ± 0.14 cm respectively. Pugliesi et al. (1992) attained the finest number of shoots from calli on MS augmented with NAA 5.4 µM and KIN 4.6 µM in *R. asiaticus*. Based on our present and previous observations, half-strength MS medium and addition of CW to the culture medium were found to be suitable for the growth of *R. wallichianus* in in vitro.

The well elongated in vitro propagated shoots (i.e. 5 cm) were rooted on a half-strength MS medium with the concentrations of 0.5–3.0 mg L\(^{-1}\) IBA and 250 mg L\(^{-1}\) activated Charcoal based on our previous results of root induction in *R. wallichianus* (Srinivasan et al. 2021). The results of root induction were expressed in Supplementary Table 2 and Fig. 2. The maximum frequency of root induction (95%) was obtained from 2.5 mg L\(^{-1}\) IBA and 250 mg L\(^{-1}\) activated Charcoal (5.1 ± 0.17 roots per shoot with 4.2 ± 0.10 cm mean root length). IBA was found to be an effective root inducing hormone in tissue culture for several plants (Ali et al. 2009; Zayova et al. 2014; Northmore et al. 2015; Chae 2016; Meynet and Duclos 1990b).

### Table 1 Effect of 10% Coconut water in combination with NAA and cytokinins on regeneration of shoots from 45 days old calli of *R. wallichianus* on half-strength MS medium after 6 week

| Growth Regulators (mg L\(^{-1}\)) | Percentage of response | Number of shoots/callus | Shoot length (cm) |
|----------------------------------|------------------------|------------------------|------------------|
| BA 0.5 – – 0.5                   | 71                     | 8.9 ± 0.17\(^{bc}\)    | 7.0 ± 0.13\(^{bc}\) |
| 1.0 – – 0.5                      | 78                     | 9.4 ± 0.12\(^{abc}\)   | 7.7 ± 0.18\(^{bcd}\) |
| 1.5 – – 0.5                      | 85                     | 10.2 ± 0.19\(^{ab}\)   | 8.2 ± 0.17\(^{bc}\) |
| 2.0 – – 0.5                      | 92                     | 10.7 ± 0.09\(^{ab}\)   | 8.6 ± 0.12\(^{ab}\) |
| 2.5 – – 0.5                      | 97                     | 11.1 ± 0.13\(^{a}\)    | 9.2 ± 0.21\(^{ab}\) |
| 3.0 – – 0.5                      | 90                     | 9.8 ± 0.21\(^{abc}\)   | 8.7 ± 0.23\(^{ab}\) |
| – 0.5 – 0.5                      | 43                     | 2.2 ± 0.14\(^{a}\)     | 6.2 ± 0.20\(^{f}\)  |
| – 1.0 – 0.5                      | 49                     | 2.7 ± 0.12\(^{hi}\)    | 6.5 ± 0.19\(^{f}\)  |
| – 1.5 – 0.5                      | 56                     | 2.5 ± 0.18\(^{hi}\)    | 6.3 ± 0.11\(^{f}\)  |
| – 2.0 – 0.5                      | 60                     | 3.7 ± 0.22\(^{hi}\)    | 6.6 ± 0.14\(^{f}\)  |
| – 2.5 – 0.5                      | 64                     | 4.4 ± 0.27\(^{hi}\)    | 7.0 ± 0.23\(^{f}\)  |
| – 3.0 – 0.5                      | 69                     | 5.3 ± 0.24\(^{hi}\)    | 7.4 ± 0.14\(^{hi}\) |
| – 0.5 0.5                        | 65                     | 6.8 ± 0.16\(^{f}\)     | 7.2 ± 0.19\(^{f}\)  |
| – 1.0 0.5                        | 73                     | 7.0 ± 0.19\(^{de}\)    | 7.5 ± 0.21\(^{f}\)  |
| – 1.5 0.5                        | 76                     | 7.8 ± 0.22\(^{de}\)    | 8.3 ± 0.18\(^{f}\)  |
| 2.0 0.5                          | 74                     | 7.1 ± 0.25\(^{de}\)    | 7.9 ± 0.11\(^{bd}\) |
| 2.5 0.5                          | 67                     | 6.7 ± 0.28\(^{f}\)     | 7.1 ± 0.17\(^{f}\)  |
| 3.0 0.5                          | 62                     | 6.0 ± 0.16\(^{fg}\)    | 6.6 ± 0.15\(^{f}\)  |

Values represent means ± Standard Error

Means followed by the same alphabets within each column are not significantly different (\(P \leq 0.05\)) according to Duncan’s Multiple Range Test.
The present result was more comparable to our prior investigation (Srinivasan et al. 2021) (Supplementary Table 2; Fig. 1 g, h & 2). The micropropagated shoots of *Thalictrum foliolosum* were rooted on ½ strength MS medium with IBA and 0.3 % activated charcoal (Mishra et al. 2020). After the successful elongation of the roots, plantlets were gradually acclimatized in the field condition. The transplantation survival rate was 94% after 60 days (Fig. 1i).

Totally fifteen RAPD (OPA-01 to OPA-10 & OPC-01 to OPC-05) and 15 ISSR (UBC-801 to UBC-815) primers were screened for the genetic fidelity assessment. Out of these primers, 9 RAPD and 8 ISSR primers produced 103 clear, unambiguous, monomorphic bands (Supplementary Table 3). The number of bands produced by RAPD primers was 56 and the banding pattern produced by a single RAPD primer ranged from 3 (OPA-07) to 13 (OPC-01) with an average of 6 bands per primer. The average sizes of the bands were ranged from 200 to 1500 bp (Supplementary Table 3) (Fig. 3a and b). The efficacy of RAPD markers in confirming the genetic stability has been well recognized by several researchers (Martins et al. 2004; Saker et al. 2006; Sharma et al. 2011; Mishra et al. 2020; Pandey et al. 2020; Sarkar and Banarjee 2020; Novikova et al. 2020; Tikendra et al. 2021). In ISSR markers, the banding patterns produced by a single ISSR primer ranged from 2 (UBC-804) to 8 (UBC-812) with an average of 5 bands per primer (Supplementary Table 3). The total number of bands produced by ISSR primers was 47 and a size range of 200–1000 bp (Supplementary Table 3). UBC-811 and UBC-812 banding profiles are expressed in Fig. 3c and d. In modern years, ISSR markers deemed as a reliable marker to assess the genetic integrity of in vitro raised plantlets for instance *Haloxylon persicum* (Kurup et al. 2018), *Eucalyptus nitens* (Ayala et al. 2019), *Albizia lebbeck* (Saeed et al. 2019), *Sapium sebiferum* (Hou et al. 2020), *Cicer arietinum* (Sadhu et al. 2020), *Pterocarpus marsupium* (Ahmad et al. 2020), *Ficus carica* (Abdolinejad et al. 2020), *Rhododendron mucronulatum* (Novikova et al. 2020) and *Dendrobium fimbriatum* (Tikendra et al. 2021). As a result, the monomorphic banding patterns produced by RAPD and ISSR markers confirm the genetic stability among the regenerants and the mother plants. This is the first report using RAPD and ISSR molecular markers for the assessment of the genetic homogeneity of tissue cultured plants in *R. wallichianus*.

In conclusion, an efficient indirect regeneration protocol from the leaf explants of *R. wallichianus* was standardized. The present proposed protocol will be effective to produce a large-scale production and conservation of *R. wallichianus*. Furthermore, the clonal fidelity studies using RAPD and ISSR markers confirmed that the mother plant and all in vitro regenerated plantlets produced by leaf explants were “true to type”.  

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**Fig. 2** Effect of half-strength Murashige and Skoog (MS) medium with various concentrations of indole-3- butyric acid (IBA) and 250 mg L⁻¹ of activated charcoal on root induction from in vitro raised shoots of *R. wallichianus*. Columns followed by same alphabets are not significantly different at *P* ≤ 0.05 according to DMRT.
Fig. 3 RAPD and ISSR profiles of mother plant and in vitro raised plantlets of *R. wallichianus*. Banding pattern attained from **a** OPA 1, **b** OPC 1, **c** UBC 811 and **d** UBC 812. Lane **L1**: 2 log DNA ladder, Lane **L2**: DNA banding profile of mother plant, Lane **L3-L7**: DNA banding profile of in vitro raised plantlets form leaf calli, L8: 2 log DNA ladder
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Author contributions PS and RT perform the study and examine the experimental data. PS prepared the manuscript. HDR gave the draw of the experimental design and complete the final version of the manuscript.

Declarations

Conflict of interest All Authors read, approved the manuscript and declare that they have no conflict of interest

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