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Bioprocess Optimization of Furanocoumarin Elicitation by Medium Renewal and Re-elicitation: A Perfusion-Based Approach

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Abstract Effect of various abiotic (methyl jasmonate, salicylic acid) and biotic (yeast extract, *Aspergillus niger)* elicitors on furanocoumarin production and in situ product removal was studied using shoot cultures of *Ruta graveolens* L. Elicitation by yeast extract (1% *w*/*v*) on day 15 was most effective. It led to 7.8-fold higher furanocoumarin production that was attained 24 h after elicitation and 43% of the product was released into the medium. Changes in the relative concentration of furanocoumarins produced depend on the elicitor used. Molar ratio of bergapten increased to 93% in response to yeast extract. With the perspective of developing a commercially feasible process, an approach for preserving viability of biomass and its reuse needs to be developed. For this, medium renewal strategy was investigated. Removal of the spent medium 48 h after elicitation allowed in situ product removal and proved effective in revival of cultures, allowing reuse of biomass. A week after medium renewal, the revived biomass was re-elicited and a second furanocoumarin production peak was obtained. A perfusion-based bioprocess optimization approach, employing elicitation coupled with medium renewal with subsequent re-elicitation, as a new strategy for improved furanocoumarin production, has been suggested.

Keywords Bioprocess optimization · Medium renewal · Re-elicitation · Furanocoumarins · Elicitors · *Ruta graveolens* L

Introduction

Furanocoumarins (FCs) are pharmaceutically important compounds widely used in the treatment of vitiligo, leucoderma, psoriasis, and multiple sclerosis [1]. They have recently been demonstrated to be potent anti-cancer and anti-HIV agents [1]. Due to these biological properties, FCs have attracted considerable attention leading to extensive investigations for their sources and availability. Successful production of FCs in vitro, at rates equal to or

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higher than in vivo plants [2, 3] has provided impetus for exploring the potential of shoot cultures for large-scale FCs production [4, 5].

For a better commercial design and process optimization, it is important to maximize biomass utilization and productivity. Elicitation has been proved to be an effective strategy for enhancing the production of secondary metabolites such as alkaloids, terpenoids, flavonoids, coumarins, phenolic compounds [6, 7]. Induction of acridone epoxides, furoquinolines, and FCs by autoclaved culture homogenate of *Rhodoturula rubra* in the cell cultures of *Ruta graveolens* was reported by Bolhmann et. al. [8]. Orlita et. al. [9–11] recently reported enhanced FC production in response to various biotic and abiotic elicitors in *R. graveolens* shoot cultures. However, they did not investigate the effect of elicitation on product release and biomass. As plant cultures grow slowly, an approach for preserving viability of biomass with the possibility of its reuse needs to be developed to achieve a commercially feasible process. For a better bioprocess control, the factors affecting elicitation and FC production need to be studied in detail.

Elicitation often results in decreased cell viability and biomass production [7]. To date, there are no reports investigating the dynamics of FC production and leaching with respect to their effect on culture viability. In this paper, we report effect of various elicitors (biotic and abiotic) on FC production and leaching.

To overcome the negative effects of elicitation, use of medium renewal strategy [7] was investigated. An approach for preserving culture viability, achieving re-growth of shoots for maximizing biomass utilization has been made for the first time. Induction of secondary metabolites in response to elicitation is transient. For further extension of elicitation, re-elicitation strategy was employed, with the view of developing a perfusion-based approach.

This is the first report detailing recovery and re-growth of biomass after primary elicitation and achieving induced secondary metabolite production by subsequent re-elicitation.

Materials and Methods

Shoot Cultures Established multiple shoots were screened on the basis of growth rate, biomass, and FC production; RS2 line was selected as reported earlier [5]. Shoot line was maintained at 24±2 °C, under a 16-h photoperiod (30 μMol m^{-2} s^{-1}) and subcultured every 3 weeks on liquid medium containing MS medium supplemented with 4.44 mM 6-benzyl-aminopurine (BAP) and 17.12 mM indole 3-acetic acid (IAA) with 3% *w/v* sucrose.

Elicitor Preparation and Elicitation Procedures MJ and SA were obtained from Sigma-Aldrich. Stock solutions of MJ and SA were prepared in methanol. Solutions were filter sterilized using a 0.45-μM membrane filter. Required dose was added exogenously to the culture medium aseptically to achieve desired concentrations.

Stock solution of yeast extract was prepared by dissolving it in water. Autoclaved yeast extract was applied to culture medium aseptically to attain final concentration of 0.5%, 1%, and 2% *w/v*.

The fungal elicitor *Aspergillus niger*, was obtained from Agharkar Research Institute, Pune. Fungal biomass was grown on potato dextrose agar (PDA). Liquid cultures were initiated from 3-week-old cultures by transferring a 1-cm^2 piece of the medium to 100 ml PDA. The flasks were incubated in dark at 24±2 °C on an orbital shaker (100 rpm) for 3 weeks. The biomass was harvested after 15 days. Harvested biomass was resuspended in distilled water (5 g in 100 ml), homogenized, and autoclaved at 120 °C for 20 min. The sterilized homogenate was centrifuged at 10,000 rpm for 10 min and the supernatant was

used as elicitor. The elicitor dose was measured as total carbohydrate content of the homogenate. Required concentrations of homogenate were added to the culture medium aseptically as glucose equivalent of desired amounts.

Two-gram shoots were inoculated in 250-ml Erlenmeyer flask containing 50 ml of medium and used for elicitation experiments. The shoots were harvested in triplicates at repeated intervals after elicitation and their fresh weights and furanocoumarin content was recorded. FCs leached in medium were also estimated. Control samples received equal amounts of the same solvent used for preparation of elicitor.

Furanocoumarin Estimation Furanocoumarins were extracted and estimated according to method described previously [5]. Briefly, dried plant material was hydrolyzed (2 N HCl at 80 °C for 20 min) and extracted with ethanol at 80 °C for 20 min followed by sonication for 20 min and then centrifuged. Filtered supernatant was injected into a chromatographic column, analyzed at 254 nm using HPLC (Merck Hitachi, UV–VIS detector, RP C18 Neucleosil). Solvent system used was methanol/water (70:30) with a flow rate of 1 ml/min. Confirmation and quantification carried out using standard Psoralen, Bergapten, and Xanthotoxin (Sigma-Aldrich, USA).

Medium Renewal The cultures were elicited with 1% *w*/*v* yeast extract on day 15, as described above. The spent medium was replaced with 50 ml of fresh medium aseptically, 48 h after elicitation and shoots were cultured for second culture cycle. Shoots were harvested at fixed intervals for monitoring effect on biomass yield.

Re-elicitation After medium renewal (17th day of culture), the second culture-cycle biomass was allowed to grow for a week and was re-elicited with 1% *w*/*v* yeast extract (24th day of culture). The shoots were harvested in triplicates at repeated intervals after re-elicitation and their fresh weights and furanocoumarin content was recorded. FCs from the spent medium were also estimated. Control samples received equal amounts of the same solvent (DW) used for preparation of yeast extract.

Statistical Analysis Each experiment was performed with three replicates and repeated twice with similar results. The mean±SE values have been calculated from the data of three experiments and were presented in the results. Statistical analysis was done for the data obtained using Student's *t* test to check differences between the treatments. The results were analyzed using two-way ANOVA (VassarStats) at significance level of 95% and critical values for Tukey HSD test were calculated for significant *F* values.

Results and Discussions

From the previous studies it was seen that the accumulation of FCs takes place after the cultures entered the stationary phase (day 14) [5]. Therefore elicitation was done on the 15th day of the culture initiation.

Effect of various biotic [yeast extract (YE), *A. niger* (AS)] and abiotic [methyl jasmonate (MJ) and salicylic acid (SA)] elicitors, on FC induction was studied to determine the optimum elicitor. In a preliminary set of experiments, FC production and leaching in relation to biomass yield was studied. It was seen that increase in FC yield and leaching in response to elicitor addition was dose dependent (Fig. 1). Abiotic elicitors MJ (10 μM) and SA (200 μM) led to 4.7-fold (697 mg/10 g DW) and 5.9-fold (878 mg/10 g DW) higher FC

production, respectively, whereas biotic elicitors YE (1%) and AS (1000 μM) led to 7.8-fold (1164 mg/10 DW) and 7.1-fold (1059 mg/10 g DW) higher FC production, respectively, as compared to control.

Beneficial effects of MJ and SA have been reported for taxane [12], ginsenosides [13], bilobalide [14], anthraquinones [15] production. Similarly, increase in silymarin production due to the elicitation by MJ and YE has been reported [16]. YE-induced FC production has been reported in cell cultures [17], roots, and shoots [18] of *Glehnia littoralis*. AS-induced production of several alkaloids and taxol [7] has been reported. AS-induced FC production in *Agelica* root discs has been reported earlier [19], however there are no reports on its effect on FC production using in vitro cultures. Induced production of linear FCs due to MJ and SA elicitation has been reported for the first time.

Leaching The main drawback in the industrial application of plant cultures is the fact that most plant products are stored intracellularly. The recovery of intracellular products, therefore, inevitably involves the destruction of biomass for harvesting the product. Leaching is beneficial as it facilitates in situ product removal of the compounds and allows reuse of biomass. In the non-elicited cultures, FCs were not released into the medium and accumulated intracellularly in the plant cultures. Therefore for obtaining the FCs, entire biomass had to be harvested. MJ at 10 μM caused 36% leaching and SA at 50 μM caused 38%, whereas YE at 1% *w*/*v* resulted in 43% and 500 μM of AS led to 48% leaching (Fig. 1a). Percentage leaching due to biotic elicitors was similar to that obtained by abiotic elicitors (40–50%).

It was observed that leaching of more than 40% led to drastic decrease in cell viability and biomass growth (Fig. 1b). The increased extracelluar FC concentration may be ascribed in part, to increase in cell membrane depermeabilization followed by cell lysis as a response to elicitors. This led to reduction in cell viability as observed by the decreased biomass yield (Fig. 1b). Though biotic elicitors were more efficient in induction of FCs, they affected biomass yield drastically. Biomass yield decreased 4.3-fold due to addition of AS, even at low concentrations (Fig. 1b). Similar observations were reported previously for various elicitors [7]. Inhibitory effects on biomass due to product leaching have been reported for as several other elicitors [20], which were similar to our results. Therefore for maximum utilization of biomass, some means to fortify the cultures were seen to be necessary. As exogenous addition of YE led to highest FC production and leaching, it was selected for further studies.

Relative Furanocoumarin Production Changes in the relative concentration of psoralen, bergapten, and xanthtoxin were observed to depend on the elicitor used. Relative FC content (molar ratio) at optimal concentrations of elicitors is presented in Fig. 2. Addition of MJ (10 μM) and YE (1% *w*/*v*) resulted in maximum induction of bergapten. Molar ratio of bergapten increased to 79% on addition of MJ and to 93% in case of YE (Fig. 2). Addition of SA (200 μM) and AS (1000 μM) resulted in increased production of psoralen and xanthotoxin. Molar ratio of psoralen increased from 7% to 37% due to addition of AS (Fig. 2). Xanthotoxin molar ratio increased to 80% due to elicitation by SA and 56% due to AS. Thus there exists a significant disparity in induction of particular FCs by different elicitors.

Similar change in molar ratios of FCs in *Angelica* root discs due to abiotic elicitation has been reported by Glowniak et. al. [19]. Changes in taxanes due to MJ and SA elicitation were noted by Wang et. al. [7]. Changes in pattern of FCs in response to various abiotic and biotic elicitors in vitro has been noted for the first time.

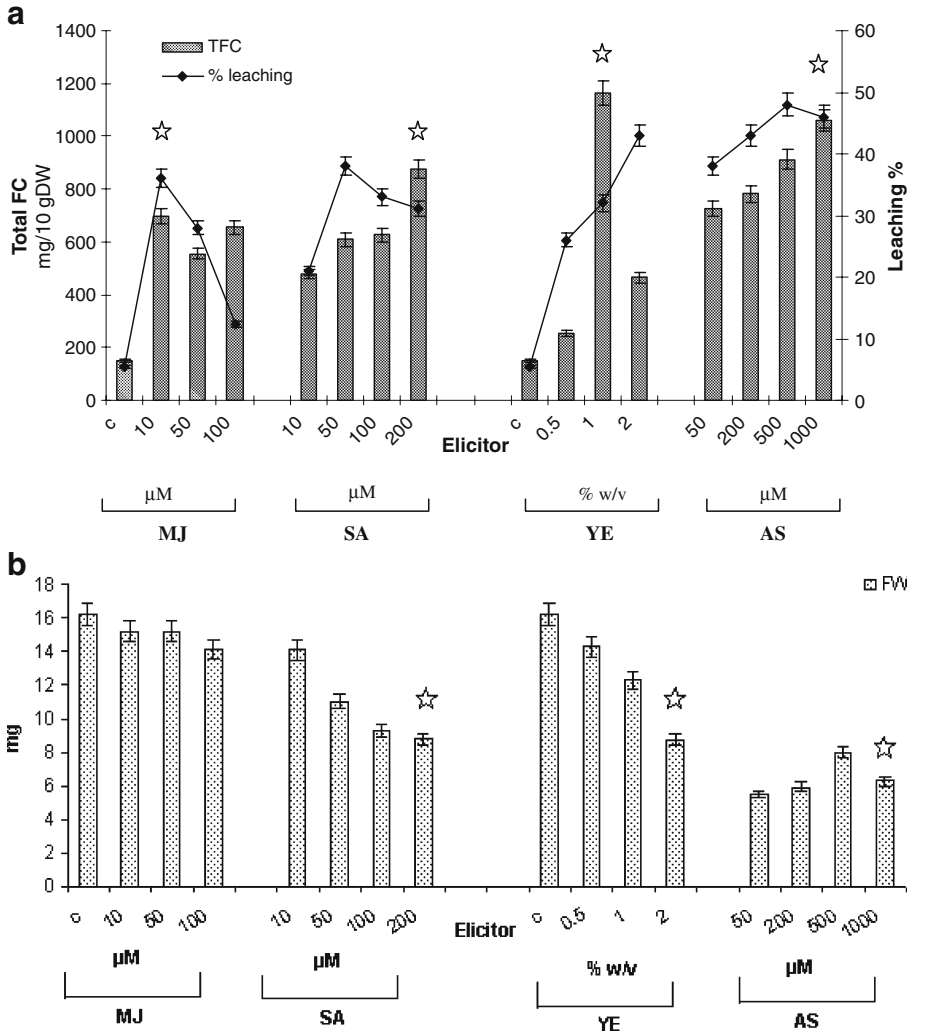

Fig. 1 **a** Furanocoumarin production and release in response to different elicitor treatments. *Y axis*: *TFC* total furanocoumarin (µg/L) represented as *bars* ▩, *Fcm* furanocoumarin leached in the medium (%) represented as *line* —◆—, values are mean of six replicates±standard deviation, *star* (☆) indicates values significant at $p \leq 0.095$ as calculated by two-way Anova. *X axis*: *MJ* methyl jasmonate treatment (10, 50, 100 µM), *SA* Salicylic acid treatments (10, 50, 100 µM), *YE* Yeast extract treatments (0.5, 1, 2% *w/v*), *AS A. niger* treatment (50, 200, 500, 1,000 µM). **b** Effect of different elicitors on biomass. *FW* fresh weight in G, *star* (☆) indicates values significant at $p \leq 0.095$ as calculated by two-way Anova, *MJ* methyl jasmonate treatment (10, 50, 100 µM), *SA* salicylic acid treatments (10, 50, 100 µM), *YE* yeast extract treatments (0.5%, 1%, 2% *w/v*), *AS A. niger* treatment (50, 200, 500, 1,000 µM)

Furanocoumarin Production Kinetics Induction of FCs in response to YE was studied in detail, as maximum elicitation and leaching was achieved due to YE. The production kinetics was studied by time course analysis for various concentrations of YE (0.5%, 1% and 2% *w/v*) and is illustrated in Fig. 3. Elicitation resulted in transient induction of FCs at all YE doses, which peaked 24 h after elicitation and decreased after 72 h (Fig. 3). Highest induction of FCs (1164 mg/10 g DW), which was 7.8-fold higher than the control was

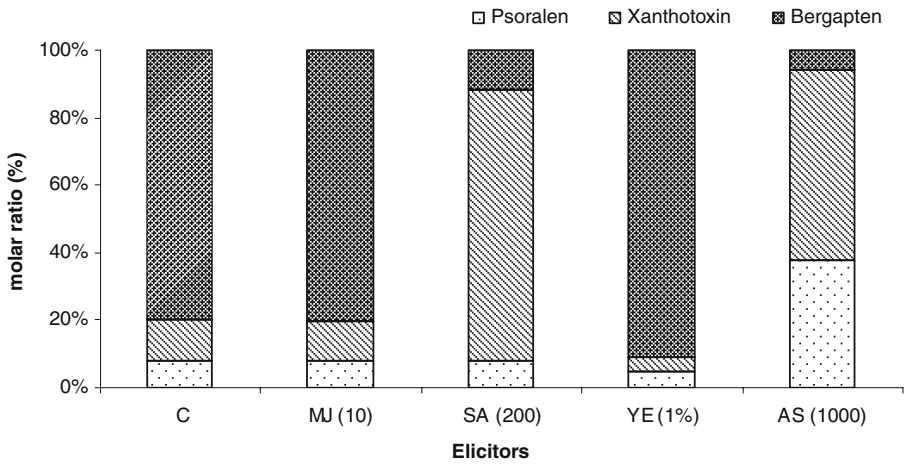

Fig. 2 Relative furanocoumarin accumulation in response to different elicitor treatments. *MJ* Methyl jasmonate 10 μM, *SA* salicylic acid 200 μM, *YE* yeast extract 1% *w/v*, *AS A. niger* 1000 μM, *TFC* total furanocoumarin (μg/L)

obtained with YE at 1% *w/v*. The maximum elicited FC was bergapten, which is commercially important (Fig. 3). The addition of YE at 1% *w/v* led to 8.9-fold increase in bergapten production. It was seen that cultures exclusively produce bergapten (molar ratio 93%) in response to YE elicitation (Figs. 2, 3). This is commercially attractive, as it may facilitate easy extraction and purification of bergapten.

Medium Renewal Elicitation due to YE at 1% *w/v* showed distinct advantages over all other elicitors, such as highest FC induction (7.8-fold) which was achieved 24 h after elicitation, exclusive bergapten production, and leaching of more than 40% of the product in the medium. Although elicitor treatment of cultures increased the FC biosynthesis, induced FC

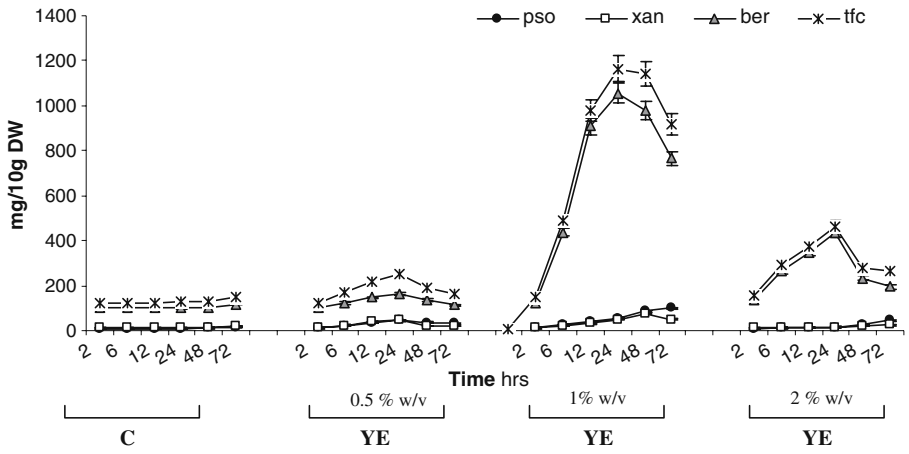

Fig. 3 Time course assay of furanocoumarin production in response to elicitation by yeast extract. *Pso* psoralen, *Ber* bergapten, *Xan* xanthotoxin, *FC* total furanocoumarins produced, *YE* Yeast extract treatment (0.5, 1 2% *w/v*),values are mean of six replicates±standard deviation

production due to elicitation could only last for a short period of time (3 days), and then it dropped rapidly (Fig. 1a). This decline in FC synthesis was accompanied by loss in cell viability (Fig. 1b). Besides elicitor toxicity, the loss of cell viability could also be ascribed to accumulation of toxic metabolites in the medium due to leaching of FCs. Therefore further process optimization required development of a strategy for biomass conservation. To overcome negative effects of elicitation, medium renewal approach was envisaged.

Renewal of the medium 48 h after elicitation was effective in preserving the culture viability. Presence of FCs in the culture medium was associated with decreased cell viability (Fig. 1a, b). The removed spent medium allowed conservation of biomass and also facilitated in situ product removal and recovery. Re-growth of biomass was achieved by renewal of the culture medium 48 h after elicitation (Fig. 4a), which has been reported for the first time. One week after medium renewal, the second culture-cycle biomass could be

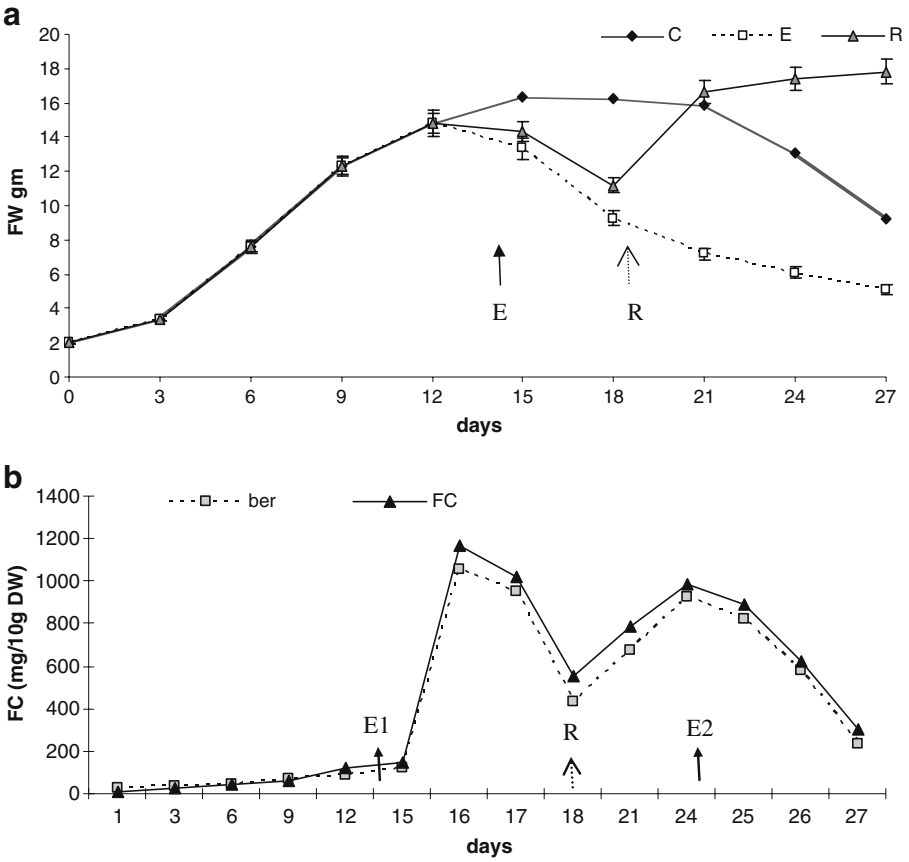

Fig. 4 **a** Effect of medium renewal on biomass in yeast extract elicited cultures. *Upwards arrow* (↑) indicates time of elicitation, ☆ indicates time of medium renewal, *C* non-elicited cultures; *E* elicitor; *R* elicitation+medium renewal, *FC* total furanocoumarins; *Ber* bergapten; *Fw* biomass; Values are mean of six replicates, *star* (☆) indicates values significant at $p \leq 0.095$ as calculated by two-way Anova. **b** Effect of medium renewal and re-elicitation on Furanocoumarin production *Upwards arrow* (↑) indicates time of elicitation, ☆ indicates time of medium renewal, *E1* First cycle of elicitation; *R* elicitation+medium renewal; *E2* Second cycle of elicitation, *FC* total furanocoumarins, *Ber* bergapten, *Fw* biomass, Values are mean of six replicates, *star* (☆) indicates values significant at $p \leq 0.095$ as calculated by two-way Anova

harvested allowing its reuse (Fig. 4a). Shoots showed growth and production characteristics similar to control (Fig. 4a). Beneficial effects of medium renewal have been reported in *Taxus chinensis* cell cultures treated by fungal elicitors [7], however such an approach has been explored for organized cultures for the first time. Successful re-growth of shoots opened up the possibility for perfusion-based approach, with or without subsequent elicitation. To assess the potential of second culture-cycle biomass to respond to elicitation, the cultures were re-elicited with YE 1% *w*/*v* 1 week after medium renewal.

Re-elicitation Induction of FCs in response to elicitation was transient; therefore for further extension of elicitation, re-elicitation strategy was employed, with the view of developing a perfusion-based approach.

The second culture-cycle biomass was re-elicited with YE 1% *w*/*v* on 24th day of culture. Pattern of FC induction was found to be similar to primary elicitation, with maximum FCs being produced 24 h after elicitation (Fig. 4b). The amount of FCs induced in secondary elicitation was 84% of that obtained in response to primary elicitation (Fig. 4b). Re-elicitation with YE led to maximum induction of bergapten, which was also similar to the primary elicitation. This indicates that the culture retained their ability to respond to elicitors. Re-elicitation allowed a second production peak, which was obtained within a week of medium renewal (Fig. 4b).

Thus, introduction of medium renewal coupled with re-elicitation strategy had many advantages: in situ product removal and recovery, re-growth of biomass and continuous production of secondary metabolites by re-elicitation.

Conclusion

Elicitation by YE (1%) on day 15 was most effective. Relative concentration of FCs depends on the elicitor used. Therefore by choosing the right elicitor, amount of the desired FC can be enhanced. Removal of spent medium 48 h after primary elicitation allowed in situ product removal and preserved culture viability. Medium renewal allowed re-growth of cultures, which has been reported for the first time. A second production peak could be obtained by re-elicitation of the second culture-cycle biomass. Thus, medium renewal and re-elicitation facilitated reuse of biomass for enhanced FC production. Bioprocess optimization in form of elicitation coupled with medium renewal with subsequent re-elicitation, as a new strategy for improved FC production has been suggested.

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