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Variability of Azadirachtin in *Azadirachta indica* (neem) and Batch Kinetics Studies of Cell Suspension Culture

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Abstract Seeds of neem were collected from different parts of India and analyzed for their azadirachtin content by High Performance Liquid Chromatography (HPLC). In order to assess the effects of genotypic and geographical variation on azadirachtin content in cell cultures, callus development was attempted in the seeds containing high and low concentration of azadirachtin. The concentration of azadirachtin in callus cultures was significantly affected by the explant source. Seed kernels with higher azadirachtin content produced higher azadirachtin content in callus cultures and lower azadirachtin content was seen in callus cultures produced from seed kernels with low azadirachtin content. The protocol for development of elite stock culture of *Azadirachta indica* was established with the objective of selecting a high azadirachtin-producing cell line. The highest azadirachtin-producing cell line was selected and the effects of different media and illumination conditions on growth and azadirachtin production were studied in shake flask suspension culture. Detailed batch growth kinetics was also established. These studies provided elite starter culture and associated protocols for cultivation of *A. indica* plant cell culture in the bioreactor.

Keywords: azadirachtin, batch kinetics, callus culture, cell line, seed variability, suspension culture

INTRODUCTION

Azadirachta indica A. Juss (neem), belonging to the family *Meliaceae*, is native to the Indian subcontinent. It grows in a wide range of agroclimatic zones, however, exhibiting its wide genetic base. It has been well known for its medicinal, agrochemical, and non-woody products since ancient times [1,2]. Today, due to its remarkable insecticidal properties shown by azadirachtin ($C_{35}H_{44}O_{16}$) and other related limonoids (AZRL), the tree has attained global importance. The use of azadirachtin and other triterpenoids as biopesticides are currently the most effective, non-toxic, and environmentally harmless means of controlling pest problems [3,4]. The worldwide attention for azadirachtin is due to its potency at relatively low concentrations (in parts per million ranges) and varying modes of action against a wide range of agricultural insects/pests associated with its safety towards non-target organisms, including human beings. It shows an array of activities against insects and works as an antifeedant, insect growth regulator, and sterilant [4,5].

With growing interest in this active, environmentally safe chemical, the supply is much less than the huge de mand. Besides, in the extraction of azadirachtin from seeds some problems remain, such as variation in azadirachtin content due to genetic variability and collection of seeds from different geographical and climatic regions [6-8]. Collection of lower quality neem seeds has led to inconsistent quality of products and an increase in the chances of fungal contamination during the processing of seeds. The need for homogenous, controlled production has prompted an investigation into the production of this active metabolite from plant cell culture [9]. Plant cell culture is a promising biotechnological approach, and has been adopted for production of various categories of secondary plant metabolites [10,11]. Although the lower yield of these compounds in cell culture has restricted the commercial utilization of this approach, several strategies have been proposed to enhance product yield and productivity in the cell culture system.

The phenomenon of variation among cell culture lines derived from the same plant species, or even the same plant but different plant parts with respect to expression of biochemical capabilities, is well documented [12,13]. This is particularly important in the case of development of the cell culture system of *Azadirachta indica,* due to known variations in azadirachtin content due to genetic variability and environmental factors [14,15]. Establishment of callus culture and, subsequently, suspension culture from the plant part, which contains the highest amount of metabolite [16] and selected high yielding genotype, is desirable to maximize the formation of par-

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ticular compounds in the cell culture system [17]. Although the variability in azadirachtin concentration in seeds is very well known, no correlation had been established between variation of azadirachtin content in seeds and in the cell culture system. Additionally, there are very few reports on the variability of azadirachtin content *in vitro* or on the dynamics of the biosynthetic capacity of *A. Indica* cells in suspension cultures. Therefore, in the present investigation, the variability of azadirachtin in seeds and in *in vitro* cultured cells was studied to select a high azadirachtin-accumulating cell line. With the above objective in mind, neem seeds from 29 provenances of India and one from Thailand were screened and selected. Seed kernels with different azadirachtin content were then used for callus development using different hormone combinations. In order to select the best *in vitro* source of azadirachtin, all calli were compared with each other to get a high azadirachtin-containing cell line. Suspension cultures were developed from the cell line that produced the highest amount of azadirachtin. The effect of culture media and illumination on cell growth and azadirachtin accumulation was studied. The detailed growth kinetics of *A. indica* shake flask suspension culture were also established. Few reports are available regarding the variability of azadirachtin content *in vitro* and on the dynamics of the biosynthetic capacity of *A. indica* cells in suspension cultures; therefore, it seemed appropriate to undertake the above task in order to establish *in vitro* biotechnological production of azadirachtin.

MATERIALS AND METHODS

Plant Material

Seed samples of *Azadirachta indica* were collected from wild plants of 26 provenances, planted in the Arid Forest Research Institute (AFRI), Jodhpur (India). Seed samples were also collected separately from different parts of India, *e.g.* Ahmedabad (Gujarat), Lucknow (Uttar Pradesh), Tamilnadu (Tamilnadu) and Trivendrum (Kerala). Leaves were derived from a tree at the Indian Institute of Technology, Delhi (India) Campus.

Extraction of Azadirachtin

Dried neem seed kernel powder (1 g) was first deoiled using hexane with constant stirring for 45 min. The hexane extract was discarded and the process was repeated with fresh hexane two more times. After filtration, the deoiled, dry mass was extracted twice with methanol for 30 min each (1 g/10 mL). Water was added to the combined filtered methanol extract at a ratio of 60:40. This aqueous methanolic layer was partitioned twice against 1:1 volume of dichloromethane. A pooled fraction of dichloromethane was evaporated under vacuum below 40°C and redissolved in methanol prior to analysis by HPLC.

Cells were dried at 30 ± 2°C until a constant weight was achieved. For the extraction of azadirachtin from cells, the deoiling step was omitted. The rest of the procedure for azadirachtin extraction was similar to that used for neem seeds.

Thin Layer Chromatography

Qualitative analysis of azadirachtin was done by thin layer chromatography on silica gel 60 F_{254} plates (Merck, Germany). The extracts were eluted with a dichloromethane: acetone (4:1) solvent system. Spot visualization was accomplished by spraying with a vanillin-sulfuric acid-95% ethanol mixture (1.5 g: 0.5 mL: 50 mL). Azadirachtin produced a blue-green spot with an R_f value of 0.36.

High Performance Liquid Chromatography

Quantitative estimation was done on an Agilent 1100 series (Agilent Technologies, USA), HPLC system equipped with quaternary pump (G1311A) and diode array detector (G1315B) with temperature controller (G1316A). Nova Pak RP-C_{18} column (Waters, USA) (250 × 4.6 mm) was used with acetonitrile:water (10:90) in the mobile phase at a flow rate of 0.5 mL/min. Column temperature was maintained at 45°C. The eluted samples were detected at 214 nm wavelength. Commercially available azadirachtin A (Sigma) was used as the standard.

Culture Initiation

Neem fruits were depulped under running tap water and the seeds enclosed in endocarp were dried overnight at ambient temperature in shade. Seeds were washed in 1% v/v Savlon (Johnson and Johnson, USA), surface sterilized in 70% v/v ethanol for 1 min, and rinsed three times with sterile distilled water (SDW). This was followed by sterilization in 0.1% w/v $HgCl_2$ for 8 min and rinsing with SDW 4~5 times. The seeds were dissected and seed kernel along with embryo was excised and used as an explant for callus induction on Murashige and Skoog medium (MS) [18] along with the following hormone combinations; (2,4-D 0.2 and 1.0 mg/L); (2,4-D 0.2 mg/L: BA 0.5 mg/L), (2,4-D 0.2 mg/L: BA 1.0 mg/L); (2,4-D 1.0 mg/L: BA 0.5 mg/L), (2,4-D 1.0 mg/L: BA 1.0 mg/L); (2,4-D 2 mg/L: BA 0.5 mg/L), (2,4-D 2 mg/L: BA 1.0 mg/L); (NAA 1 mg/L: BA 0.5 mg/L), (NAA 1 mg/L: BA 1.0 mg/L); (NAA 2 mg/L: BA 0.5 mg/L), (NAA 2 mg/L: BA 1.0 mg/L); (IBA 1.0 mg/L: BA 0.5 mg/L), (IBA 1.0 mg/L: BA 1.0 mg/L); (IBA 2 mg/L: BA 0.5 mg/L), (IBA 2 mg/L: BA 1.0 mg/L). Callus was also developed from leaves. Leaves were thoroughly washed in 1% v/v Savlon for 5 min and thoroughly rinsed with SDW. Surface sterilization was done in ethanol (70% v/v) for 30 sec and in $HgCl_2$ (0.1 % w/v) for 3 min, followed by three times washing with SDW after each step. Leaves were cut into sections (approx. 1 × 1 cm) and incubated on MS medium with the following combinations of growth hormones, (IBA 4.0 mg/L: BA 1.0 mg/L), (IBA 4.0 mg/L: BA 2.0 mg/L), (IBA 4.0 mg/L: BA 4.0 mg/L), (IBA 8.0 mg/L: BA 1.0 mg/L), (IBA 8.0 mg/L: BA 2.0 mg/L), (IBA 8.0 mg/L: BA 4.0 mg/L) (NAA 4.0 mg/L: BA 4.0 mg/L). The cultures were incu-

bated at 25 ± 2°C temperature under 16/8-h light/dark regime. After callus induction, the biomass was transferred aseptically into fresh media and subcultured regularly. The callus cultures were grown under 16/8-h light/dark regime. Cell suspensions of *A. indica* were developed on MS medium with NAA 2 mg/L: BA 1.0 mg/L and containing 3% sucrose. Before starting experimental treatments, the time course of suspension culture growth and azadirachtin production was evaluated to establish the length of time required for batch cultivation of liquid state culture. Subculturing was done at a regular interval on the 10th day.

Effect of Different Culture Medium in Suspension Culture

Five commonly used standard plant cell culture media *viz.* MS, B5, White, Nitsch, and Eriksson [19] were tested for the growth and azadirachtin production in *A. indica* suspension culture. The *A. indica* cells were inoculated to give 2 g/L final (DW) of cells in 250-mL Erlenmeyer flasks containing 50 mL of each medium. Incubation was done under the same culture condition as previously described. The flasks were harvested after 12 days to analyze for dry cell weight and azadirachtin.

Effect of Illumination on Cell Growth and Azadirachtin Production

A. indica cell suspension cultures were cultivated under a 16/8-h light/dark regime and in complete darkness to assess the effect of light and dark on cell growth and azadirachtin production in MS medium. Dry cell weight and azadirachtin content were analyzed after 12 days.

Growth Kinetics of *A. indica* Suspension Culture

To study the growth kinetics of *A. indica* cells, 2 g/L (DW) cells were inoculated in 50 mL media in 250-mL Erlenmeyer flasks. The cultures were incubated on gyratory shakers and incubated at 125 rpm and 25 ± 2°C in 24 h dark. Each individual flask was harvested at a regular interval (2 days) of time and analyzed for dry cell weight, residual sugar, pH, and azadirachtin content.

Measurement of Dry Cell Weight and Residual Sugar

The cells were centrifuged at 3,000 rpm, washed with distilled water, centrifuged again, and dried at 28 ± 2°C temperature to determine dry cell weight. The reducing sugar was estimated as glucose by the dinitrosalicylic acid method [20].

RESULTS AND DISCUSSION

Azadirachtin Content Variation in Seeds of Different Provenances

Neem seeds collected from 30 provenances were evaluated to study the variability of azadirachtin content due to genetic and environmental factors. The azadirachtin content of seeds collected from different provenances is arranged in ascending order of azadirachtin A concentrations (Table 1). The investigation revealed a large variation in azadirachtin content among the seed material obtained from different parts of India. Significant variation was observed to the extent of 0.21 to 5.1 mg/g per kernel. The seeds obtained from the Arid Forest Research Institute, Jodhpur (26 provenances out of 30 provenances tested) were exposed to the same edaphic and environmental conditions of temperature, rainfall, and humidity. The level of variation of azadirachtin among the seeds, however, was significantly higher, with a minimum of 0.21 mg/g (Ai-33) and maximum of 3.99 mg/g (Ai-21) indicating that the synthesis of azadirachtin is largely dependent upon the genotype of neem seeds. These results are in accordance with another study in which the individual trees of a particular agroclimatic zone (with similar environmental conditions) showed different levels of azadirachtin content. It was, therefore, concluded that there are individual genetic differences among neem trees and that synthesis of azadirachtin is not only dependent upon environmental differences in temperature, rainfall, and humidity [15]. On the other hand, seeds from different agroclimatic zones, namely Ahmedabad (Gujarat), Lucknow (Uttar Pradesh), Trivendrum (Kerala), and Tamilnadu (Tamilnadu) also indicated variation ranging from 1.07 mg/g (Ahmedabad) to 5.1 mg/g (Trivendrum), exhibiting either the effect of different genetic makeup or of environmental conditions. Variability in azadirachtin content due to different environmental conditions such as rainfall, drought, humidity, and seasonal differences of various geographic regions of India and the world have been reported in some studies [7,8]. Therefore, the sizeable variations in azadirachtin content in the present study may be attributed to both the different genetic make up and to varying the environmental conditions of different geographical regions. The highest amount of azadirachtin (5.13 mg/g) was obtained from Trivendrum (Kerala) seeds and sufficiently large content (3.22 mg/g) was obtained from seeds of Tamilnadu (fourth highest among all the seeds tested), the parts representing the southern region of India. This was in accordance to the study in which a state-wise compilation of the azadirachtin content was performed and it was concluded that the southern peninsula has comparatively higher content and yields of azadirachtin [21].

Azadirachtin Content Variation in Callus Cultures

Callus culture from seed kernels and leaves of the local area (Delhi) were developed. For the development of callus culture, different hormone combinations were tested as mentioned in the materials and methods section. Cultures from four seed kernels (Ai-24, Ai-25, Ai-41 and Ai-43, representing higher, as well as lower, azadiracachtin-containing seeds) which showed callus development on the medium with same hormonal combination, *i.e.* on MS + NAA 2 mg/L: BA 1.0 mg/L, were selected and designated as four different cell lines; AG-24, AG-25, AG-41 and AG-

Table 1. Variability of azadirachtin content in seeds of different provenances

S. NO.	Seeds kernel	Azadirachtin A (mg/g)
1	Ai-33 (J)	0.21 ± 0.14
2	Ai-39 (J)	0.85 ± 0.07
3	Ai-5 (J)	0.98 ± 0.05
4	Ai-41 (ABD)	1.26 ± 0.18
5	Ai-34 (J)	1.30 ± 0.03
6	Ai-10 (J)	1.49 ± 0.04
7	Ai-36 (J)	1.65 ± 0.09
8	Ai-37 (J)	1.66 ± 0.08
9	Ai-45 (THL)	1.79 ± 0.03
10	Ai-19 (J)	1.81 ± 0.08
11	Ai-17 (J)	1.81 ± 0.08
12	Ai-2 (J)	1.87 ± 0.05
13	Ai-3 (J)	1.92 ± 0.05
14	Ai-24 (J)	1.93 ± 0.09
15	Ai-40 (J)	2.11 ± 0.10
16	Ai-31 (J)	2.13 ± 0.11
17	Ai-26 (J)	2.13 ± 0.02
18	Ai-20 (J)	2.23 ± 0.005
19	Ai-42 (LKW)	2.39 ± 0.03
20	Ai-25 (J)	2.55 ± 0.07
21	Ai-28 (J)	2.63 ± 0.08
22	Ai-35 (J)	2.65 ± 0.27
23	Ai-12 (J)	2.80 ± 0.10
24	Ai-6 (J)	2.85 ± 0.06
25	Ai-22 (J)	2.93 ± 0.08
26	Ai-38 (J)	3.01 ± 0.01
27	Ai-44 (TN)	3.22 ± 0.19
28	Ai-4 (J)	3.36 ± 0.05
29	Ai-21 (J)	3.99 ± 0.01
30	Ai-43 (TRIV)	5.13 ± 0.01

Each value represents the mean ± S.D of three samples.
J-Jodhpur; ABD-Ahmedabad (Gujarat); THL-Thailand; LKW-Lucknow (Uttar Pradesh); TN-Tamilnadu; TRIV-Trivendrum (Kerala)

43. Other seed kernels which gave rise to callus on different hormone combinations were not accounted for in the present comparison. This was done to minimize the variation caused by different hormonal combinations and to confirm that the difference in azadirachtin content in cell lines originated due to varying concentrations of azadirachtin in the parent explant and was not due to hormone treatment. The cell line, which originated from leaves (AGL), was obtained on MS + IBA 8 mg/L: BA 4 mg/L. The azadirachtin contents of selected cell lines are shown in Fig. 1. Significantly higher differences were found in the azadirachtin content among cell lines obtained from seeds of different regions and leave explants. A maximum concentration of 1.89 mg/g of azadirachtin A was detected in the AG-43 cell line developed from Ai-43 seed kernels (Trivendrum, Kerela), having the highest azadirachtin content of the seed kernels studied. This was approximately 19 times higher than the lowest azadirachtin producing cell line, Ai-41 (Jodhpur), and also significantly greater than the cell line developed from leaves (0.23 mg/g). Similarly, variation in azadirachtin content was observed from these cell lines derived from leaf and bark explants of two different regions [22]. Variation in azadirachtin content in different cell lines derived from seeds of different genetic makeup, different geographical origin, and from different plant parts indicated that the product concentration in explant influences the accumulation of secondary metabolites in the cell line developed. A similar observation was made in *Linum album* where a concentration of 5-methoxy podophyllotoxin was found to depend on the genotype used for the initiation of the culture [23]. This variation may be attributed to the high heterogeneity in metabolic productivity of constituent cells of the explant source, which is being taken for cell line development. Development of cell lines from selected high yielding genotypes and further screening and cloning of high-yield cells from a group of cells can greatly enhance the overall product yield. There are a few reports in the literature regarding the selection of cell lines for better yielding subclones [24-28], but in most cases, the screening was done for the cells producing coloured compound or for cells with highly fluorescent compound. These methods of screening can be done visually by just selecting the more pigmented part of cells or with the help of flow cytometry, respectively. This cannot be applied for the cells, like azadirachtin, which produce colourless compounds.

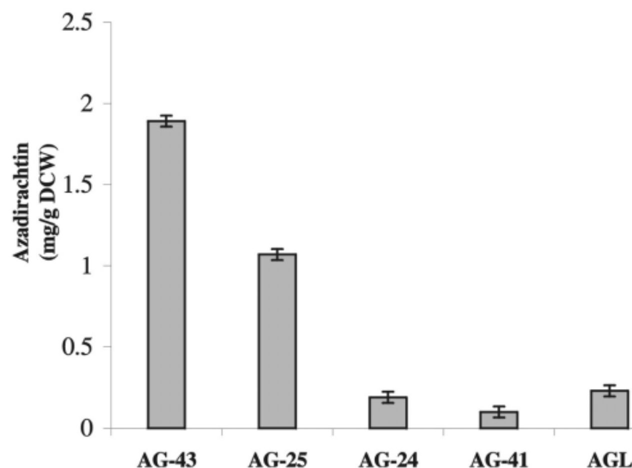

Fig. 1. Azadirachtin content in different cell lines of *A. indica*. Each value represents the mean ± S.D of three samples.

Effect of Different Culture Medium in Suspension Culture

Different culture medium has been used for the growth and secondary metabolite production of various plant cell culture systems. Macro and micronutrients have been

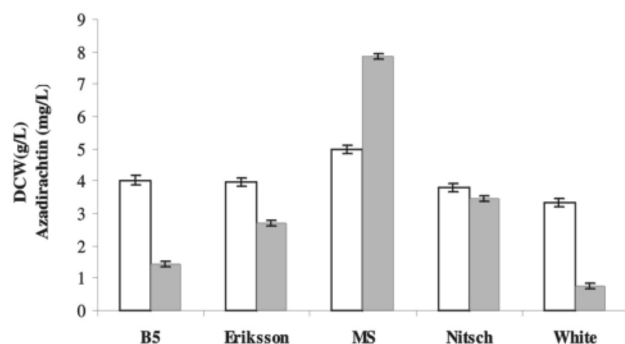

Fig. 2. Effect of different culture media on growth (DCW) and azadirachtin production in *A. indica* suspension cultures (□ DCW ■ Azadirachtin). Each value represents the mean ± S.D of three samples.

Table 2. Effect of illumination on cell growth and azadirachtin production in cell suspension culture of *Azadirachta indica.*

Light/dark regime (h/day)	DCW (g/L)	Azadirachtin (mg/L)
16/8 h	4.98 ± 0.064	7.87 ± 0.041
24 h darkness	5.94 ± 0.10	9.35 ± 0.089

Each value represents the mean ± S.D of three samples.

reported to impart considerable influence on growth and secondary metabolite formation in cultured cells. In general, increased levels of nitrate, ammonium, and phosphate tend to support rapid cell growth, whereas the depletion of some nutrient leads to growth limitation with a concomitant enhancement of secondary metabolite [17]. To select the most appropriate media for both cell growth and product formation in *A. indica* suspension cultures, the effects of various culture media were studied. Fig. 2 shows the effects of five commonly used culture media on cell growth and azadirachtin production by *A. indica* in suspension culture. Azadirachtin accumulation was observed in all the media tested. The MS medium demonstrated the highest growth of 4.98 g/L and azadirachtin production of 7.87 mg/L.

Effect of Light Illumination on Cell Growth and Azadirachtin Production

The synthesis of secondary metabolite is affected by environmental regulation. Light is an important parameter which can affect the biosynthesis and accumulation of product. Therefore, the effects of different intervals of light irradiation were examined. In the present study, cell growth and azadirachtin were found to be increased in 24 h darkness intervals, compared to 8 h darkness and 16 h light. Around 19% increase in both growth and azadirachtin production was observed when *A. indica* cells were cultivated in the dark (Table 2). Azadirachtin A has been reported to be labile to sunlight and pure azadirachtin A was found to degrade rapidly in the presence of sunlight [29]. It appears that light affects the expression of enzymes involved in primary and secondary metabolite pathways. More studies involving the activities of the respective enzymes would be required to confirm this postulation.

Kinetics of Growth, Azadirachtin Biosynthesis, and Sugar Consumption of *A. indica* Suspension Culture

The kinetics of growth, azadirachtin, residual sugar, and pH were investigated in shake flask cultures of *A. indica*. The time course study on growth and azadirachtin revealed that the biomass in suspension culture increased up until the 12th day, which was associated with an increase in azadirachtin synthesis indicating growth-associated product formation (Fig. 3). The maximum specific growth rate μ_{max} was found to be 0.16 per day. The maximum azadirachtin content of 9.35 mg/L (1.58 mg/g) was obtained on 12th day, with maximum cell growth (5.94 g/L), which declined thereafter. The decrease in the azadirachtin content after the 12th day of cultivation could be explained either by a low production rate, by the initiation of a degradation process, or by a change in the chemical structure, leading to other non-detectable compounds. The pH of the shake flask suspension culture medium was monitored subsequent to each sampling. It was observed that after an initial drop from the second to the sixth days of culture, the pH subsequently increased during the late log (8th day to 12th day) and stationary phase of growth. This may be due to the differential consumption of ammonium and nitrate ions during the growth cycle. Similar pH profiles, in which pH of the medium was first decreased and then increased towards the end, had been reported in the *Panax quinquefolium* cell line for saponin production [30]. The sugar consumption profile revealed that approximately half the sugar was left unconsumed in the medium even after the growth had ended at 16 days. These results were in contrast to our previous studies in which MS medium did not induce the formation of azadirachtin and product formation had only been detected in White's medium on the 9th day of cultivation, thus necessitating the use of a two-stage process for the production of azadirachtin [31]. This difference in the pattern of azadirachtin accumulation may be due to the different cell lines involved in the studies. In the previous study, the cell line had been derived from the nodal segment (Delhi University, Delhi, India). In the present study, however, the seed kernel-derived cell line (of Trivendrum) was used. Azadirachtin production from plant cell culture had also been previously reported in some other studies. However, these studies only dealt with callus induction and culture maintenance [22,32,33]. In another study, the amount of azadirachtin bioproduction in callus culture was found to be 0.006% on dry wt. basis, which was significantly lower than in the present study [34]. Suspension cultures were developed for demonstration of the antifeedant effects of *in vitro* culture extract [35] and to study permeabilization of azadirachtin in medium [36]. Detailed growth kinetics have not yet been reported. The product formation kinetics of azadirachtin in cell culture have been reported, in

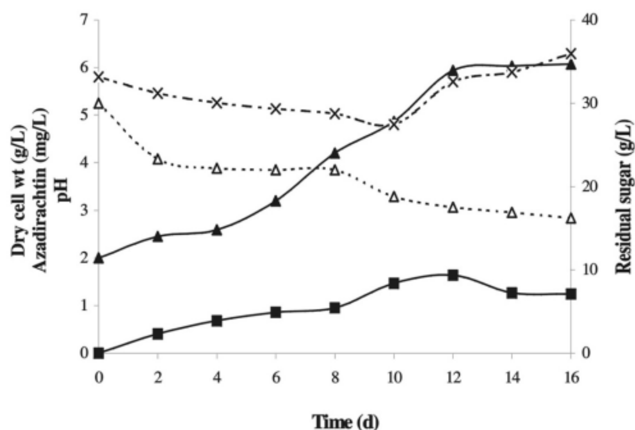

Fig. 3. Growth and production profile of *A. indica* in shake flask suspension culture. ▲ Dry cell weight; ■ azadirachtin; △ residual sugar; x pH.

which the maximum amount of azadirachtin in cells was found to be 9.31 ± 2.40 mg/L, and the effect of various precursors, elicitors, permeabilizing agents, and growth retardants, which further enhanced the yield, were studied [37]. Hairy root cultures have also been established for azadirachtin production [38], but the yields have been 27 μg/g, as compared to 1.58 mg/g from the present study. In the present study, a high productivity of 0.77 $mgL^{-1}d^{-1}$ (almost comparable to the highest literature reported value) [37] was obtained; this may be attributed to vigorous selection and development of protocols of suspension culture from a high azadirachtin-containing cell line. Further improvements in yields through the varying of the medium composition, statistical media optimization, and the addition of elicitor and precursors are in progress. These may lead to a substantial increase in production and establish the use of this protocol as a preferred choice for *in vitro* mass production in bioreactors.

CONCLUSION

In conclusion, a large variation in azadirachtin content in seeds, 0.21 to 5.1 mg/g of the kernel (of different genotypes and with origins in different geographical regions) and their respective callus cultures (0.1 to 1.89 mg/g dry wt. basis) were found. A cell line with higher initial yield of azadirachtin was selected and shake flask suspension culture was established. It featured maximum growth of 5.94 g/L and azadirachtin concentration of 9.35 mg/L in 12 days in MS medium under complete darkness. These studies are not only essential for determining the optimal culture conditions for the production of biologically active compounds from *in vitro* culture, but also important for developing a commercially viable process, maximizing the cost effectiveness in the scaling up of such products. Further optimization studies of culture parameters to increase the growth/product yield in shake flask and regarding scale up for mass scale bioreactor cultivation are in the progress.

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NOMENCLATURE

μ_{max} Maximum specific growth rate

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