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Large scale production of Ruta graveolens. L shoots using aerated bioreactor

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Abstract
Ruta graveolens is important medicinal and aromatic plant of pharmaceutical value. To meet the industrial requirements and optimize production and decrease cost of seedlings, the bioreactor helps lot. The main aim of this work was to compare in vitro cultivation of Ruta graveolens. L different systems of liquid culture with simple aerated bioreactors. The in vitro growth of the shoots was promoted in traditional system and in aerated bioreactor. Both propagation systems used the basic MS liquid medium supplemented with 4.44 µM BA+2.84 µM IAA. The cultures were kept into culture room for 30 days with controlled temperature at 25 ±2 ^{0}C under white cold light (3000 lux) with photoperiod of 16 hours. And evaluation was done. Among the two systems, bioreactor system showed the best results for shoots and biomass production compared with the traditional system. So the aerated bioreactor system turns into effective technique to produce shoots of R. graveolens in large scale.

Keywords: *in vitro* cultivation, Aerated bioreactor, *Ruta graveolens. L*

Introduction
The micro propagation technique is still costly due to intensive hand manipulation of the various culture initial stage of establishment and response is slow and the survival of the plants in the final stage *ex vitro* is often poor, Mechanization and automation of the micro-propagation process can greatly overcome the limitations imposed by existing conventional labour-intensive methods. Various liquid medium culture techniques have therefore been developed to reduce labour costs, stimulate growth and improve multiplication rates and to increase culture uniformity by the elimination of nutritional gradients (Etienne *et al*. 1997) [9]. These techniques were reported for some plants and were shown to reduce hand manipulation and thus reduce *in vitro* plant production costs. When it is required to increase production to large-scale, the bioreactor systems are options to be considered, because they reduce the production costs of seedlings

Progress in tissue culture automation depend on the use of liquid cultures in bioreactors, which allow fast proliferation, mechanized cutting, separation, and automated dispensing (Sakamoto *et al*. 1995) [20]. Bioreactors are usually described in a biochemical context as self-contained, sterile environments which capitalize on liquid nutrient or liquid/air inflow and outflow systems, designed for intensive culture and affording maximal opportunity for monitoring and control over micro environmental conditions. Bioreactor of different types extensively studied by different authors for production shoots and biomass and secondary metabolite. Shoot cultures cultivated in bioreactor showed the synthesis of lower terpenoids. hoot cultures of *Spathiphyllum cannifolium* (Dewir *et al*. 2007) [6] and *Stevia rebaudiana* (Sreedhar *et al*. 2008) [22] were carried out in bubble column bioreactors in order to produce flowers and biomass, respectively. Organ culture of *Lavandula officinalis* was cultivatedin 5 L bubble column bioreactor to obtain rosmarinic acid and organ culture of *Hypericum perforatum* in 2 L stirred tank bioreactor to produce hypericin (Wilken *et al*. 2005) [24]. Shoot culture of *Ananas comosus* was performed in a 10Lairlift bioreactor by Firoozabady and Gutter son (2003) [10].

Ruta graveolens L. is well known medicinal and aromatic evergreen perennial herb of Rutaceae. The plant is having wide spectrum of different phytoconstituents. Several investigations have been conducted on the chemical constituents of *R. graveolens* (Benazir *et al;* 2011) [2]. *R. graveolens* was found to contain highest concentration of furocoumarins (Poutaroud *et al.* 2000) [19]. Furocoumarins (FCS) have gained wide applications in Pharmaceutical industry. The whole herb is, antispasmodic and carminative, expectorant, ophthalmic and rubefacient abortifecent, anthelmentic (Ivanova *et al.* 2005) [13]. Furocoumains e.g. Xanthotoxin, Burgaptin, Isopimpinelin have been applied in the treatment of skin diseases e.g. Psoriasis, Mycosis fungoids or in pigmentation disorder e.g. Vitilago (Ekierts *et al.* 2005) [8].

The volatile oil from *R. graveolens* possesses phototoxical, bacteriostatistical and anthelmentic activities (Petit-Paly *et al.* 1988) [17]. Fungicidal activity of the essential oil of *R. graveolens* and 8-MOP has been reported (Oliva *et al.*; 1999; Oliva *et al.* 2003) [15, 16]. Rutin- a compound isolated from *Ruta,* which has been suggested to have an anti-oxidant properties and to reduce triglycerol level (Bernardo *et al.* 2002) [3]. Wide applications of *Ruta graveolens* L. in pharmaceutical industry has led to increased interest in large scale plant production, with emphasis on use of in vitro cultures. Attempts have been made previously to develop protocols for the micropropagation of *Ruta graveolens* (Castro and Barros, 1997; Faisal *et al*., 2005, 2006; Sekar. T and Gopalkrishnan M. (2007) [5, 21]. Bohidhar *et al*., 2008, Tejavathi *et al.* 2010) [4, 23]. Diwan and Malpathak (2008) [7] reported one-step protocol with improved regeneration efficiency for multiple shoots induction employing liquid culture systems. They scaled up shoots in liquid culture system from 250 mL to 2 L in Erlenmeyer flask and further scale up of 5 L, in glass culture vessel. Gontier *et al.;* (2005) [12] developed an efficient low cost bioreactor for production of furocoumarins with *R. graveolens* L. shoot cultures. The aim of present investigation was to compare the traditional systems of *in vitro* propagation with simple aerated bioreactor for *Ruta graveolens* for large-scale shoot biomass production.

Materials and Methods

Establishment and maintenance of *in vitro* shoot cultures

Healthy shoots maintained on MS solid medium with 4.44 µM BA + 2.84 µM IAA were inoculated in liquid MS medium with the same composition of PGR with 3 % sucrose and pH adjusted to 5.7. Shoot cultures were agitated on gyratory shaker at 100 rpm under fluorescent light using 16 hr photoperiod and temperature was maintained at 23 $\pm 2^0$C. Shoots were maintained regularly by subculturing every 15 days and these shoots were used for inoculating bioreactor. The multiple shoots of *Ruta graveolens* were scaled up to 500 ml conical flask prior to the cultivation in bioreactor for 30 days. When 250 ml flask was used 3 gm of shoot were inoculated in 50 ml MS medium and in case of 500 ml flask, 5gm of shoots for 100 ml medium.

Bioreactor system: Air driven bioreactor is the simplest type of bioreactor in which air filter-sterilized air is passed into the vessels bottom and aeration is done to medium. Since this type of bioreactor are easy to run and cost effective. Simple aerated bioreactor system with capacity 2 litre and 5 litres were designed and fabricated at the KET's V. G. Vaze College, Mulund. The reactors were used for the mass cultivation of *Ruta graveolens*.

Bioreactor accessories: An air compressor (High speed appliances Mumbai) was used for compressed air. Air was drawn from atmosphere into compressor cylinder through efficient air filters removing dust particles.

Control panel and air sterilization system: Outlet of the compressor was fitted with metal pipes for the passage of air from compressor to vessel containing sterilized water. The moist air saturated in vessel containing sterilized water was passed to vessel containing to bioreactor vessel. Air was processed before coming in contact with sterile shoots. Air pressure of 1.0 bar was maintained. Sterile air inlet and outlet were connected with venting filter Midisart® 2000 (Sartorus staedim biotech, Goettingen, Germany) to maintain sterility.

Bioreactor vessel: Aerated bioreactor was fabricated with 2 litre and 5 litre capacities. Both the bioreactor vessels were designed in such way the reactor vessel with four ports. Two ports for air inlet and outlet and other two ports for medium inlet and out let and air inlet and outlet were connected to hydrophobic air filter (Midisart® 2000).2 Litre bioreactor was conical shaped with height of vessel 23 cm and diameter of base 18 cm with air tight lid of 7.5 cm. The lid consisted of four ports, an air-inlet, air-outlet, medium-inlet, and medium-outlet. Air was circulated with the help of a circular sparger with 5.5 cm diameter which was placed 1 cm from the bottom of the reactor (Fig.1.1).

Whereas 5 litre bioreactor was 31 cm in height and 17 cm diameter with lid (Fig.1.2). The air tight lid of bioreactor vessel also consisted of four ports, an air- inlet, air- outlet, medium-inlet, and medium-outlet. Air was circulated with the help of a circular sparger with diameter 12 cm diameter which was placed 2 cm from the bottom of the reactor below the perforated steel grid. The air-inlet, air-outlet ports were connected to Teflon micro porous air filter (Midisart® 2000) with a pore size of 0.2µ, to maintain sterility. The sterile compressed air was first passed into vessel filled with sterilized water. This was done to retain moisture level of air which prevented drying of cultures. The sterile moist air then introduces in the bioreactor vessels through the sparger. The continuous outflow of air was maintained through air- outlet port. All bioreactor systems were connected with the help of autoclavable silicon tubing's.

Culture conditions: Both the bioreactors were incubated in culture room at 25 ± 2^0C under 16 hrs photoperiod with cool, white fluorescent tube light (3000 Lux) with 80% relative humidity for 30 days.

Results and Discussion

Establishment and scale up of multiple shoots

Shoot cultures isolated from differentiated shoot grown on MS agar medium supplemented with 4.44 µM BA + 2.84 µM IAA were successfully cultivated in MS liquid medium supplemented with same hormone supplements. Shoot cultures were scaled up from 250 ml conical flask to 500 ml conical flask. This was kept for 30 days for acclimatization and then was transferred into the bioreactor.

When 250 ml flask containing 3 gm of initial inoculum on 50 ml MS liquid medium with 4.44 µM BA + 2.84 µM IAA was harvested after 15 days, 14 gm of biomass was recovered Shoot cultivated in 500 ml flask with 5 gm of initial inoculum on 200 ml MS medium with same hormone composition were incubated for 30 days. After harvesting on 30th day, 20 gm of final biomass was obtained. Bioreactors with same media composition were inoculated with 15 days old multiple shoot of *R. graveolens* from conical flasks which were maintained on gyratory shaker. For 2 litre bioreactor 10 gm of healthy shoots were inoculated in 400 ml media and for 5 litre bioreactor 16 gm of shoots were inoculated in 1000 ml media under aseptic conditions. The inoculated shoots acclimatize in two days to bioreactor conditions and then they start to multiply vigorously. At the end of first week the base of bioreactor vessel gets covered with the shoots. At the end of 30 days 500ml flask yield 20gm of shoots measuring 5-6 cm in height (Fig.2A), shoots

were showing hyperhydricity with dark green leaves as shoots were submerged in media. In 2 litre bioreactor the yield obtained measured 180gm of shoots with 10 to 11 cm length. The shoots in this bioreactor were green healthy with little vetrification were observed (Fig.2B). In 5 litre bioreactor vessel 330 gm of shoot biomass was obtained. These shoots measured 15-18 cm in length these shoots were erect and more clustered with leaves broader (Fig.2C, D) than normal shoots. These shoots obtained were without vitrification. Cultures obtained from 5 lit bioreactor system showed better quality and higher multiplication rates, than 2lit, since it was partly submerge in liquid media and the shoots grown in 5 litre bioreactor were grown on perforated steel grid. Shoot culturing in the aerated bio reactor models resulted in a 4.1 folds in 2 litre bioreactor vessel and 5.2 folds in 5 litre bioreactor compared to 500ml conical flask culture. Aerated bioreactor system proved significantly in large scale production of *Ruta graveolens.* The high efficiency of plant propagation using bioreactors has been revealed by the work of many researchers. Phatak and Heble (2002) [18] successfully scaled up *Mentha arvensis* shoot biomass by 3.7 folds in 2 litre aerated bioreactor by using MS basal liquid medium containing 5mg/l BA + 0.5mg/l NAA. Similarly Joshi and Paratkar (2010) [14] also succeeded in large scale production of *Eclipta Alba* shoot biomass by using 2 litre aerated bioreactor. They observed 6 fold increases in biomass production by using MS basal liquid medium containing 0.44µM BA. Bioreactor cultivation for shoot cultures of *Artmissia annua* has been demonstraterd by Fulzele and Heble (1994) [11].

Table 1.1: Comparison of Biomass in Different Culture System

Type of Reactor	Initial Biomass to liquid ratio (gm/ml Fresh weight)	Incubation period (Days)	Final Biomass (gm)
500ml Flask	0.05	30	20
2 Litre Bioreactor	0.025	30	180
5 Litre Bioreactor	0.016	30	330

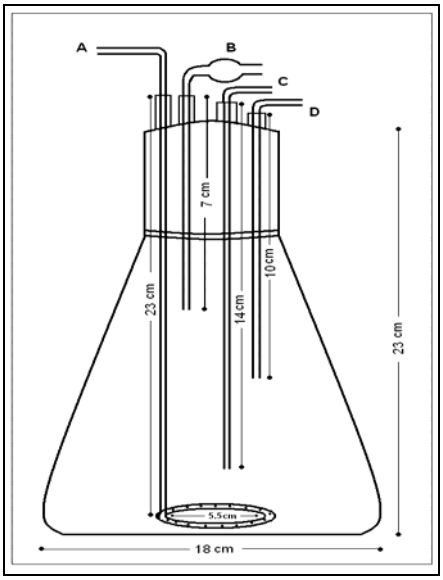

Fig 1.1: Schematic diagram of 2 litre bioreactor.

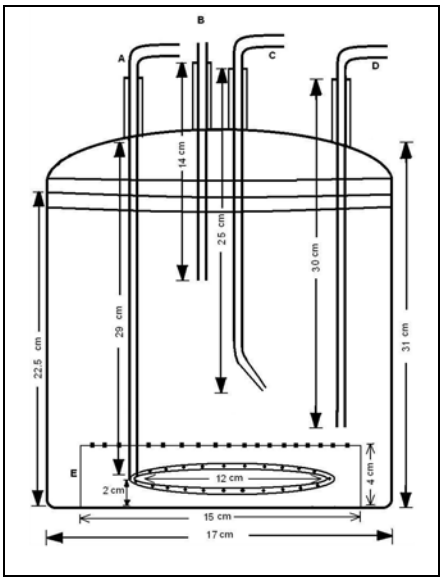

Fig 1.2 Schematic diagram of 5litre bioreactor

A: - Sparger
B: - Media inlet port
C: - Air exhaust port
D: - Additional outlet

A: - Sparger
B: - Media inlet port
C: - Air exhaust port
D: - Additional outlet port
E: - Perforated steel grid port

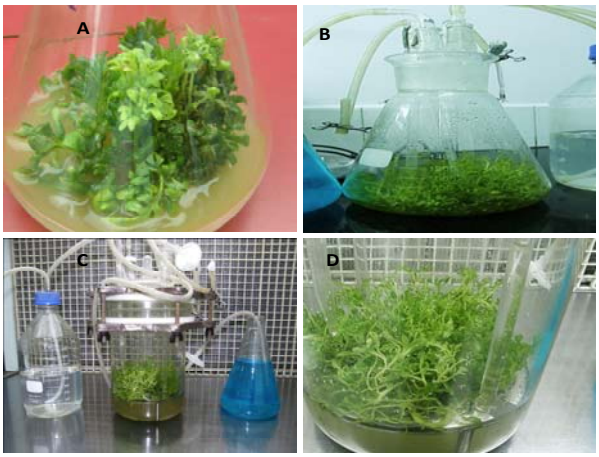

Fig.2 A-500ml culture flask with shoots.
B - 2.Lit. Bioreactor assembly with shoots
C - 5. Lit. Bioreactor assembly
D - 5. Lit. Bioreactor assembly with shoots

Conclusion

Shoot culturing in the simple aerated bioreactor systems resulted in a 4.1 folds increase in 2 litre bioreactor vessel and 5.2 folds increase in 5 litre bioreactor compared to 500 ml conical flask culture. The increases in the shoot biomass suggest that the permanent ventilation and the absorption of nutrients from the liquid media are responsible for the favourable effects. We can conclude that our system allowed an efficient nutrient uptake and a good aeration. Thus, provides good growing conditions and makes it possible to achieve high multiplication rate of shoots. The bioreactor system makes it possible to produce large quantities of plants in a relatively small area. Thus aerated bioreactor system proved significantly effective in large scale production of *R. Graveolens*

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