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

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Research Article

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Abstract

Ranunculus wallichianus is a medicinally important plant and an endemic species to 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 (1.0 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 half-strength MS medium with 2.0 mg L^{-1} of 2, 4-D. Then, the *in vitro* raised organogenic callus was cultured on half strength MS medium containing various concentrations (1.0 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 maximum number of shoots formation (11.1 ± 0.13 shoots/culture with 9.2 ± 0.35 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 rooted in half strength MS medium with 2.5 mg L^{-1} IBA + 250 mg L^{-1} activated charcoal shows high frequency of root formation. The well rooted plantlets were successfully hardened and acclimatized with the survival rate of 94%. Clonal fidelity of *in vitro* raised plantlets was assessed by using DNA based RAPD and ISSR molecular markers. The total of 56 and 47 monomorphic bands were obtained from RAPD and ISSR markers respectively. This present *in vitro* propagation protocol system could be an effective for the conservation of *R. wallichianus* with their genetic purity and its further investigations.

Main Text

Ranunculus wallichianus Wight & Arnn (Wallich butter cup) is an endemic medicinal plant that belongs to the Ranunculaceae family (Mathew 1996; Kumar 2004). The genus *Ranunculu*s has diverse pharamacological 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 to conservation of *R. wallichianus*. *In vitro* multiple shoot induction via calli is a promising 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 were achieved in our previous study (Srinivasan et al. 2021). Since, no indirect organogenesis of *R. wallichianus* has been reported earlier from leaf explants.

Somaclonal variations may be occurred in *in vitro* propagated plantlets due to various kinds of biotic and 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 and Youssef 2019; Saeed et al. 2019). Among the various genetic markers, RAPD and ISSR have been predominantly ideal because of their reproducible, reliability, simplicity and cost-effectiveness (Mehrotra et al. 2012). The aim of the present investigation was to develop protocol for large-scale propagation of *R. wallichianus* through indirect organogenesis from leaf explants and to assess the genetic stability of regenerants by using RAPD and ISSR molecular markers.

The leaf explants were collected from the Pampar Shola of Kodaikanal, India and surface sterilized by 1% (w/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 laminar air flow chamber and washed with autoclaved double distilled water for 5 min to eliminate the excess of $HgCl_2$. The leaf explants were cultured on MS (Murashige and Skoog 1962) medium containing 30 g L^{-1} sucrose and 8 g L^{-1} agar with 1.0 to 3.0 mg L^{-1} 2, 4-D and NAA and the pH 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 μEm^2s^{-1} Photon Flux Density. For multiple shoot regeneration, the 45 d of well proliferated callus was transferred to the half-strength MS medium containing various concentrations (1.0 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 d of culture.

The *in vitro* raised shoots (3.0-5.0 cm long) were excised and transferred to half-strength MS medium containing 250 mg L^{-1} activated charcoal with 0.5-3.0 mg L^{-1} IBA for *in vitro* rooting. The percentage of rooting, mean number of roots per shoot and mean root length was recorded after 2 wk of transfer onto the rooting medium. Finally, the *in vitro* rooted plantlets were acclimatized. The plantlets were placed in the pots containing 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 equipped with a quarter strength of M.S. liquid medium for a wk and maintain under a cultivation chamber at 25 ± 2 °C under white fluorescent tube light (50 μEm^2s^{-1} PFD). At end of fourth week, the well adapted plantlets were transferred to pots containing normal soil and maintained under greenhouse and 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 Cetyl-Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle 1987). The quality and quantity of the DNA isolated was checked using Agarose (0.8%) gel electrophoresis stained with Ethidium Bromide (0.5 μg μL^{-1}) 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 were 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 Gel documentation system (Bio-Rad, USA).

All data were analyzed with SPSS Software package (version 17.0; SPPS INC., Chicago, IL, USA). The mean number of shoots, Shoot length, mean number roots, root length with standard error was calculated after 6 wk. The sample means were measured by using one way ANOVA followed by Duncan's Multiple Range Test (DMRT) at 5% probability level ($P \leq 0.05$).

Callus induction was obtained from leaf explants on half strength MS medium fortified with 1 to 3 mg L^{-1} 2,4-D and 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 maximum of 92 percentages result with yellowish green compact nodular callus after 6 wk. (Fig 1b). Numerous studies were reported on highest occurrence of callus induction with 2,4-D (Natarajan and Konar 1970; Meynet and Duclos 1990b; 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 produce optimum result with 67 percentages of results at 2.5 mg L^{-1} with friable green callus. Sarkar and Banerjee (2020) developed a friable callus in *Solanum erianthum* on medium supplemented with NAA alone. While none of the explants were responded on full strength MS medium. A comparison efficiency of hormone 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 an ideal for the induction of yellowish green compact nodular organogenic callus from leaf explants of *Ranunculus wallichianus*.

45 d old calli was transferred to half strength MS medium containing various concentrations (1.0 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 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, in the present study the highest regeneration of shoots (97%) from calli obtained in 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, 1e & 1f). The number of shoots per callus was 11.1 ± 0.13 with 9.2 ± 0.35 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 1990a). Klimek-Chodacka (2020) recently stated the medium combined with BA and NAA (BN medium) was more effective for 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 shoots from calli on MS augmented with NAA 5.4µM and KIN 4.6µM in *R. asiaticus*. Based on our present and previous observations half MS medium and addition of CW to culture medium was 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 half strength MS medium with 2.5 mg L^{-1} IBA + 250 mg L^{-1} activated Charcoal based on our previous results (Srinivasan et al. 2021). The maximum frequency of root induction was 95 % (5.1 ± 0.19 roots per shoot with 4.2 ± 0.21 cm mean root length) which was more comparable to our prior investigation (Srinivasan et al. 2021) (Fig 1g, 1h & 2). After the successful elongation of the roots, plantlets were gradually acclimatized in the field condition. The transplantation survival rate was 94 percentages after 60 days (Fig. 1i).

Totally fifteen sets of RAPD (OPA-01 to OPA-10 & OPC-01 to OPC-05) and ISSR (UBC-801 to UBC-815) primers were screened for the genetic fidelity assessment. 9 RAPD and 8 ISSR primers produced 103 clear, unambiguous, monomorphic bands. The number of bands produced by RAPD primers were 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 200-1500 bp (fig 3a & 3b). RAPD analysis showed no polymorphism in several plant species such as *Prunus dulcis* (Martins et al. 2004), *Phoenix dactylifera* (Saker et al. 2006), *Jatropha curcas* (Sharma et al. 2011), *Thalictrum foliolosum* (Mishra et al. 2020), *Solanum viarum* (Pandey et al. 2020), *Solanum erianthum* (Sarkar and Banarjee 2020), *Rhododendron mucronulatum* (Novikova et al. 2020) and *Dendrobium fimbriatum* (Tikendra et al. 2021). In ISSR markers, the banding patterns were ranged from 2 (UBC-804) to 8 (UBC-812) with an average of 5 bands per primer and size range of 200-1000 bp (Fig 3c & 3d). In modern years, ISSR markers deemed as a reliable marker to assess the genetic stability of 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 pattern produced by RAPD and ISSR markers confirms the genetic stability among the regenerants and the mother plants.

In conclusion, an efficient indirect regeneration protocol from the leaf explants of *R. wallichianus* was standardized. To the best of our knowledge, indirect organogenesis of *R. wallichianus* has not been achieved earlier. The present proposed protocol will be an effective to produce a large scale production and conservation of *R. wallichianus*. The clonal fidelity studies using RAPD and ISSR confirm all *in vitro* regenerated plantlets were “true to type”.

Abbreviations

BA – 6-Benzyladenine

KIN – Kinetin

TDZ – Thidiazuron

CW – Coconut Water

2,4-D – 2,4-dichlorophenoxyacetic acid

NAA - 1-Napthyl Acetic Acid

IAA – Indole-3-Acetic Acid

IBA – Indole-3-Butyric acid

DNA – Deoxyribonucleic acid

RAPD – Random Amplified polymorphic DNA

ISSR – Inter Simple Sequence Repeats

Declarations

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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 edited the final version of the manuscript.

Compliance with ethical standards

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

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Tables

Table 1. Effect of 10% Coconut water and cytokinins on regeneration of shoots from 45 d old calli of *R. wallichianus* on half-strength MS medium after 6 wk.

Growth Regulators (mg L^{-1})				Percentage of response	Number of shoots/callus	Shoot length (cm)
BA	KIN	TDZ	NAA			
0.5	-	-	0.5	71	8.9 ± 0.17^{bc}	7.0 ± 0.13^{def}
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^{a}
3.0	-	-	0.5	90	9.8 ± 0.21^{abc}	8.7 ± 0.23^{ab}
-	0.5	-	0.5	43	2.2 ± 0.14^{i}	6.2 ± 0.20^{g}
-	1.0	-	0.5	49	2.7 ± 0.12^{hi}	6.5 ± 0.19^{fg}
-	1.5	-	0.5	56	2.5 ± 0.18^{hi}	6.3 ± 0.11^{g}
-	2.0	-	0.5	60	3.7 ± 0.22^{gh}	$6.6 \pm 0.14f^{g}$
-	2.5	-	0.5	64	4.4 ± 0.27^{ghi}	7.0 ± 0.23^{def}
-	3.0	-	0.5	69	5.3 ± 0.24^{fgh}	7.4 ± 0.14^{cde}
-	-	0.5	0.5	65	6.8 ± 0.16^{def}	7.2 ± 0.19^{def}
-	-	1.0	0.5	73	7.0 ± 0.19^{cde}	7.5 ± 0.21^{cde}
-	-	1.5	0.5	76	7.8 ± 0.22^{bcd}	8.3 ± 0.18^{bc}
		2.0	0.5	74	7.1 ± 0.25^{cde}	7.9 ± 0.11^{bcd}
		2.5	0.5	67	6.7 ± 0.28^{def}	7.1 ± 0.17^{def}
		3.0	0.5	62	6.0 ± 0.16^{efg}	6.6 ± 0.15^{fg}

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.

Figures

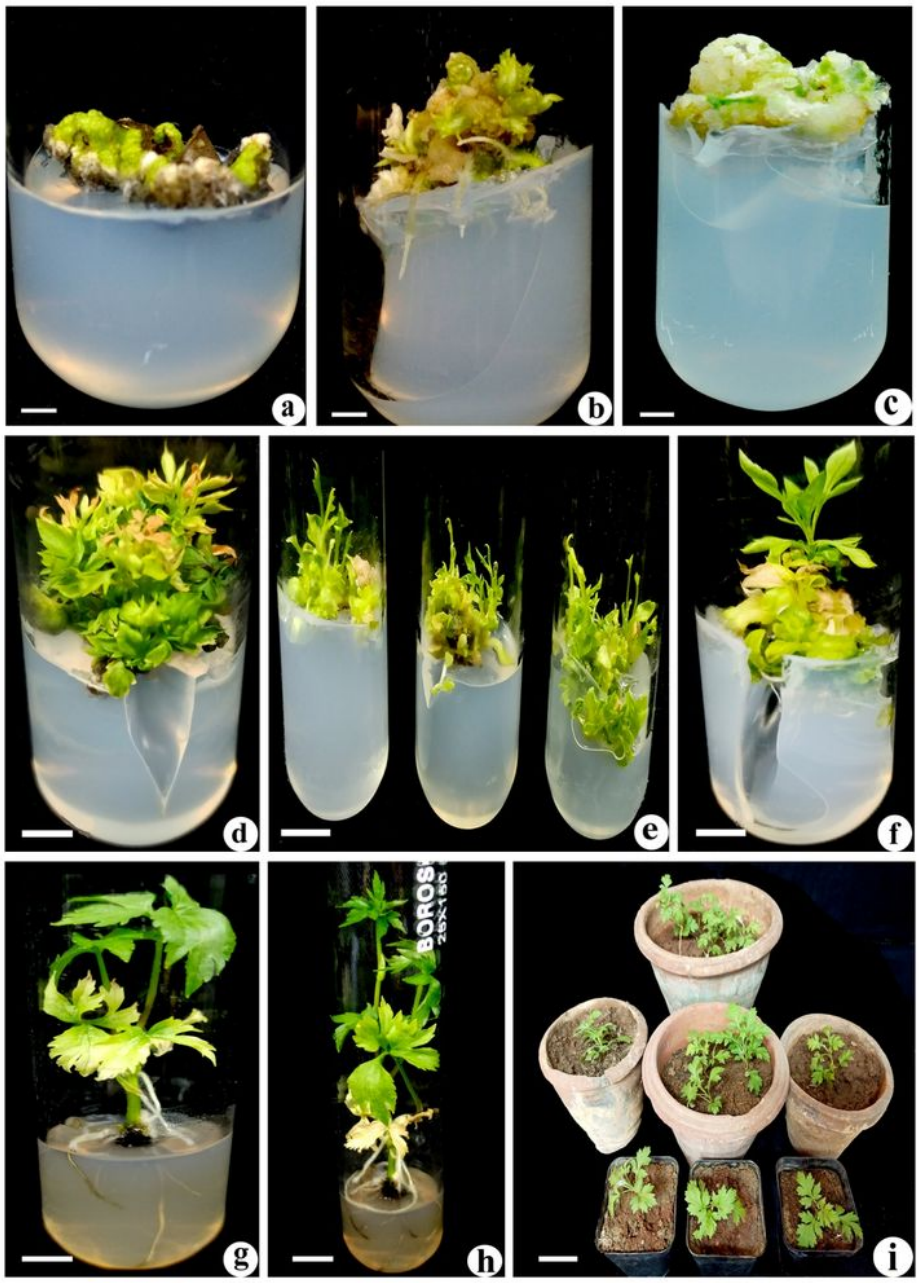

Figure 1

In vitro callus induction and shoot regeneration from leaf explants of R. wallichianus. a. Callus initiation on half-strength Murashige and skoog (MS) medium supplemented with 2,4-D 2 mg L-1 after 7 d. b. Yellowish green, compact nodular callus induction on half-strength MS medium with 2,4-D 2 mg L-1 after 6 wk. c. whitish green friable callus formation on half MS medium with 3.0 mg L-1 of NAA after 6 wk. (Scale bar-5mm) d. Shoot differentiation on half-strength MS medium containing 2.5 mg L-1 BA with 0.5

mg L-1 NAA and 10% CW after 7 d of transfer (Scale bar- 5mm).e. Microshoot formation from calli after 10 d (Scale bar-1 cm). f. Shoot elongation from calli after 20 d of culture (Scale bar-5mm). g. Root initiation half-strength Murashige and Skoog (MS) medium with 2.5mg L−1 of indole-3- butyric acid and 250 mg L−1 of activated charcoal after 10 d of transfer (Scale bar – 1 cm). h. Well elongation of roots after 3 wk (scale bar - 2 cm). i. Hardened plantlet transferred to pots for survival after 2 months (scale bar - 6 cm).

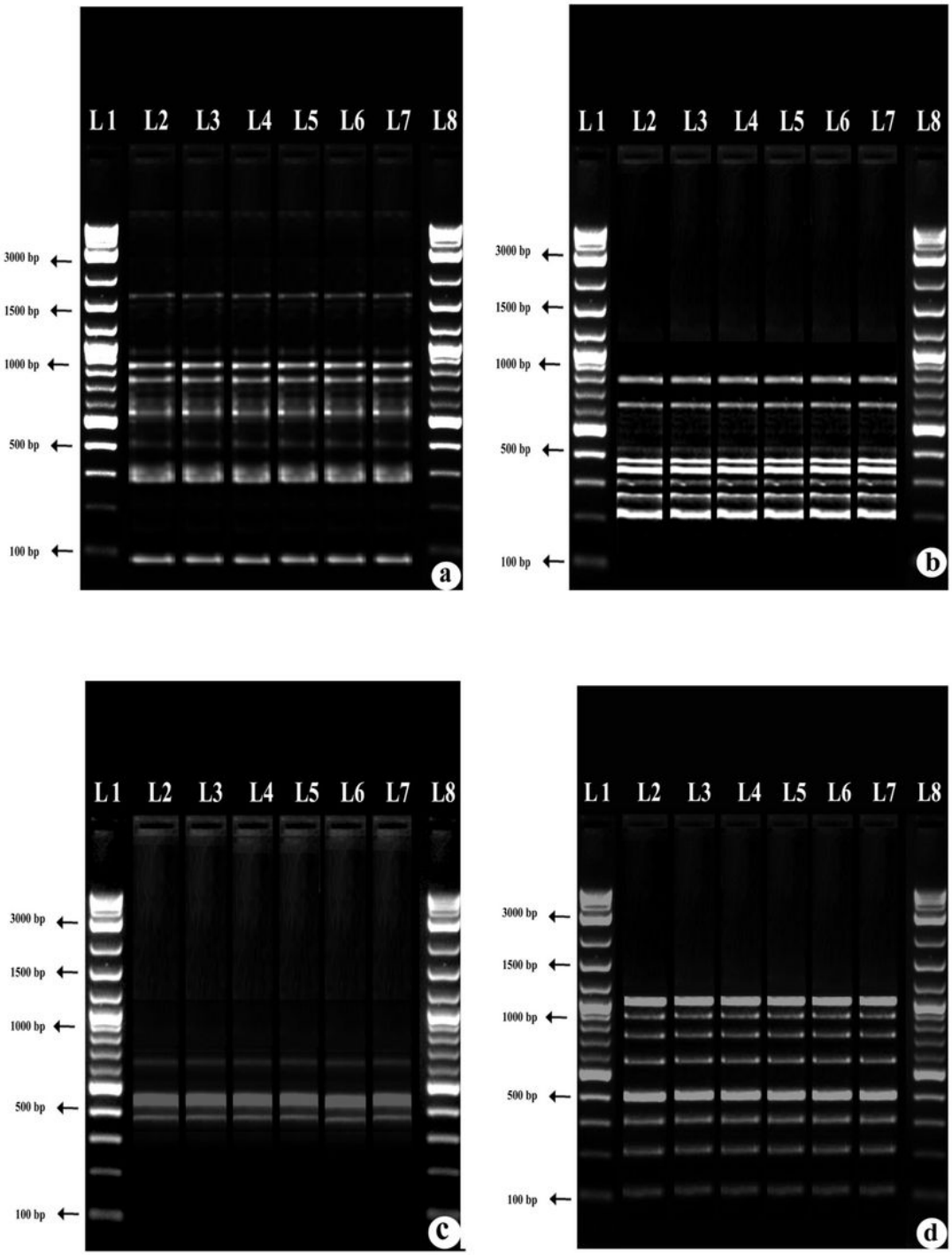

Figure 2

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.

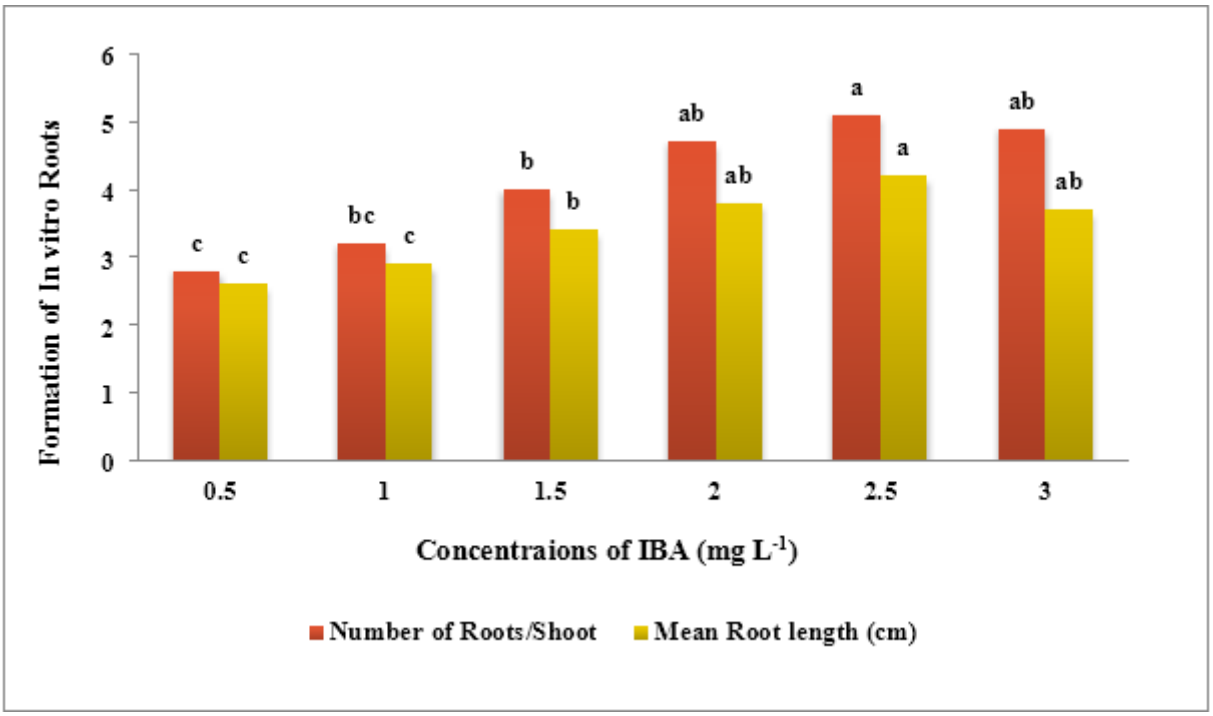

Figure 3

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.