of shoots formed, weight of biomass, number of leaves, height of shoot, etc.

2.4 Elicitations of *Rhodiola imbricata* shoot cultures

In vitro grown shoots were used for carrying out elicitations with chemical and physical elicitors under optimized culture conditios (Table 3) and the data was collected for all the required parameters for control and different elicited cultures.

2.4.1 Chemical elicitors

The in-vitro grown shoots have been cultured on MS media supplemented with growth hormones and different concentrations of chemical elicitors (Table 3). The chemical compounds have been used in mentioned concentrations and supplemented in the media as per the protocol.

2.4.2 Physical Elicitors

Electric Shocks

Finely excised shoots submerged in MS media were induced to electric shocks using a DC voltmeter at 5 mA, 10 mA, 20 mA, 30 mA, 50 mA, 75 mA, 100 mA, 125mA, 150mA, 200mA for continuous 2 minutes. The current was measured using an Amp meter. These cultures were later transferred to solid MS media in plant tissue culture chambers in conditions mentioned above.

Photosynthetic Lights

Plants transferred on MS media were kept in incubation in a incubater shaker (provided by New Brunswick) integrated with Photosynthetic Growth Lamp of 4000 lux intensity. The control for the same was shoot cultures grown in White Fluorescent Light (WFL) under optimized conditions.

Ultraviolet Light

Finely excised plantlets were exposed ultraviolet light kept at 30 cm above in a closed chamber for 10 minutes, 20 minutes, 30 minutes. These plantlets were later transferred to solid MS media and incubated at optimized conditions.

2.5 Quantification of Salidroside in *Rhodiola imbricata* tissues

2.5.1 Standard Preparation

A standard stock solution was prepared by dissolving 1 mg of Salidroside (Chromadex) in 1 ml of 80% methanol. These stock solutions were diluted twice to prepare 50 ppm standard working solutions and stored in an HPLC vial at 4 ° C.

2.5.2 Sample Preparation

Fresh tissues in vitro grown in vitro control and tested shoots and callus were taken for salidroside analysis were collected and stored at -80 ° C. Approximately 100 mg of crushed tissue from each sample was collected and filtered with 100% 1:15 methanol (w / v). The Sonicator water bath was used to incubate the mixture at 30 ° C for 15 minutes. The mixture was filtered through a 0.2 µm filter apparatus and the resulting extract was stored in HPLC vials at 4 ° C.

2.5.3 Chromatography Conditions

The analyzes were performed using a Waters HPLC system, equipped with HPLC 515 water pumps, a Waters 717 automatic sampler, a Waters 2996 photodiode array detector and the Empower software. The stationary phase used was the Waters Spherisorb reverse phase C18 column (4.6 mm x 250 mm, 5 µm). The temperature of the column oven was adjusted to 25 ° C. Various mobile phase compositions (methanol, acetonitrile and Milli Q water at pH 5.8) with different flow rates were tested to resolve the standard Salidroside mixture. The diode array detector was used to detect the salidroside (at 225 nm) while the injection volume was maintained at 10 µl. The peak area data and the salidroside retention time were recorded. These experiments were performed in triplicate and repeated three times. The concentration (mg / g) of the compounds was calculated using the formula 1.

3. Result

3.1. Development of Callus cultures and shoot cultures

Out of tested 10 media combinations (Table 4), callus growth was observed in 4 media combinations *viz.* AA2, AA3, AA4, AA8 at 15 ± 2°C and 25± 2°C. Callus growth was initially observed at 15 ± 2°C (10-30 days) as compared to 25± 2°C (20 -50 days). MS medium supplemented with TDZ (1 mg/L) was found to be the best for regeneration of callus within 10 -1 5 days at 15 ± 2°C with 81-92% of calli from old callus as well as excised leaves and shoots. Leaf explant was found to be the best for initiation of callus in 10-15 days with 91±0.67% of calli. Within 4 weeks of culture, complete callus mass was obtained from leaf explants. The callus mass was maintained by

subculturing on AA2 after 4 -5 weeks. Regeneration was initiated from calli with a green appearance. (Figure 2).

3. 2 Regeneration of shoots from callus and its multiplication

Out of tested 10 media combinations (Table 4) used to subculture growing callus cultures, callus was regenerated into shoots in 3 media combination *viz.* AA1, AA3, and AA7 at 15 ± 2°C and 25± 2°C. Shoot regeneration was observed initially at 15 ± 2°C (15-20 days) as compared to 25± 2°C (30-40 days). MS medium supplemented with BAP (1 mg/l) + IBA (2 mg/l) was found to be the best for shoot regeneration from calli within 15 - 20 days at 15 ± 2°C with 15.22±0.01 shoot number and average shoot length of 2.5±0.01 (Figure 3). Regenerated shoots of callus were transplanted in a different combination of media (Table 4). Of the 10 media combinations tested, the shoot was multiplied by 3 media combinations AA1, AA3 and AA7 at 15 ± 2 ° C and 25 ± 2 ° C. The multiplication of shoots was observed before at 15 ± 2 ° C (18-23 days) compared to 25 ± 2 ° C (25-30 days). Furthermore, at 15 ± 2 ° C, the number and duration of the outbreaks were much greater. The MS media supplemented with BAP (1 mg / L) + KN (2 mg / L) was the best for propagating shoots in 18-23 days with 5.74 shoots and an average length of 3.22 at 15 ± 2 ° C (Figure 4).

3.3 Optimization of Liquid Media for callus induction and growth and shoot regenration

Out of tested 7 media combinations (Table 5), callus was regenerated in 3 media combinations *viz.* AAL3, AAL5, AAL6 at 25 ± 2°C shaker. MS medium supplemented with BAP (1 mg/L) + IBA (2 mg/L) was found to be the best for initiation and growth of callus within 10 -1 5 days at 25 ± 2°C. Green callus was observed within 15 days without browning of medium and good yield with respect to biomass was collected that is (87±0.95 g) from initial biomass of sub cultured callus 0.43±0.03.Liquid media significantly enhanced plant biomass by 203.1 fold. The average callus biomass obtained in solid media is 16±0.43 g. Thus, the relative enhancement achieved in liquid media in comparison to solid media is 5.35 folds more. The regenerated shoots 21 in number from optimized liquid cultures would used for further multiplication and up scaling experimentation. (Figure 5).

3.4 Elicitation and Quantification

Phenotypic effects of different elicitors were noted and HPLC was performed for quantifying salideroside quantification. Different mobile phase compositions (methanol, MilliQ Water and acetonitrile of different pH values) were tested to resolve Salidroside standard mixtures. The optimum resolution could be achieved using MilliQ Water (B), and Acetonitrile (A) as mobile phase with isocratic elution: 15A/85B for Salidroside with flow rate of 10 mL min^{-1}. (Figure 6), (Table 6, 7), (Graph 1).

5. Discussion

The cultivation of medicinally important Tran-Himalayan plant, *R. imbricata* is very difficult in its natural environment. There are many problems encountered in mass propagation of this plant. The employment of labour is one of the most difficult problems due to expensive availability at high altitudes severe damage to the health of the workers working under these extreme climatic conditions. Even if feasible, the destruction caused to the vulnerable ecosystem would be so drastic that it will simply add the plant varieties to Red list.

Thus, a better way to approach this issue would be the use of biological engineering technologies, such as plant tissue culture and metabolic engineering to obtain medicinally important biological components synthesized by the human intervention. And this is the basic idea behind doing this project.

The rationale behind this study was to develop an *in vitro* system for micropropagation of *R. imbricata* and using this system for enhanced biosynthesis of salidroside using different elicitors. The best medium for callus regeneration was found to be MS medium supplemented with TDZ (1mg/L). Depending upon the endogenous concentration of growth hormones applied to different explants under same in vitro conditions, different responses were noted with respect to plant regeneration [16]. The best growth was reported in MS medium supplemented with BAP (1 mg/l) + IBA (2 mg/l), which is in accordance with results earlier reported [15]. According to the

results obtained in the elicitations, the significant growth enhancement was seen in plants of *R. imbricata* grown in photosynthetic light. The increased plant height, stem girth and multiple shooting was reported for the first time as the effect of being grown in photosynthetic light. The earlier studies find the mention of increased growth and development rate but no morphological changes [17]. For *R. imbricata*, the effect of differently coloured lights was also studies for callus cultures showing enhanced growth in red light and increased accumulation of salidroside in blue light [18]. As for electricity, there was 3 fold increase in the leaf size, which is similar to the findings of Evans Kaimoyo. The results also supported the enhancement of salidroside but not as high as reported earlier [19].

The in vitro grown shoots of *R. imbricata* gave significantly high content of salidroside as compared to salidroside content in field grown root extract. According to the study done by Sahil kapoor *et al* [20], the salidroside content present in field grown parts was 1.08mg/g whereas the amount of salidroside present in *R. imbricata* tissue cultured shoots grown in MS media supplemented with BAP (1 mg/l) + IBA (2 mg/l) was 3.6 mg/g, which is nearly 3.4 folds higher. This finding as it is solves the basic purpose of our study.

In addition to this, the elicited plants also showed enhanced salidroside amount where callus elicited with electric shock gave a positive response. The amount achieved was greater than control callus grown in optimized MS media. But the results achieved were not as impressive as reported by Evans Kaimoyo *et al* in *Pisum sativum* [19]. When the salidroside content of callus cultures grown in Liquid MS media supplemented with similar growth hormones as in optimized solid MS media, slight enhancement was observed. Despite the same media composition, the increased metabolite concentration holds a strong ground for up scaling the research. The similar findings were also reported in suspension cultures of *Rhodiola sachalinensis* by Jay Xu [21]. UV also proved to be an efficient elicitor in enhancing the concentration of salidroside which is in accordance with the research done by Y. K. Bernard [22]**.** In addition to this, the liquid culture optimization to enrich the biomass of the plant was successfully done with 5.35 fold increase in the callus in comparison to callus grown on solid media. The similar findings were also reported by D. Popli *et al.* (2016) [23].

So, the present findings provide platform to upscale the study to bioreactor level. Thus help to achieve desirable plant growth and marker production can be carried out at commercial scale in order to meet the demands of pharmaceutical industries.

6. Conclusion

Through this experiment, we have optimized the liquid medium for improving the growth and development of callus. It has enhanced by 5.35 folds within 20-25 days as compared to controls grown in solid media.

Moreover, we have used 9 different elicitors for enhancing the concentration of marker compound that is salidroside under in vitro conditions but photosynthetic light and UV gave some significant elicitation along with good growth and development, whereas some positive morphological changes such as elongated shoot with thick girth were observed in cultures grown in electric shock as an elicitor.

Hence, the current study possesses the robust potential in large scale propagation of this plant and its secondary metabolite production. It also provides a platform for up scaling this research to bioreactor level so that desirable plant growth and marker compound production can be carried out at commercial scale.

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enriching biomass of *Dictylorhiza hatagirea*," *International Journal of Tropical Agriculture*, vol. 1, pp. 1271-1276, 2016.

Figures

Figure 1 Plantlets of *R. imbricata* grown and acclimatized in the green house of the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, India.

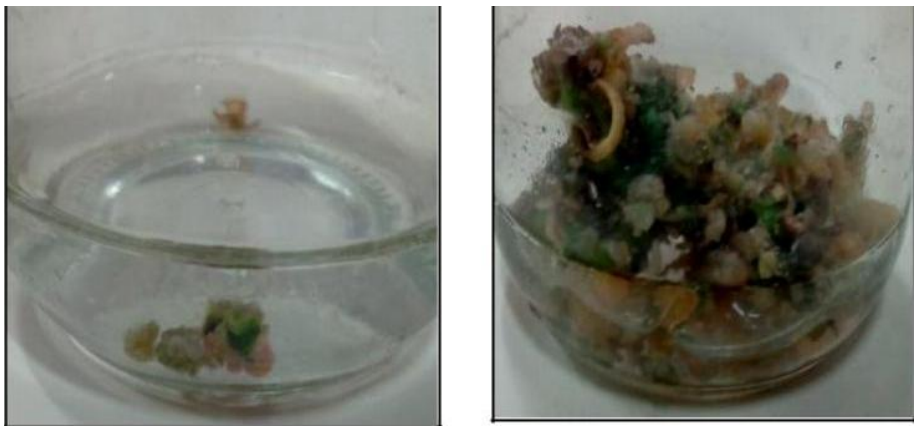
Figure 2: *R.imbricata* callus grown in MS media + TDZ (1 mg/L)

(a) **(b)** **(c)**

Figure 3: Regeneration of shoots from callus of *R.imbricata* (a) After 7 days (b) After 15 days (c) After 30 days of incubation in 15 ± 2°C plant tissue culture chambers.

(a) **(b)** **(c)**

Figure 4: (a) Initiation of multiple shooting in *R.imbricata* (b) Multiple shooting in *R.imbricata* after 7 days (c) Multiple shooting after 30 days of incubation in 15 ± 2°C plant tissue culture chambers.



(a) **(b)**

Figure 5: (a) Callus Growth in Liquid media after 5 days (b) Callus Growth in Liquid media after 3 weeks in plant *R. imbricata*

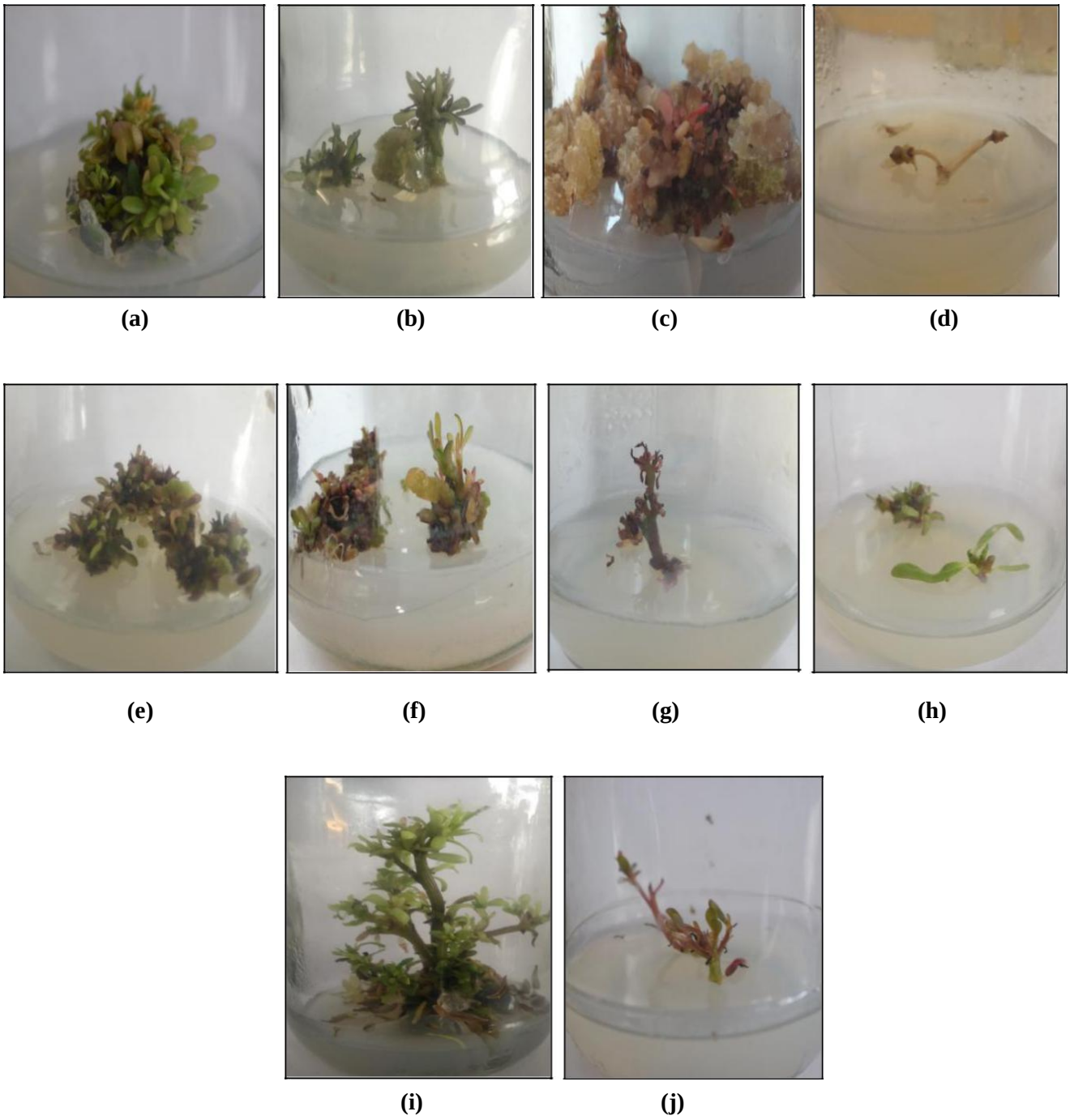


Figure 6: In- Vitro grown shoots of *R.imbricata* under different elicitors (a) Control (b) Cinnamic acid (c) Chitin (d) L-Phenylalanine (e) Pectin (f) Methyl Jasmonate (g) Yeast Extract (h) Electric Shocks (i) Photosynthetic Light (j) Ultraviolet Rays

Tables

Table 1: MS media supplemented used for callus induction and shoot regeneration in *Rhodiola imbricata*.

S.No.	Medium Name	MS media composition
1.	AA0	MS
2.	AA1	MS + BAP (2 mg/L) + KN (2 mg/L)
3.	AA2	MS + TDZ (1 mg/L)
4.	AA3	MS + BAP (1 mg/L) + IBA (2 mg/L)
5.	AA4	MS + IBA (1 mg/L) + 2,4-D (1.5 mg/L)
6.	AA5	MS + BAP (0.5 mg/L) + IBA (2 mg/L) + GA3 (2 mg/L)
7.	AA6	MS + IBA (4 mg/L)
8.	AA7	MS + BAP (1 mg/L) + KN (2 mg/L)
9.	AA8	MS + KN (2 mg/L) + IBA (1 mg/L)
10.	AA9	MS + BAP (2 mg/L) + GA3 (2 mg/L)

Table 2: Different liquid media composition for callus induction and regeneration in *R.imbricata* tissue samples.

S.No.	Medium Name	MS media composition
1.	AAL0	MS
2.	AAL1	MS + BAP (1 mg/L) + KN (1 mg/L) + TDZ (0.5mg/L)
3.	AAL2	MS + TDZ (1 mg/L)
4.	AAL3	MS + BAP (1 mg/L) + IBA (2 mg/L)
5.	AAL4	MS + IBA (1 mg/L) + 2,4-D (1.5 mg/L)
6.	AAL5	MS + BAP (0.5 mg/L) + IBA (2 mg/L)
7.	AAL6	MS + IBA (0.5 mg/L) + IBA (2 mg/L) + KN (2mg/L)

Table 3 : Chemical and Physical components used as elicitors along with their tested concentrations/ranges in *R.imbricata*.

S.No	Elicitor (Physical)	Tested factors
1	Cinnamic Acid	1.0 mM/L, 1.5 mM/L and 2 mM/L
2	Chitin	0.5 g/L, 1.5 g/L and 2.0 g/L
3	L- Phenylalanine	0.5 mM/L, 1 mM/L and 2 mM/L
4	Pectin	0.5 g/L, 1.5 g/L and 2.0 g/L
5	Methyl Jasmonate	0.25 mM/L, 0.5 mM/L and 1 mM/L
6	Yeast Extract	0.5 g/L, 1.0 g/L and 2.0 g/L
7	Electric Shocks	50 mA - 200mA for 2 minutes
8	Photosynthetic Light	4000 Lux Intensity
9	Ultrasound	20,000 Hz for 1 min, 1.5 min, 2 min
10	Ultraviolet Rays	10 minutes, 20 minutes, 30 minutes

Table 4: Effect of MS media supplemented with different growth hormones on callus induction and growth followed by shoot regenration in *R. imbricata*.

S.No.	Medium Name	Callus Growth (Days)	Callus Colour	Percent Survival	No. of Days For Regeneration	No. of Shoots
1.	AA0	30 – 35	Pale Green	30±0.34 %	--	--
2.	AA1	--	--	--	23 – 28	4±0.5
3.	**AA2**	**7 - 15**	**Dark Green**	**91±0.67 %**	--	--
4.	AA3	20 - 25	Pale Green	75±0.4 %	**25 - 30**	**8±0.75**
5.	AA4	18 - 25	Creamy	45±0.5 %	--	--
6.	AA5	--	--	--	--	--
7.	AA6	--	--	--	--	--
8.	AA7	--	--	--	18 - 25	3±0.5
9.	AA8	21 - 30	Yellowish Green	65±0.5 %	20 - 25	4±0.75
10.	AA9	--	--	--	--	--

Table 5: Liquid Media optimized with respect to days of regeneration and Biomass yield of *R.imbricata*

S.No.	Medium Name	Days For Regeneration	Biomass (g)
1.	AAL0	28 - 40	8±0.93
2.	AAL1	27 - 35	19±0.67
3.	AAL2	--	--
4.	**AAL3**	**20 – 25**	**87±0.95**
5.	AAL4	--	--
6.	AAL5	23 - 30	39±0.38
7.	AAL6	25 - 31	21±0.76

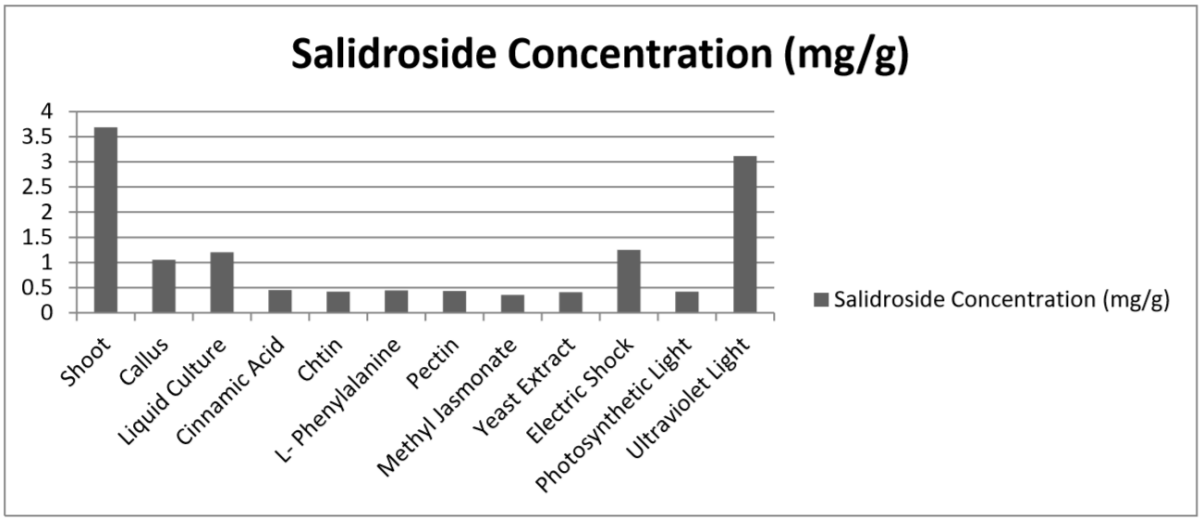
Table 6: Effects of chemical elicitors on in - vitro grown shoots of *R.imbricata* and salidroside quantified.

S.No	Elicitor	Conc. Tested	Conc. Optimiz Ed	No. of Shoots	Avg. Shoot Size (cm)	No. of Leaves	Average leaf size (cm)	Biomass (g)	Leaf Color	Part Used for extraction	Salidroside Content (mg/g)
1	Cinnamic acid	1.0 mM/L, 1.5 mM/L and 2 mM/L	1.5mM/ L	3±0.67	3±0.81	20±0.83	1±0.22	7±0.58	Dark Green	Shoot + Callus	0.454 ± 0.03
2	Chitin	0.5 g/L, 1.5 g/L and 2.0 g/L	2.0 g/L	1±0.67	0.7±0.0 5	5±0.16	0.5±0.032	5±0.49	Red, Green	Shoot + Callus	0.424 ± 0.05
3	L-Phenylalanine	0.5 mM/L, 1 mM/L and 2 mM/L	1.0 mM/L	1±0.16	0.3±0.0 6	3±0.83	1±0.033	0.7±0.05	Pale green	Shoot	0.444 ± 0.06
4	Pectin	0.5 g/L, 1.5 g/L and 2.0 g/L	2.0 g/L	2±0.34	0.8±0.0 5	11±0.5	0.9±0.062	6±0.67	White, red	Shoot + Callus	0.433 ± 0.02
5	Methyl Jasmonate	0.25 mM/L, 0.5 mM/L and 1 mM/L	0.5 mM/L	1±0.5	0.5±0.0 06	12±0.16	1±0.33	5±0.59	Green, red, brown	Shoot + Callus	0.355 ± 0.03
6	Yeast Extract	0.5 g/L, 1.0 g/L and 2.0 g/L	2.0 g/L	1±0.16	3±0.05	8±0.5	1±0.033	5±0.244	Red, brown	Shoot	0.408 ± 0.04
7	Electric Shocks	50 mA - 200mA for 2 minutes	150 mA for 2 Minute S	2±0.16	1±0.03	4±0.85	3±0.053	5±0.96	Green	Callus	1.249 ± 0.09
8	Photosynthet ic Light	4000 Lux Intensity	--	8±0.33	6±0.63	31±0.8	1±0.28	10±0.96	Green	Shoot + Callus	0.420 ± 0.05
9	Ultraviolet Rays	10 minutes, 20 minutes, 30 minutes	30 Minute S	4±0.16	2±0.83	3±0.83	0.9±0.063	4±0.13	Green, Red	Shoot	3.117 ± 0.08

Table 7: Concentration of Salidroside from in-vitro plants of *Rhodiola imbricata*

S.No	Normal	No. of Shoots	Avg. Shoot size (cm)	No. of Leaves	Average leaf size (cm)	Biomass (g)	Leaf Color	Part Used for extraction	Salidroside Content (mg/g)
1	Shoot	8±0.75	5±0.65	22±0.83	2±0.52	6±0.58	Dark Green	Shoot	3.687 ± 0.07
2	Callus	--	--	--	--	--	Green	Callus	1.054 ± 0.04
3	Liquid Culture	--	--	--	--	--	Dark Green	Callus	1.202 ± 0.05

Graphs



Graph 1: Comparative graphical representation of quantified salidroside amount from different tissues of *R. imbricata.*

Formula

Formula 1

PA of sample/PA of standard) x (1/IV) x (Volume of sample/weight of sample) x dilution factor

Where, PA – Peak Area

IV – Injection Volume