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Research Article

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Effect of chitin and chitosan on the biosynthesis of rutin in shoot cultures of *Ruta graveolens* L.

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ABSTRACT

The objective of the present study was to study the effect of biotic elicitors (chitin and chitosan), to enhance the production of flavonoid rutin in shoot cultures of *Ruta graveolens* L. The *in vitro* shoots were cultured on solid and liquid medium augmented with different concentrations of chitin and chitosan (0, 0.01%, 0.1% and 0.5%). The highest content of rutin (38.11±0.77 mg/g DW) was shown by shoot suspension cultures cell line fortified with 0.1% chitin and the lowest rutin content (14.44±0.86 mg/g DW) was observed in cell line supplied with 0.5% chitin. The maximum fresh weight 167.23±1.66 g/L and dry weight 17.36±0.55 g/L was also observed at same concentration of chitin. While in the case of chitosan, the maximum content of rutin (36.75±0.75 mg/g DW) was shown by shoot suspension culture cell line fortified with 1.5 mg/L BAP (6-Benzyl amino purine)+1.0 mg/L IBA (Indole-3-butyric acid) and 0.1% Chitosan. The rutin content increased by increasing the concentration of chitin and chitosan from 0.01% to 0.1% but further increase (0.5%) of the elicitor showed decrease in rutin content but still above the level of the control cell line. These results clearly suggest that both the biotic elicitors can enhance the production of rutin in shoot cultures but chitin at 0.1% w/v induced maximum production.

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INTRODUCTION

Plants are an important source of medicinally important compounds. Many plant products are used as pharmaceuticals, pigments, herbicides, etc. Plant cell culture has been used for the production of various valuable phytochemicals. *Ruta graveolens* L. (*Rutaceae*), the common rue, is an endemic of the Mediterranean region. It has been introduced to and cultivated in many parts of the world because of its medicinal properties (Kong *et al.*, 2003). Rutin is a citrus flavonoid glycoside found in many plants such as *Viola tricolor* (3.36%), *Cappris spinosa* (0.28%), Apple (0.17%), *Lycopersicon esculentum* (0.002%-0.009%) and many more (McGregor & McKillican, 1952; Atanassova & Bagdassarian, 2009). Ruta was shown to consist of bioactive compounds that plays a key role in protecting plants against harmful ultraviolet radiation or pathogens and is also used to control various diseases such as hypertension, arteriosclerosis, diabetes, and obesity (Martens & Mithofer, 2005; Mehta *et al.*, 2010). *Ruta graveolens* L. and *Fagopyrum esculentum* (Sofic *et al.*, 2010) are identified as the biggest plant sources of rutin (or rutoside).The phytochemical profile of rue is quite complex. It shows presence of more than 120 compounds of different classes of secondary metabolites such as acridone alkaloids, coumarins, essential oils, flavonoids, and furoquinolines (Oliva *et al.*, 2003). Many of these compounds are physiologically active and therefore of immense pharmaceutical interest. Rue extracts have potent anti-cancer activity exhibited through strong anti-proliferative and anti-survival effects on cancer cells (Fadlalla *et al.*, 2011). Psoralenes, among the main constituents of rue, are known for their photosensitization effects, which can produce a very strong undesirable syndrome in the form of photodermatitis. They are also used for therapeutic purposes in photochemotherapy to treat cutaneous T-cell lymphoma and granuloma annulare (Browne *et al.*, 2011). Recently, it has been reported that (+)-rutamarin is a dual inducer of both GLUT4 translocation and expression and therefore ameliorates glucose homeostasis in insulin-resistant mice (Zhang *et al.*, 2012).

Elicitation is one of the most successful biotechnological approaches employed to enhance the production of active

principles in cell suspensions (Dixon, 2001). Abiotic stress like drought can modify plant metabolism, resulting in increased biosynthesis of bioactive compounds. The amount of phytoconstituents found in plants is influenced by the growth conditions. Plants grown under favourable conditions of growth produce a higher quantity of phytocomponents than plants grown under stress conditions. Drought stimuli significantly enhanced the concentration of secondary metabolites under field conditions (Khan *et al.*, 2011). Consequently, using elicitors like osmotic stress could enhance the production of secondary metabolites under *in vitro* conditions (Smetanska, 2008). Recent developments in the elicitation of cell cultures have provided new approaches for the production of these secondary compounds. Their synthesis and accumulation in cell cultures can be activated by the augmentation of elicitors to the culture medium. The application of biotic elicitors in plant cell cultures constitutes an excellent system to enhance the synthesis of secondary metabolites with phytoalexinic properties (antimicrobial and antioxidative) as well as to get more insight into the regulation of their biosynthetic pathways. Exogenous biotic elicitors include compounds released by microorganisms and other pathogens or formed by the action of plant enzymes on microbial cell walls, e.g., microbial enzymes, fungal and bacterial lysates (*Bacillus* sp.), yeast extracts, and polysaccharides from microorganism cell walls (Ramirez-Estrada *et al.*, 2016). Considering the medicinal importance of rutin, the present study was undertaken to evaluate the effect of biotic elicitors' chitin and chitosan on the *in vitro* growth of *R. graveolens* shoot cultures and the biosynthesis of flavonoid rutin.

MATERIALS AND METHODS

Shoot Cultures

Shoot cultures of *Ruta graveolens* L. were established by culturing nodal and Internodal segments (10-15 mm long) from 4 week-old aseptically germinated plants on MS Media enriched with 3% sucrose supplemented with 1.5 mg/L BAP in combination with 1.0 mg/L IBA for shoot induction. The pH of media was adjusted to 5.8 using 0.1 N NaOH or HCl prior to autoclaving for 15 min at 120 °C and 10.8 kPa. The cultures were maintained at 23±2 °C under a 16-hour photoperiod.

Regents and Materials

The Murashige and Skoog's Culture media, Plant growth regulators and Standard rutin were purchased from Himedia, Mumbai, India. Chitin, chitosan and HPLC grade methanol were purchased from S-D fine Chemicals limited, Mumbai India.

Establishment of Static and Shaking Shoot Cultures.

After 35 days of growth, 10 grams of the developing shoots raised by direct organogenesis were subcultured in culture vials containing solid medium to initiate the Static shoot cultures and 250 mL Erlenmeyer flasks containing 70 mL of liquid media to initiate shoot suspension cultures. Both types of cultures were fortified with a combination of 1.5 mg/L BAP and 1.0 mg/L IBA. The shoot suspension cultures were continuously agitated on an orbital shaker incubator at 120 rpm for 4 weeks at 23±2 °C with a photoperiod of 16 hours of light (100 mmol m^{-2} s^{-1}) and 8 hours of darkness. Sub-cultures were carried out after every 14 days.

Elicitor Treatment Method

Both biotic elicitors were filter-sterilized through a 0.45 µm Millipore filter. The elicitation was carried out by adding 0.01, 0.1 and 0.5 % (W/V) chitin and chitosan, during the 4th week (6 days before the end of the culture) of the experiment separately for shoot suspension cultures as well as for static shoot cultures. The 4th week shoots were chosen for elicitation because it has been revealed that best results are achieved during this period (Orlita *et al.*, 2008b). Each experiment was carried out in triplicates.

Extraction of Shoot Cultures

After 35 days of growth, the *in vitro* shoots were taken out, completely washed with distilled water to discard any medium and were gently pressed between Whatman filter paper to remove water; the fresh weights (FW) were recorded followed by drying in oven at 45 °C until constant dry weights were achieved for consecutive days and then the dry weights were also determined (Mohamed & Ibrahim, 2011). About 150 mg of plant powder of shoot cultures of *R. graveolens* was accurately weighed in a flask. To which 20.0 mL of (40%) aqueous methanol was added and the flask was shaken at 120 rpm in an orbital shaking incubator for 24 hours at 25 °C. The contents of the flask were filtered through Whatman filter paper No. 41 and were used for HPLC analysis of rutin (Dighe *et al.*, 2011).

Determination of Rutin by HPLC

HPLC quantification of rutin was performed by the method described by Kale and Laddha (2012), with some modifications (Figures 1-15). The analysis was carried out with a Shimadzu HPLC (Kyoto, Japan) equipped with quaternary pumps, degasser coupled to a photo-diode-array detector and injection value with a 20 µL loop. Rutin was detected at 257 nm. Chromatographic separations were performed on a C18 (250 mm × 4.6 mm), 5 µm column using a solvent system consisting of methanol: water: acetic acid (45: 54: 1 v/v) in an isocratic mode for rutin. The mobile phase was filtered through a 0.45 µm membrane filter (millifore, Bedford, MA, USA) before analysis. Separation was carried out with an injection volume of 20 µL, a flow rate of 1 mL min-1 with 5 minutes of run time. All the injections were performed in triplicate. The retention time for standard rutin was 2.3 minutes (Figure 1). The chromatographic peaks of the analytes were confirmed by comparing their retention time with those of pure standards. Quantification was carried out by the integration of the peak using the external standard method. Class WP software (version 6.1) from Shimadzu was used for instrument control, data acquisition and data processing. Quantitative determinations were made by taking into account the peak area of the standard at a particular retention time

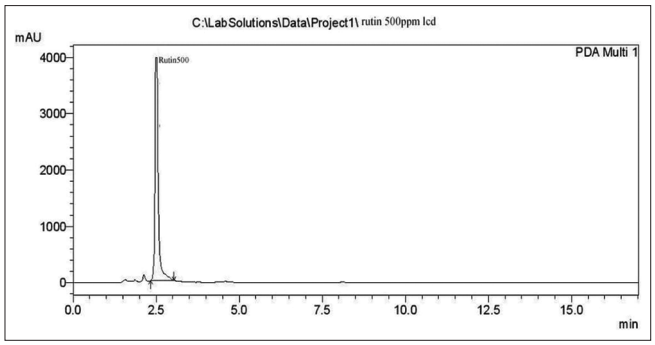

Figure 1: HPLC chromatogram of standard (rutin)

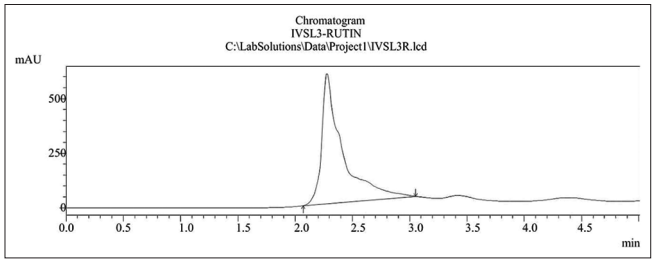

Figure 2: HPLC chromatogram of rutin in methanolic extract of Shoot Cell line IVSL3 of *R. graveolens* L.

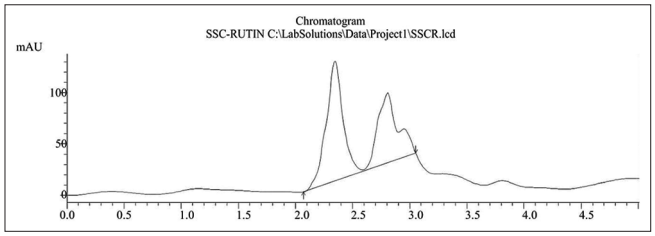

Figure 3: HPLC chromatogram of rutin in methanolic extract of Shoot Suspension culture (SSC) of *R. graveolens* L.

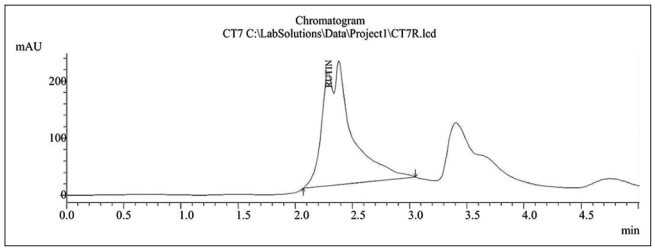

Figure 4: HPLC chromatogram of rutin in methanolic extract of elicitated cell line CT7 of *R. graveolens* L.

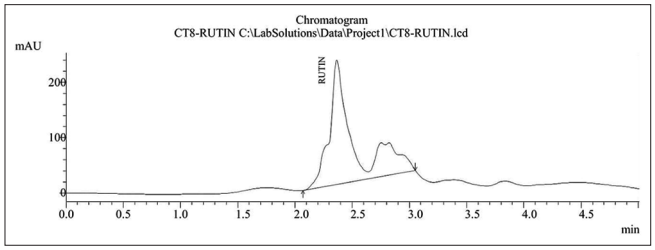

Figure 5: HPLC chromatogram of rutin in methanolic extract of elicitated cell line CT8 of *R. graveolens* L.

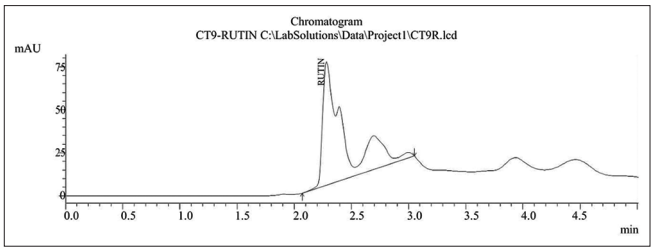

Figure 6: HPLC chromatogram of rutin in methanolic extract of elicitated cell line CT9 of *R. graveolens* L.

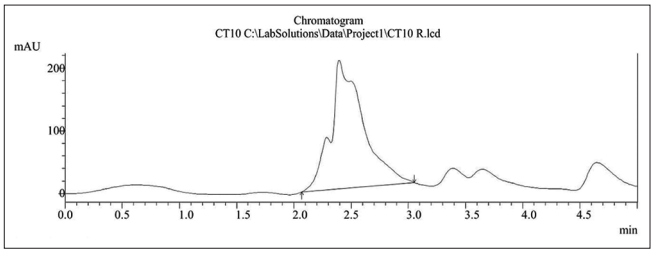

Figure 7: HPLC chromatogram of rutin in methanolic extract of elicitated cell line CT10 of *R. graveolens* L.

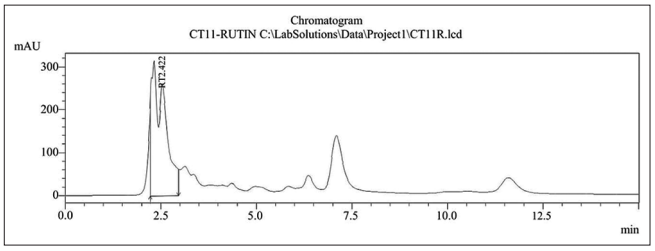

Figure 8: HPLC chromatogram of rutin in methanolic extract of elicitated cell line CT11 of *R. graveolens* L.

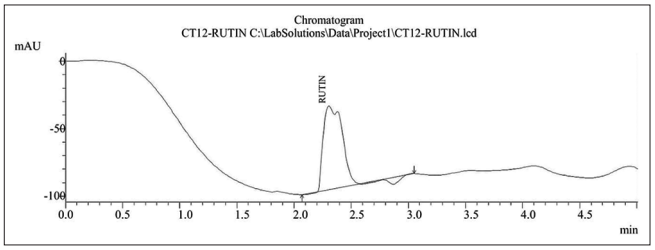

Figure 9: HPLC chromatogram of rutin in methanolic extract of elicitated cell line CT12 of *R. graveolens* L.

versus concentration and expressed in milligrams per gram of dry weight.

Statistical Analysis

The experiments were carried out in triplicates and were repeated thrice. The results were analyzed using one-way analysis of variance (ANOVA) and significance was determined at $P<0.05$. The data were analyzed statistically using SAS System ('Local', W32_VSPRO) software and means were compared using Duncan's Multiple Range Test (DMRT). Variability in data has been expressed otherwise as mean±standard deviation.

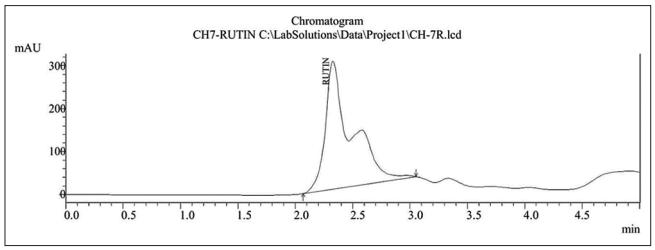

Figure 10: HPLC chromatogram of rutin in methanolic extract of elicitated cell line CH7 of *R. graveolens* L.

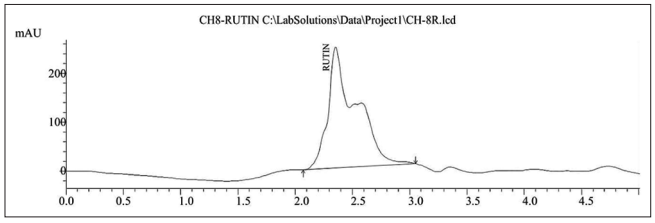

Figure 11: HPLC chromatogram of rutin in methanolic extract of elicitated cell line CH8 of *R. graveolens* L.

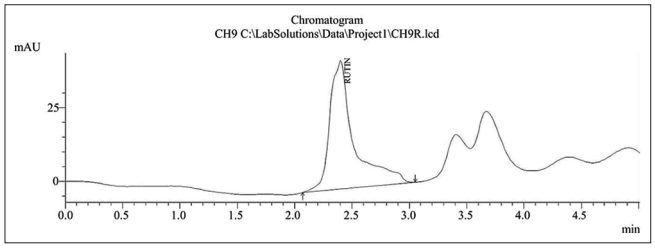

Figure 12: HPLC chromatogram of rutin in methanolic extract of elicitated cell line CH9 of *R. graveolens* L.

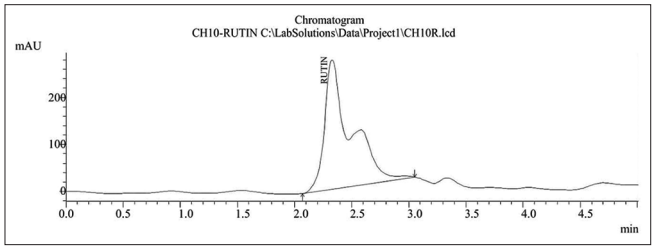

Figure 13: HPLC chromatogram of rutin in methanolic extract of elicitated cell line CH10 of *R. graveolens* L.

RESULTS AND DISCUSSION

Commercial production of bioactive compounds is often hindered because of practical problems, such as low and variable yields, limited availability of natural resources, environmental conditions etc. *In vitro* cultures provide an alternative to avoid problems associated with field production. They offer a system in which cells are cultured, and the yield of phytochemicals is independent of environmental changes. Use of elicitors in *in vitro* cultures may enhance the production of phytochemicals to a large extent (Lambert *et al.*, 2011).

The effect of biotic elicitors (chitin and chitosan) on biomass growth and rutin production in static (MS solid media) and

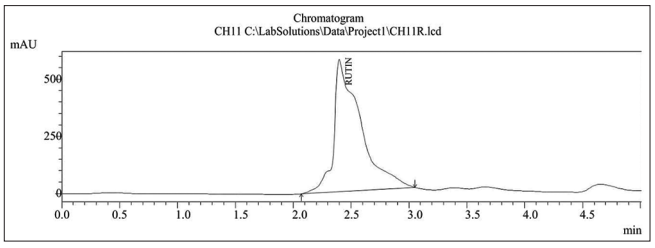

Figure 14: HPLC chromatogram of rutin in methanolic extract of elicitated cell line CH11 of *R. graveolens* L.

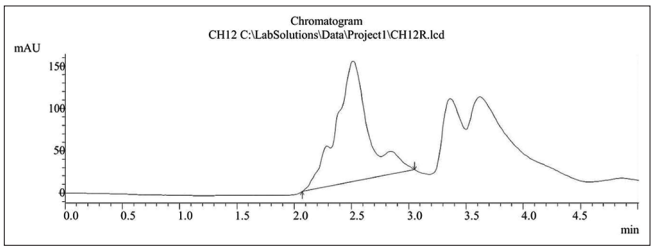

Figure 15: HPLC chromatogram of rutin in methanolic extract of elicitated cell line CH12 of *R. graveolens* L.

suspension cultures (MS liquid media) was studied. The previously established shoot cell lines (IVSL3 (Figure 2) for static shoot culture cell lines and SSC (Figure 3) for shoot suspension culture cell lines) with high rutin content were selected for the initiation of elicitated shoot cultures. These cell lines were sub cultured in MS solid and liquid media enriched with different combinations/concentrations of plant growth regulators. The growth rates of cultures were initially slow but as time proceeded, they increased significantly and accumulated large amounts of biomass over a period of 4 weeks. Medium enriched with BAP 1.5 mg/L in combination with IBA 1.0 mg/L showed higher multiple shoot growth rates than the medium containing any other combination or the control. These cultures were analyzed at regular intervals for 5 weeks.

Similarly fresh multiple shoot cultures of selected plants were analyzed at regular intervals for 5 weeks. Since the beginning of the 2nd week a linear increase in shoot biomass was observed which reached a maximum value on the 4th week. The liquid MS media enriched with 1.5 mg/L BAP+1 mg/L IBA was found to be the most effective for shoot biomass production than all the other treatments or control. The physiological state of the cells at the moment of transfer from growth to production medium is crucial. The optimum age of the culture for elicitation is different in different plant cell systems (Kang *et al.*, 2009). In most of the cases, response of the cells was maximum if they were challenged at the end of the growth phase or at the onset of the stationary phase (Khosroushahi *et al.*, 2006). On the contrary, taxol and baccatin were produced during the initial stationary phase of growth in *Taxus baccata* cell cultures (Barradas-Dermitz *et al.*, 2010). The response to elicitation is dependent on the growth phase of the culture that affects the quantitative response as well as the product pattern (Vázquez-Flota *et al.*, 2009). The amount of metabolite production varied with duration of incubation with elicitors (Karwasara *et al.*, 2010).

After confirming the ability of static as well as the suspension cultures to produce rutin in optimized MS medium, elicitation process was carried out to enhance rutin biosynthesis. The biotic elicitors (chitin and chitosan) at different concentrations (0.01%, 0.1%, and 0.5%) were used to elicit the shoot cultures in solid as well as in liquid medium. The Static shoot culture and shoot suspension cultures have different control IVSL3 and SSC respectively. Elicitation was carried out in twelve shoot cell lines. Among the 12 cultures 6 were in solid media and 6 in liquid media. All the shoot cultures were enriched with 1.5 mg/L BAP + 1.0 mg/L IBA as these combinations were the most successful for shoot growth. The idea behind using media of different strengths (Solid and Liquid) was to assess the effect of media strength on the biomass and rutin content of shoot cultures.

As indicated in Table 1, the biotic elicitors, chitin and chitosan, when supplied to MS media enriched with 1.5 mg/L BAP+1.0 mg/L IBA) showed an increase in rutin content both in solid as well as in liquid cultures. The shoot culture cell lines when elicitated by different concentrations (0.01%, 0.1%, and 0.5%) of chitin and chitosan showed variation in their rutin content. The highest content of rutin (38.11±0.77 mg/g DW) among the cell lines treated with chitin was shown by shoot suspension culture cell line CT11 (Figure 8) fortified with 0.1% chitin followed by static shoot culture cell line CT8 (34.58±1.09 mg/g DW) (Figure 5) also supplied with same concentration of chitin and lowest rutin content (14.44±0.86 mg/g DW) was observed in cell line CT12 (Figure 9).

The application of chitin 0.01% doubled the yield of Bergapten and Rutacultin and increased the production of Xanthotoxin and Isopimpinelin 5- and 9-fold respectively as compared with the control value. Such an effective increase in the production of nearly all Coumarins suggests that these compounds may be participating in the natural resistance mechanisms of *R. graveolens*. However, when chitin was supplied at higher level (0.1%), it was observed that most of the analysed coumarins are synthesized to a lesser extent than when lower concentration (0.01%) was used (Orlita *et al.*, 2008c). Increasing the level of chitin to 0.5% decreased the rutin yield but not below the control. The maximum fresh weight of 167.23±1.66 g/L and dry weight of 17.36±0.55 g/L was observed in cell line CT11 (Figure 8) treated with 0.1% chitin while as lowest fresh weight of 90.66±1.52 g/L and dry weight of 7.30±0.43 g/L was observed in cell line CT9 (Figure 6) supplied with 0.5% chitin. Cell line CT11 enriched with 0.1% chitin and 1.5 mg/L BAP+1.0 mg/L IBA proved best for increasing the fresh weight production as far as elicitation with chitin is concerned but there was hardly any difference between the dry weights of cell line CT11(Figure 8) and control cell line SSC (Figure 3). Orlita *et al.* (2008a) also observed increase in the growth of *in vitro* shoots when supplied with 0.1% chitin as compared to non elicitated cultures.

The biotic elicitor Chitosan when applied in different concentrations (0.01%, 0.1%, and 0.5%) to shoot culture cell lines showed variation in their rutin content. The highest content of rutin 36.75±0.75 mg/g DW among the cell lines treated with chitosan was observed in the shoot suspension culture cell line CH11 (Figure 14) fortified with 1.5 mg/L BAP+1.0 mg/L IBA and 0.1% Chitosan followed by static shoot culture cell line CH7 25.26±0.53 mg/g DW (Figure 10) supplied with 0.01% of chitosan while as lowest rutin content 13.27±0.52 mg/g DW was observed in cell line CH9 (Figure 12) supplied with 0.5% chitosan as well as 1.5 mg/L BAP+1.0 mg/L IBA. Orlita *et al.* (2008c) treated *R. graveolens* shoot cultures with chitin and chitosan that resulted in a significant increase in the concentration of several Coumarins. The application of the 0.1% chitosan doubled the yield of Furanocoumarins like Bergapten, Xanthotoxin or Isopimpinelin in comparison to 0.01% chitosan which is similar to the results obtained in

Table 1: Elicitation of shoot cultures of *Ruta graveolens* L. for rutin content using biotic elicitors' chitin and chitosan

Culture Type	PGR	Chitin (%)	Sample Name	Fresh Weight (g/L)	Dry Weight (g/L)	Rutin content (mg/g DW)
Static Shoot Culture	BAP 1.5 (mg/L)	0	Control	92.66±1.04^{j}	8.90±1.01^{fg}	17.16±0.29^{g}
	+		(IVSL3)			
	IBA 1 (mg/L)	0.01	CT7	99.50±1.50^{i}	8.60±0.69^{fgh}	19.63±0.56^{e}
		0.1	CT8	137.03±1.26^{d}	15.56±0.40^{b}	34.58±1.09^{b}
		0.5	CT9	90.66±1.52^{j}	7.30±0.43^{hi}	16.19±0.29^{gh}
Shoot Suspension Culture	BAP 1.5 (mg/L)	0	Control (SSC)	142.33±1.15^{c}	17.16±1.04^{a}	19.96±0.75^{de}
	+	0.01	CT10	152.33±1.04^{b}	17.26±1.07^{a}	20.85±0.62^{d}
	IBA 1 (mg/L)	0.1	CT11	167.23±1.66^{a}	17.36±0.55^{a}	38.11±0.77^{a}
		0.5	CT12	120.83±1.15^{e}	9.76±0.37^{ef}	14.44±0.86^{i}
		Chitosan %				
Static Shoot Culture	BAP 1.5 (mg/L)	0	Control	92.66±1.04m	8.90±1.01^{i}	17.16±0.29^{f}
	+		(IVSL3)			
	IBA 1 (mg/L)	0.01	CH7	114.83±1.60^{j}	12.10±1.21^{gh}	25.26±0.53^{b}
		0.1	CH8	117.50±1.32^{i}	13.43±0.69^{fg}	21.54±0.58^{d}
		0.5	CH9	74.36±1.70p	5.43±0.40^{k}	13.27±0.52^{h}
Shoot Suspension Culture	BAP 1.5 (mg/L)	0	Control (SSC)	142.33±1.15^{d}	17.16±1.04^{b}	19.96±0.75^{e}
	+	0.01	CH10	165.16±1.04^{a}	16.50±1.32^{bc}	22.87±0.58^{c}
	IBA 1 (mg/L)	0.1	CH11	159.50±1.32^{b}	15.16±0.76^{cde}	36.75±0.75^{a}
		0.5	CH12	128.50±1.44^{g}	8.46±0.45^{i}	14.92±0.53^{g}

Values represent mean±standard deviation. Means with the same letter within columns are not significantly different at $P<0.05$ using Duncan's multiple range test (DMRT).

our study though the compound concerned is a flavonoid and not a coumarin. When the concentration of Chitosan was increased from 0.1 to 0.5%, the yield of rutin was reduced to a lower amount as compared to the control which suggests that above 0.1% these fungal elicitors might be losing their elicitation property.

The maximum fresh weight 165.16±1.04 g/L and dry weight 16.50±1.32 g/L (slightly lower than that of control cell line SSC) was observed in cell line CH10 (Figure 13) supplied with 0.01% chitosan while as lowest fresh weight 74.36±1.70 g/L and dry weight 5.43±0.40 g/L was observed in cell line CH9 (Figure 12) supplied with 0.5% chitosan. Cell line CH10 enriched with 0.01% chitosan and 1.5 g/L BAP + 1.0 g/L IBA proved best for biomass production as far as elicitation with chitosan is concerned. Bahabadi *et al.* (2011) reported an increase in Podophyllotoxin production in *L. album* cell cultures when treated with chitin and chitosan. Similarly improvement in several other metabolites in different plant species were reported by early workers like Paclitaxel production in cell suspension cultures of *T. chinensis* (Zhang *et al.*, 2000; Zhang & Xu, 2001), Phenylethanoid glycoside accumulation in *Cistanche deserticola* cell suspension cultures (Cheng *et al.*, 2006), Anthraquinones, Phenolics and Flavonoids in *Morinda citrifolia* adventitious roots (Baque *et al.*, 2012), flavonoid content in *Andrographis paniculata* cell suspension cultures (Mendhulkar & Vakil, 2013), phenols and terpenoids in *Ocimum basilicum* plant (Kim *et al.*, 2005) and phenolic compounds in cell suspension cultures of *Vitis vinifera*. All these studies support the findings of the present investigation. Several studies have indicated that chitosan's presence induces a natural defence response in many plants (Rabea *et al.*, 2003). Elicitation of a cellular suspension of the brassicaceous flowering plant *Farsetia aegyptia* with chitosan was found to induce the biosynthesis of various Phytoalexins (Al-Gendy & Lockwood, 2005).

Chitosan has been used to produce menthol in a suspended culture of *Mentha piperita*. The yield of Anthraquinone and Indirubin *in vitro* cultures of *Polygonum tinctorum* was effectively increased when supplied with Chitosan at a concentration of 0.02% (Kim *et al.*, 2007).

CONCLUSION

Hence, from the present study, it can be concluded that application of elicitors in the culture media had a significant effect on shoot biomass as well as on the amount of rutin. It can also be concluded that both the elicitors chitin and chitosan) were successful in enhancing the rutin content of the shoot cultures cell lines of *R. graveolens* L. but the shoot suspension culture cell line CT11 supplied with chitin 0.1% proved best for rutin biosynthesis and biomass production. More than two fold increases in rutin content was found in the shoot suspension culture cell line CT11 as compared to the control whereas Chitosan also increased the rutin content of cell lines but less than chitin. Elicitors at higher levels were not as successful as they were at lower levels in enhancing the amount of rutin in different cell lines. A decline in the amount of rutin was found at higher concentrations of elicitors. Liquid cultures showed higher amount of biomass and rutin content as compared to solid cultures. These results of improving effect of biotic elicitors on *in vitro* cell lines of *R. graveolens* have thrown up many questions which need thorough investigation for the betterment of mankind.

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