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Micropropogation of *Alpinia purpurata* using low cost media for quantification of rutin

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ABSTRACT

Rutin is the natural flavonoid with antihyperglycemic, antihypertensive, and antioxidative properties. To study in vitro production of rutin, we established plant tissue culture techniques for micropropogation of Alpinia purpurata. The specific objectives of this study were to test some of the simple methods and cheaper alternatives for micropropagation of Alpinia purpurata for determination of rutin. The accumulation of flavonoids was evaluated in organogenic cultures of Alpinia purpurata using High Performance Thin layer Chromatography (HPTLC). Alpinia purpurata were cultured on Murashige and Skoog, 1962 (MS) agar medium supplemented with low cost alternatives like coconut water, marketed sugar, commercial corn flour as alternative gelling agents, benzylaminopurine (BA), Kinetin, NAA (Napthalene-3-acetic acid), IAA (Indole-3-acetic acid) and 2, 4-D (2, 4-Dichlorophenoxyacetic acid) at the different concentration range for callus, shoots and root initiation. With a combination of low cost additives in MS media the best result of Alpinia purpurata was found in 2, 4-D (2 ppm) and kinetin (2ppm) for callus initiation, maximum number (9-11) of shoots were observed in medium with NAA (0.1ppm) and BA (3.0 ppm). Roots initiation was found in IAA at the concentration of 3ppm. The results of HPTLC methods revealed that rutin content in the leaves extract of Alpinia purpurata was more in low cost tissue culture grown plant micropropogated with 20% coconut water, 3% marketed sugar and 100 gm/l gelling agent than naturally grown plants. It indicate that low cost media in tissue cultures of Alpinia purpurata could be a valuable alternative approach for rutin production

Keywords: BA: 6-benzyl amino purine, IAA: Indole acetic acid; NAA: Naphthalene acetic acid, MS: Murashige and Skoog, 2, 4-D: 2, 4-dichlorophenoxy acetic acid.

INTRODUCTION

Tissue culture techniques are widely used to grow many different plants for commercial and research purposes. Presently the secondary metabolites were obtained by conventional method of cultivation, collection, extraction and isolation, which has its limitations like unpredicted environmental conditions, time consuming, desired quality, and a gap between the demand and supply. Therefore micropropagation through plant tissue culture can be an attractive alternative method. By suitable manipulation of hormones and contents of the medium, it is possible to initiate the developments of roots, shoots and complete plants from callus cultures. [1]

It has been estimated that a 50% reduction in cost of micropropagated plants would increase the market demand by 10 times. [2] Use of alternative and where possible locally available equipment and resources to reduce the unit cost of tissue culture products without compromising the quality of the plants *viz* using natural daylight to promote photoautotrophic growth. [3] It is recommended that table sugar be considered as low-cost substitute for potato

micro-propagation. [4] Factors like infrastructure, equipments, energy and consumables affect the cost and it will be replaced by low cost alternatives, so the aim of our experiments was to lower the price of micropropagated plantlets by carrying out low cost alternatives in different stages of growth of *Alpinia purpurata* and determine the concentration of rutin at different stages of growth.

Rutin is a naturally occurring bioflavonoid having broad range of physiological activities. It has been found to occur in large quantities in some plants. Phytochemical studies on *Alpinia purpurata* revealed that it possess flavonoids, rutin, kaempferol-3-rutinoside and kaempferol -3-oliucronide. [5] In the last two decades, high performance thin layer chromatography (HPTLC) has emerged as an efficient tool for the phytochemical evaluvation of herbal drugs. [6-7] Therefore, an attempt was made to compare the rutin content in the low cost tissue culture extract of *Alpinia purpurata* with conventional extracts using HPTLC. Hence a simple, specific, and sensitive HPTLC method was developed and validated according to the ICH guidelines.

MATERIALS AND METHODS

The procedure for *in vitro* culture of *Alpinia purpurata* for production of rutin and kaempferol-3-O-glucuronide by tissue cultures of *Alpinia purpurata* (Vieill) K. Schum were standardized earlier. [8] Modifications in the conventional *in vitro* culture technique were adopted in this study as described below.

2.1 Plant material

Plant material of *Alpinia purpurata* (Vieillard) K. Schumann was collected from the Jawaharlal Nehru Tropical Botanic & Research Institute, Palode, Thiruvananthapuram. The Director, Jawaharlal Nehru Tropical Botanic & Research Institute, authenticated the samples.

2.2 Surface sterilization of explants

Rhizome buds of about 1-2 cm were cleaned and washed in tap water to which a few drops of a laboratory detergent was added, followed by 3-4 washes with water. Under sterile conditions they were transferred to an autoclaved bottle and treated with 0.1% HgCI2 (w/v) for 10 minutes followed by 3-4 washes with sterile water.

2.3 Culture media preparation

All culture media were based on the Murashige and Skoog's (1962) (MS) medium. Other additives like marketed sugar, coconut water, gelling agent and plant growth regulators were added as required for the experiments. The pH of the media was adjusted using 1 N or 0.1 N HC1 or NaOH.

2.4 Incubation of cultures

The cultures were maintained in culture room under a regime of 16 hr photoperiod (intensity 40µE cm2 /min/sec) at 25 ± 1^0C.

2.5 Callus initiation

For callus initiation the rhizome bud of *Alpinia purpurata* was transferred in solid basal MS media containing different combination of growth hormones and other low cost additives.

2.6 Shoot multiplication

The healthy callus was transferred in different low cost culture media to establish shoot cultures. Media was adjusted to pH 5.7 before autoclaving. After 42 days, the shoots were measured for growth and subsequently subculture. For each treatment 20 replicates were used and each experiment was repeated at least three times.

2.7 Root induction

Isolated shoots derived from media containing 3 ppm of (NAA) and 0.1 ppm of (NAA) was then placed on rooting medium, M_1 and M_2. Media M_1 and M_2 contain different concentration of IAA.

2.8 HPTLC fingerprinting analysis

Standard solution of rutin and *in vitro* grown solution of *Alpinia purpurata* from different stages were loaded as 5mm band length in the 10 x 10 Silica gel TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour) with respective mobile phases and the plate was developed in the respective mobile phase up to 90mm. The

developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in Photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at White light, UV 254nm and UV366nm. The developed plate was sprayed with respective spray reagent and dried at 100º C in Hot air oven.

RESULTS

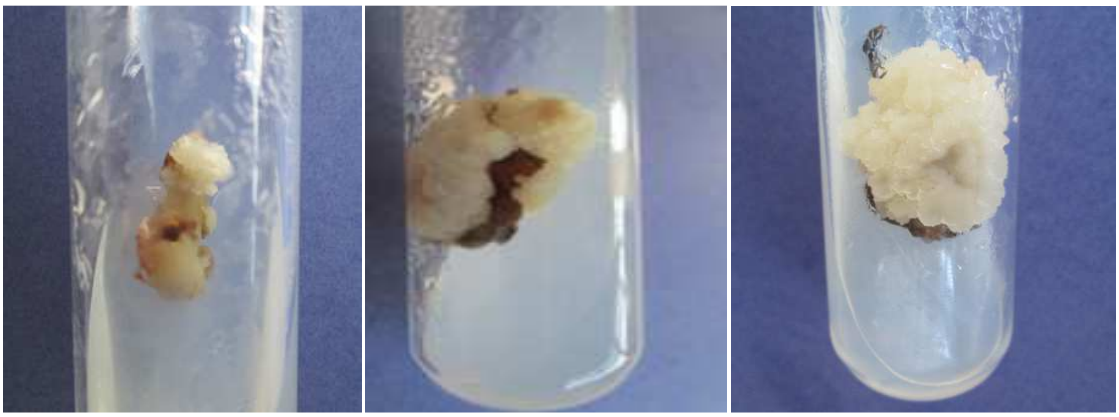
3.1 Callus initiation

Different media composition was tried for callus initiation from rhizomes explant of *Alpinia purpurata* on MS medium supplemented with growth hormones and other low cost additives. The growth of callus was observed in each medium and the maximum growth pattern was observed in MS medium supplemented with 2, 4-D (2ppm), kinetin (2ppm) and 15% coconut water as compare to other media. The results were shown in Table 3.1. These compositions were given healthy, green, friable callus within a period of 28 days. The growth of callus was observed as fresh weight (FW) and dry weight (DW) measured every week. Both fresh weight (FW) and dry weight (DW) increased over the period of incubation.

Table 3.1 Effect of phytohormones on callus growth of *Alpinia purpurata*

Media	Coconut water (%)	Hormones	Conc.(ppm)	FW (mg)	DW (mg)
MS	15	NAA + BAP +kinetin	1:2:1	1540	120
MS	15	NAA + BAP	2:2	240	15
MS	15	NAA + BAP + kinetin	1:2:2	1625	80
MS	15	BA + kinetin	2:2	550	20
MS	15	NAA + BA	1:2	650	22
MS	15	2,4-D +kinetin	2:2	2260	540
MS	15	2,4-D +BAP	1:2	840	45
MS	15	NAA + BAP+NAA	2:2:2	75	10

Fig 3.1 Different Stages of Callus initiation in *Alpinia purpurata*



3.2 Initiation of shoots

For the initiation of shoots, two week old callus obtained in MS medium supplemented with 2, 4-D (2ppm), Kn (2ppm) and 10% coconut water was transferred in various shooting medium. Shoot initiation was observed after one month of incubation. Maximum number (9-11) of shoots was observed in medium with NAA (0.1ppm) combination with BA (3.0ppm) after incubation.

Table 3.2 Shoot proliferation of *Alpinia purpurata*

Sr. No.	Hormones	Conc. (ppm)	Observation
1	NAA+BA	0.1+1	Cell mass was less, less granular callus, no rooting formation.
2	NAA+BA	0.1+2	After sub culturing the callus in same media 2-3 small shoots arises from some test tubes, shorter, thicker shoots about 0.5 mm-2 cm heights.
3	NAA+BA	0.1+3	Superficial callus granular, core compact, more callus, rooting was observed with some test tube.

Fig 3.2 Shoot proliferation of *Alpinia purpurata*.

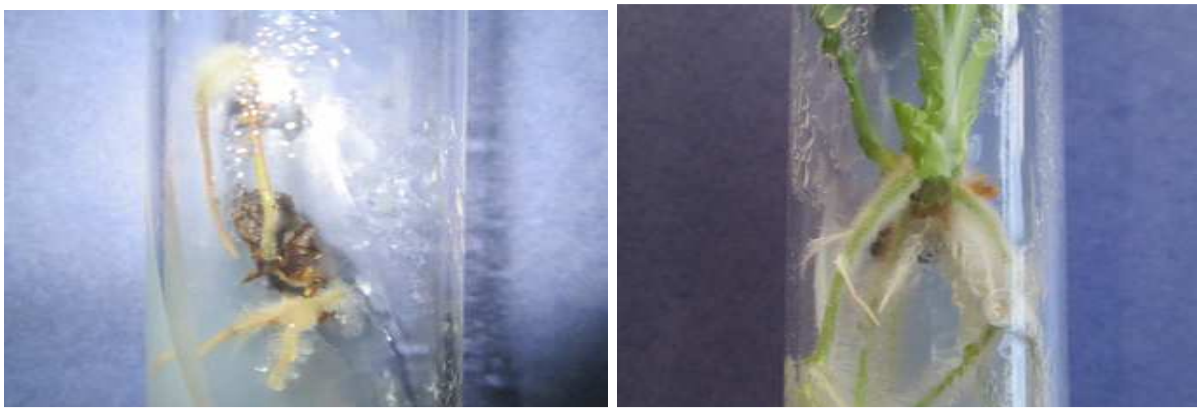
3.3 Initiation of roots

The effect of IAA on root induction from *Alpinia purpurata* shoots using MS rooting media (M_1 and M_2) is presented in Table 3.3. The use of IAA (M_1 medium) greatly improved rooting on all shoots with maximum rooting percentage (89.32%) found in MS media containing 3 ppm of Indole acetic acid (IAA). At higher concentration of IAA (8 ppm and 10 ppm) the percentage of rooting decreased slightly due to negative effect.

Table 3.3 Root formation of *Alpinia purpurata*

S. No.	Medium	IAA (PPM)	% rooting	
			After 15 days	After 30 days
1	MS (M_1)	1	21	43
2	MS (M_1)	2	13.48	76.19
3	MS (M_1)	3	32.34	89.32
4	MS (M_1)	4	23.8	71.42
5	½ MS (M_2)	1	----	18.18
6	½ MS (M_2)	2	17.64	34.20
7	½ MS (M2)	3	32.21	45.30
8	½ MS (M2)	4	21.30	44.57

Fig 3.3 Root initiation of *Alpinia purpurata*



3.4 HPTLC Method development and validation for rutin

Out of number of solvent systems tried, the one containing tolune-acetone-formic acid (4.5:4.5:1 v/v) gave the best resolution of rutin with the retention factor (Rf) of 0.17 from the sample extract. Linear regression revealed good relationship between the concentration of standard solutions and the peak response within the concentration range of 100 to 2000 ng/spot with a correlation coefficient (r^2) of 0.999 (y=2.544x + 676.4) for rutin. The LOD and LOQ were obtained with the signal-to-noise ratio of 3.3 and 10. The LOD and LOQ were found to be 28.08 and 85.10 ng/spot for rutin, this indicated that the new method exhibited a good sensitivity for the quantitation of rutin. The precision and the repeatability at three different concentration levels reflect the robustness of the method. The standard deviation of peak areas was calculated for each condition and percentage Relative Standard Deviations (% RSD) was found to be less than 2%. These low values of % RSD was indicative of the robustness of the method. After the addition of standard rutin to same amount of the sample solution at three different concentration levels, the percentage recovery of rutin was found to be 100.79%, 99.90% and 98.66% with an average of 99.78%.

Fig 3.4.1 Linearity of rutin

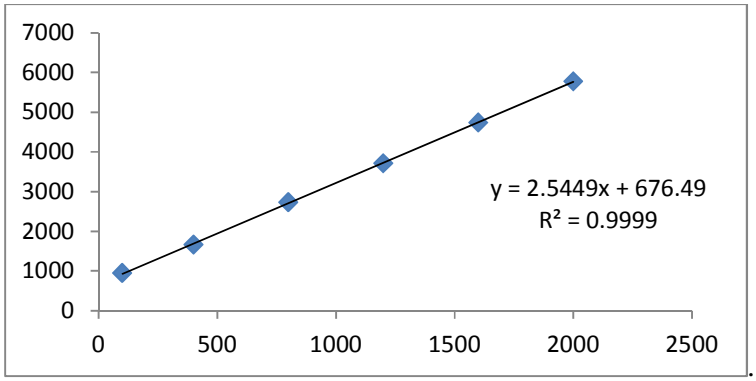


Table 3.4.1 Intraday and interday precision of rutin

Standard drug	Nominal concentration [a]	concentration obtained [a]		Precision obtained [a]	
		Intra day	Inter day	Intra day	Inter day
Rutin	400	391.35	399.34	0.92	0.86
	800	799.24	814.57	0.94	0.82
	1200	1207.26	1216.30	0.57	0.77

Table 3.4.2 Robustness for rutin

Parameters	Rutin	
	SD of concentration found	% RSD
Mobile phase (Ethyal Acetate) composition (± 0.1 mL)	11.53	0.42
Amount of mobile phase (± 5 %)	20.50	0.75
Time from band application to chromatography (+ 10 min)	25.63	0.94
Time from chromatography to scanning (+ 15 min)	18.52	0.67

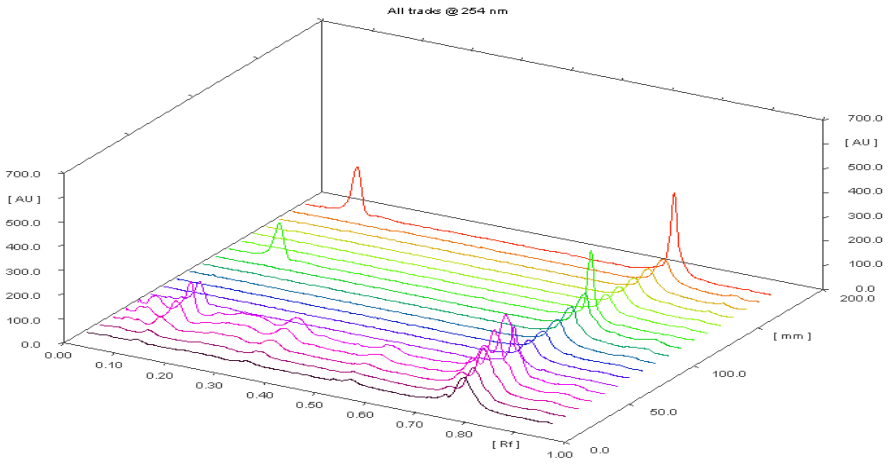
3.4.3 Accuracy study for rutin

Amount Taken	Amount added	Amount found		% Recovery ± % R.S.D.	
Rutin	Rutin	Rutin	SD	Rutin	RSD
300	240	544.26	4.141373331	100.79	0.760916781
300	300	599.42	3.545007667	99.90	0.59140287
300	360	651.18	8.45811116	98.66	1.298891391

Table 3.4.4 Quantification of rutin from normal grown plant of *Alpinia purpurata*

Normal grown plant part of *Alpinia purpurata*	Extract	Content of rutin (%w/w)
Leaves	Hexane	4.32
	Ethyl acetate	3.46
	Methanolic	7.32

Fig 3.4.2 HPTLC profile of rutin and quercetin in hexane, ethyl acetate and methanolic extracts of *Alpinia purpurata*



3.5 Quantification of rutin in natural grown and tissue culture medium

The rutin content increases with the percentage of coconut water in both conventional and modified MS medium. Conventional MS medium showed less accumulation of rutin while at modified MS medium with 15% v/v percentage of coconut water, tap water in place of distilled water, 3 % marketed sugar in place of sucrose and 100 gm/l commercial corn flour in place of agar showed best results for rutin.

Table 3.5.1 Quantification of rutin in the leaves of natural grown plant of *Alpinia purpurata*

Extract used	Rutin (%W/W)
Leaves	14.93±0.36

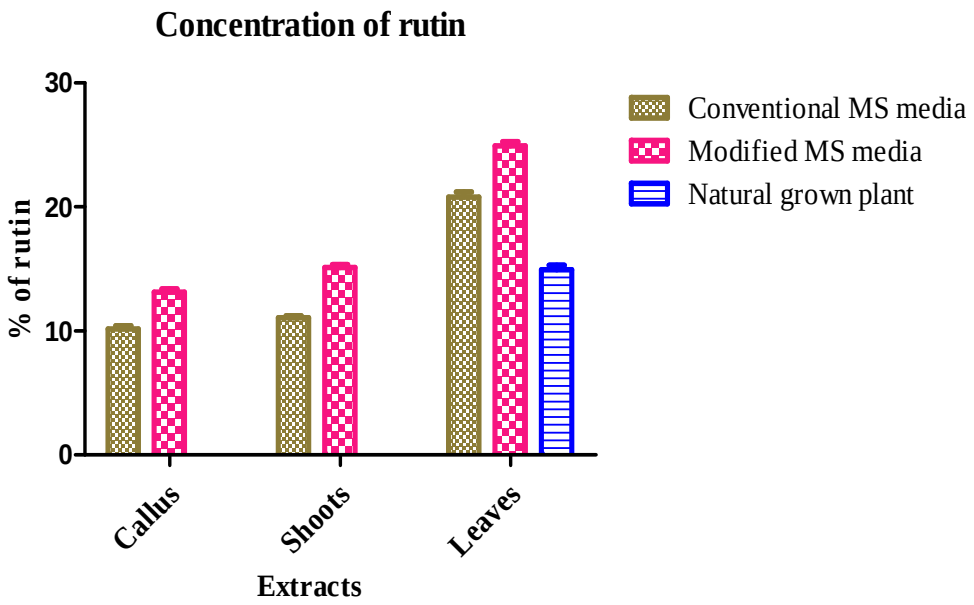
Results are mean ±SEM of three parallel measurements (n=3)

Table 3.5.2 Quantification of rutin in conventional MS medium and modified medium

Extract used	Conventional MS medium (distilled water + sucrose + Agar)	Modified MS medium (Tap water + marketed sugar + corn flour + coconut water)
	Rutin (%W/W)	Rutin (%W/W)
Callus	10.16±0.24	13.13±0.25
Shoots	11.06±0.14	15.11±0.24
Leaves	20.79±0.43	24.93±0.32

Results are mean ±SEM of three parallel measurements (n=3)

Fig 3.5 Determination of concentration of rutin in natural grown and tissue culture grown plant of *Alpinia purpurata*



DISCUSSION

Now a day there is big potential to produce healthy planting material, so the use of low cost tissue culture-based plant micropropagation has emerged as one of the most important agro-technologies. However, the lack of information about its survival and the benefits of these technologies and the cost of *in vitro* plant production are obstacles for its right to use to growers and farmers in developing countries. Thus, efforts to develop low-cost technologies to the people are necessary. [9] Use of table sugar reduced the cost of the medium between 34 and 51%. Supply of sugar to the culture medium promote plant growth *in vitro* and give back for the low or negative net photosynthetic rate as a result of poor photosynthetic ability thus increasing the survival rates of tissue sections cultured *in vitro*. [10] The cost of tissue culture of banana was reduced by 90% by replacing the tissue culture sucrose grade with a commercial sugar. [11] It is recommended that table sugar be considered as low-cost substitute for potato micro-propagation and it was significantly lowered the production input costs by 34 to 51% when compared with the analytical grade sucrose. [4] Water is one of the major medium components. Distilled water obtained by electrical distillation is costly and required a sophisticated distillation apparatus. However, rain water or tap water can be used as a substitute in banana, ginger and strawberry respectively. [12-14] Coconut water is traditionally used as a growth supplement in plant tissue culture. The wide applications of coconut water can be justified by its unique chemical composition of sugars, vitamins, minerals, amino acids and phytohormones. [15] Coconut water has been shown to induce division of mature cells. [16-17] The growth of spinach tissue on a medium supplemented with 10% to 15% (v/v) mature coconut water increased the weight of spinach callus after 5 weeks. [18] Plant growth regulators are key media components. The use of agriculture products containing plant growth regulator in plant tissue culture media has been reported previously. [19] The substitution of macronutrients and micronutrients with the alternatives reduced the cost by 94.2 and 97.8%, respectively. Substitution of gelling agents (agar and gerlite) with support matrices (glass beads, cotton wool and vermiculite), conventional equipments (autoclave, culture bottles, micropipette and measuring cylinder) with easily accessible alternatives (pressure cooker, jam jars, insulin and vet syringes) reduced costs by 94.2 and 85.9%, respectively

The time required for sample analysis in HPTLC is much less compared to HPLC. In HPLC, one sample is injected at a time and after every injection there is a washing period. On the other hand, in HPTLC more than one sample is applied on a plate and quantitated in a single run. [20] The HPTLC densitometric technique is therefore suggested for the determination of rutin in low cost *in vitro* grown plant of *Alpinia purpurata.*

According to the results obtained by our study the concentration of rutin was increased in low cost grown plant of *Alpinia purpurata*.

CONCLUSION

Plant tissue culture technique with low cost produced healthy planting material throughout the year and also it represents an improvement over traditional methods. The high survival rates of 100% and the achievement of vigorous plantlets were indications that change in the variables does not affect the growth and production of secondary metabolites. The concentration of secondary metabolites was also changed with change in different growth parameters or by different variables. So there is need to try low cost growth regulators, infrastructure, equipments and energy on micropropogation technique.

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