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Review

Production of biopesticide azadirachtin using plant cell and hairy root cultures

The extensive use of nondegradable chemical pesticides for pest management has developed serious environmental hazards. This has necessitated the urgent need to switch over to an alternative mode of biopesticide development for mass agriculture and field crop protection. *Azadirachta indica* A. Juss (commonly known as neem) houses a plethora of bioactive secondary metabolites with azadirachtin being the most active constituent explored in the sector of ecofriendly and biodegradable biopesticides characterized by low toxicity toward nontarget organisms. It has been reported that the highest content of azadirachtin and related limonoids is present in the seeds, available once in a year. Moreover, the inconsistent content and purity of the metabolites in whole plant makes it imperative to tap the potential of *in vitro* plant tissue culture applications, which would allow for several controlled manipulations for better yield and productivities. This review gives a summarized literature of the applied research and achievements in plant cell/hairy cultures of *A. indica* A. Juss mainly in context with the biopesticide azadirachtin and applications thereof.

Keywords: Azadirachtin / Differentiated plant cell tissue/hairy root / *In vitro* mass propagation

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1 Introduction

Neem (*Azadirachta indica* A. Juss, family Meliaceae) is a reserve of about 300 important potentially active compounds responsible for the several biological functions of the plant such as blood purification, anti-inflammation, antitumor, bactericidal properties, and as insect repellent [1–3]. The limonoids (tetraterpenoids) constitute one-third of the total metabolites present [4, 5]. Of these the most noteworthy ingredients include azadirachtin, an insecticide (mainly Azadirachtin A). This compound is generally used in commercial insecticides [6], whereas others such as salanim and nimbin are used as a spermicide and anti-inflammatory in vertebrates [7–9]. The use of *A. indica* derivatives as a biopesticide has conspicuous advantages of easy availability, biodegradability, low toxicity toward nontargets such as pollinators and vertebrates, systematic action, low persistence levels in the environment, and ability to control a wide range of insects/pests including *Siphonaptera* and

Thysanoptera [1, 6, 10]. The plant extract consists of a mélange of complex bioactives all of which affect different portions of the insect life cycle, thereby, preventing the pests from developing a resistance toward it (Agro Forestry Tree Database). Azadirachtin is reportedly active on about 500 insect species [11].

As far as the commercial aspect is considered the major product of interest is the neem seed oil. Neem insecticide was registered under the name Margosan-O by the environmental protection agency (EPA) in the United States way back in 1985 [12]. Since then several neem-based products have been registered under various trade names, “Green Gold R neem extract,” “Align TM,” “Azatin R,” and “Turplex TM.” Various companies are also actively involved in the formulation of neem-based products that include Neemix 90EC (azadirachtin concentration reported to be 90 g/L), Neemmzid, Trilogy 90EC, Bio-neem, Turplex, and Bollwship (Thermo Trilogy); Fortune Aza and Fortune biotech (Fortune); NeemAzal (Trifolio-M), Kayneem (Krishi Rasyan), Neemolin (Rallis), Surfire, and Neemachtin (Consep), and Nimbecidine (T stanes), all obtained by extraction from the natural plants [13]. The main bottleneck in the conventional process of extraction of azadirachtin is the variability imposed by the heterozygosity in seeds resulting in fluctuating secondary metabolite content [14–16] and uncontrollable seasonal and geographical constraints.

Azadirachtin is mainly localized in the seed kernels (0.2–0.6%) that remain viable for a limited span of time with a decrease

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Abbreviations: AZRL, azadirachtin and related limonoids; DW, dry weight; MS medium, Murashige and Skoog medium; ON medium, Ohyama and Nitsch's

of 32% in around 4 months of storage precluding the *ex situ* processes of crop improvement and collection of germplasm [17–19]. Another major concern is the amount of aflatoxins (present on the seed surface, primarily arising out of microbial growth on sugars leaking out from seed burst) that get extracted along with compounds of interest [18]. Moreover, the structural complexity hinders the chemical synthesis [5, 18].

In this regard, plant cell/hairy root culture offers several foreseeable benefits to overcome the above-mentioned obstacles along with facilitating mass scale production in bioreactors. Although dynamic research efforts have been directed toward the development of successful *in vitro* cultures for *A. indica* not much data is available on the same with most of the reports focused on extraction/analysis as well as the biological and chemistry aspects. The present review is aimed at critically assessing the recent developments in the zone of plant cell and hairy root cultures for improved yield of azadirachtin. Wherever not mentioned, azadirachtin refers to the total extract. Several other relevant reviews have been cited for the reader's reference.

2 Elucidation of mechanism of action

All parts of the *A. indica* tree possess insecticidal activity but it is the seed kernel that is the most effective. The total pesticidal active ingredients are together called “triterpene” more specifically “limnoids.” The four best limnoids are as follows: azadirachtin, salannin, meliantriol, and nimbini. Azadirachtin itself comprises of a group of compounds of which, Azadirachtin A is found in plenty and is biologically the most potent [6]. Till date nine types of azadirachtin, Azadirachtin A to Azadirachtin I, have been isolated from the neem seed extract [20].

Azadirachtin (molecular formula $C_{35}H_{44}O_{16}$) is a highly oxidized tetranortriterpenoid with the surface covered with several intimately associated reactive groups, namely acetate, tiglate ester, methyl esters, alcohol, epoxide, and vinyl ether. This overabundance of the reactive groups results in a complicated chemistry. The full structure and chemistry has been elucidated recently by Ley and co-workers [21]. Azadirachtin is known to have a rapid effect by mimicking the activity of endocrine and neuroendocrine system [22], thereby, interfering with the insect life cycle at different time points [8]. Observations of the behavioral pattern of some parasitic larvae namely, *Spodopteralittoralis* and *Mamestrabrassicae* have suggested that azadirachtin might function by acting as a feeding deterrent [8, 23, 24]. This property adversely affects the ecdysteroid and juvenile hormones thus preventing pest proliferation. The sensitivity of various fungi, protozoans, and viruses toward this compound has also been indicated [18, 25]. In addition, it markedly reduces the viability of the eggs.

Analysis at the molecular level revealed the blockage of the cell cycle (due to inhibitory effect of azadirachtin on microtubule polymerization) [26] and onset of apoptosis that explains the retarded development of the pests [27–29]. Azadirachtin being a highly reactive molecule has several cellular components as targets both in the nucleus and cytoplasm. It causes a direct damage to the DNA [30] apart from altering the activity of various genes and proteins [25–31]. Besides its use as a biopes-

ticide, the potential of azadirachtin has also been extensively explored in inhibiting the reproductive cycle in malaria parasite, as a larvicide and in the inhibition of dengue virus type-2 replication [18, 32]. It also functions against the common ectoparasite, *Argulus sp.*, known to infect ornamental fishes [33].

3 Trends in azadirachtin production from plant tissue culture

3.1 Biosynthesis in undifferentiated cultures

Plant tissue culture technology possesses an immense potential for a sustainable production of high-valued metabolites [34]. *In vitro* azadirachtin production from undifferentiated callus and cell suspension cultures has been previously studied by Allan and co-workers [35]. The effect of calli differentiation and the source of parent explants have been examined by Nair et al. [36]. These authors by utilizing 13 explants estimated a maximum Azadirachtin A amount of 11 790 $\mu\text{g/g}$ dry weight (DW) in the callus derived from intermodal segments. Shifting to suspension culture, however, reduced the biosynthetic capacity. It was inferred that some kind of a cell to cell contact mechanism might have played a role in the earlier callus culture that was lost on transfer to the suspension medium. Another interesting observation in the study was that although the flower buds otherwise accumulated very small concentrations of Azadirachtin A, the callus raised from different floral parts showed multi-fold increase in the content that emphasized on the major role played by differentiation. Singh and Chaturvedi [16] quantified higher levels of azadirachtin in callus derived from redifferentiated zygotic embryo cultures (2.33 mg/g DW) compared to that from dedifferentiated callus obtained from leaves. In this case too, the content was reported to be strongly regulated by the degree of differentiation with higher amounts of the compound in the redifferentiated calli. Even then the accumulation of the azadirachtin was several folds lower than in the seed (7.41 mg/g DW), leaf (5.49 mg/g DW), and ovary (1.38 mg/g DW) of the whole plant. Prakash et al. [37] developed callus cultures via seeds procured from various geographical locations characterized by different azadirachtin content. The azadirachtin accumulation in the callus was found to be strongly linked to the content in the parent seed kernel indicating the influence of the genetic makeup, geography, and explant source. Variability was observed in the azadirachtin content among the cell lines developed from seeds of different locations as well as from the leaf explants. Maximum concentration of Azadirachtin A (1.89 mg/g) was detected in the AG-43 cell line derived from the most potent seed kernels Ai43 procured from Trivandrum, Kerala, which reportedly had the highest azadirachtin content (5.13 mg/g DW) of all the seed kernels examined. The content was approximately 19 times higher than that in the lowest producing cell line, Ai-41 (Jodhpur) (0.21 mg/g DW) and also significantly greater than the cell lines obtained from leaves (0.23 mg/g).

In a very recent study on *A. indica*, NAA was found to be more favorable for callus induction as opposed to 2,4-D (2,4-dichlorophenoxyacetic acid). When MS (Murashige and Skoog) medium was supplemented with 1-naphthaleneacetic

acid (NAA) (2 mg/L) + 6-benzylaminopurine (BAP) (0.5 mg/L) leaf callus proliferated. Authors also observed the presence of rhizogenesis and embryonic masses, which indicated the possible morphogenic potential of the developed leaf callus. However, the azadirachtin content was not quantified [38]. Spieth et al. [39] used 4200 explants under different light and dark rhythms. Successful callus induction and proliferation was achieved in MS media with NAA and BAP under 16/8 h light/dark photoperiod in 90% of the explants. The formation of transformed callus infected with *Agrobacterium tumefaciens* has been earlier described [40], however no data on azadirachtin accumulation are available till date. As a result comparisons based on azadirachtin content with nontransformed cultures are not possible.

The biosynthesis of secondary metabolites although genetically controlled is considered to have an exquisite sensitivity to alterations in nutritional and environmental factors [41] (Table 1). In one of the early studies, Raval et al. [42] tested the effect of basal media with an aim to achieve enhanced azadirachtin and related limonoids (AZRL). After initial experimentation, the authors observed that while MS medium resulted in highest biomass of the suspension, White's medium was favorable for the metabolite accumulation. Catabolite repression was cited as the possible reason for absence of the bioactive compound in the otherwise most commonly used MS and B5 recipes. A modified MS medium with altered nitrate and phosphate content was then devised using response surface methodology, resulting in only slight enhancement in productivity. This tempted the authors to switch from single to two-stage cultivation also designed using statistical methods. The cultures were transferred from growth to production medium on the ninth day of cultivation resulting in 2.75 mg/L of the bioactive compound. As a benchmark the authors used the azadirachtin in the seed kernels that was in the range of 2–6 mg/g. Although a significant improvement was achieved on switching and modifying the medium, the yield was still on the lower side compared to the intact plants. Another innovative attempt in the above study was the side by side online OTR (oxygen transfer rate) measurement carried out to understand the culture dynamics in a specially designed shake flask vessel, RAMOS. On similar lines, Prakash and Srivastava [43] developed a medium recipe adopting tools such as Placket Burman design and response surface methodology resulting in increased biomass growth of 15.02 g/L as well as azadirachtin biosynthesis of 2.98 mg/g DW (45 mg/L) in suspension cultures. Moreover, these authors by their experiments reported glucose as a better carbon source compared to sucrose. Furthermore, ammonium concentration was observed to inhibit the azadirachtin synthesis while increase in nitrogen content demonstrated a progressive enhancement in the bioactive compound. The impact of medium recipe has been highlighted in other studies as well [44]. Cell suspensions derived out of the neem variety Crida-8 showed extreme sensitivity to the carbon source. A total absence of azadirachtin was reported on reduction in sucrose concentration to 15 g/L. On the other hand, a complete removal of phosphate enriched the intracellular concentrations of the biomolecule to 6.98 mg/L of which 6.94 mg/L was of Azadirachtin A and remaining was of azadirachtin B. On alteration of the nitrate levels keeping the ammonium level constant, a progressive enhancement in the

content followed. At 4:1 nitrate ammonium ratio, a 1.5-fold increase in total azadirachtin (A + B) and enhanced cell mass (8.5%) was reported. Eventually, coupling of the desirable nitrate to ammonium ratio with complete phosphate removal in MS medium led to a 36% increase in the biomass (59.36 g/L). However, the intracellular azadirachtin content was drastically reduced with most of it released in the medium. Spieth et al. [39] suggested optimizing the cell suspension media separately for growth and production. In order to meet this complex challenge a fully automated high-throughput microbioreactor system was put forward as a future scope of the study that would allow for a fast and controlled batch and fed-batch screening in 48-well microtiter plates.

The key to successful commercialization of the plant tissue culture technology is by reducing the capital and operating cost. Exogenous additions such as elicitors and biosynthetic pathway intermediates/precursors could be a good ground of study for improved yield making it competitive with respect to other routes of production [45]. In one such study, *A. indica* calli grown in liquid woody plant medium supplemented with casein acid hydrolysate, followed by elicitation with methyl jasmonate yielded highest azadirachtin concentration of 0.2470 $\mu\text{g/g}$ [5]. This corresponded to about 57% of the content in the native plant seed used as the starting parent material for initiation of the *in vitro* cultures. Parameters such as rotational speed and exposure time to the elicitors were also reported to control the yield.

For the first time, a research involving cyanobacterial elicitors (*Anabaena* sp. and *Nostoc carneum*) was reported for azadirachtin production [46]. Higher protein accumulation was observed in elicited cultures compared to control, which was indicative of higher growth since actively growing cells participate in protein synthesis. The cyanobacterial elicitor *Anabaena* sp. was capable of increasing the biomass by fivefolds which was otherwise deemed difficult by the authors resulting in a maximum azadirachtin concentration of 0.32 $\mu\text{g/L}$ on the eighth day of cultivation.

Independent screening experiments with elicitors such as jasmonic acid, methyl jasmonate, salicylic acid, yeast extract, and yeast extract carbohydrate fraction resulted in two to three fold increase in cell suspension cultures. Considering that each elicitor would affect the cell physiology and defense response in a different manner, response surface methodology was adopted by the authors to study all possible interactions with less experimentation. A synergistic response on exposure to a combination of these elicitors was observed resulting in a fivefold increase in azadirachtin response (15.9 mg/g DW) as compared to control [47]. With more insight into the effect of exposure time 17.4 mg/g (DW) of azadirachtin was demonstrated. Various precursors were also supplemented to maintain their optimum/required concentration for increased flow toward the azadirachtin pathway. After several tests sodium acetate was observed to be the most potential precursor (162 mg/L, 9.6 mg/g). Sodium acetate on entering the cell converts first to acetic acid and acetyl coenzyme A, thereby, promoting the energy metabolism [48].

Azadirachtin is known to accumulate intracellularly, precluding cell immobilization and continuous removal, that can be conveniently achieved by the use of cell wall permeabilizers [18]. These compounds are generally organic solvents whose selection is based on the log *P* (the octanol/water partition coefficient

Table 1. Advances in azadirachtin production from cell suspension and hairy root cultures

Strategy	Type of culture	Azadirachtin	Reference
Medium optimization	Suspension/callus	2.79 mg/L	[42]
	Suspension/callus	45 mg/L	[43]
	Hairy root	0.0016% DW	[41]
	Hairy root	73.84 mg/L	[53]
Elicitor addition			
<i>C. Purpurea</i>	Hairy root	0.074% DW	[41]
Jasmonic acid	Hairy root	0.095% DW	[41]
Salicylic acid	Hairy root	0.14% DW	[41]
<i>C. lunata</i>	Hairy root	7.1 mg/g	[55]
Salicylic acid	Hairy root	4.95 mg/g	[5]
MeJA	callus/suspension	0.2470 μ g/g	[46]
<i>Anabaena sp.</i>	callus/suspension	0.32 μ g/L	[47]
Elicitor combination	callus/suspension	17.4 mg/g	[55]
Precursor addition			
Cholesterol	Hairy root	3.1 mg/g	[55]
Sodium acetate	Suspension	9.6 mg/g	[48]
Permeabilization			
Chitosan	Hairy root	4.6 mg/L (Int)	[49]
DMSO	Hairy root	4.2 mg/L	[49]
Triton X 100	Hairy root	10 mg/L (total)	[49]
<i>n</i> -Hexadecane	Suspension	3.8 mg/g (Int)	[48]
Gas phase effect			
Air flow rate	Hairy root	46.02 mg/L (3.5mg/g)	[56]
Oxygen enrichment	Hairy root	78 mg/L (5.9mg/g)	[57]
Model-based cultivation			
Fed batch bioreactor	Suspension	82 mg/L (4.1 mg/g)	[51]
Continuous culture cell retention	Suspension	380 mg/L (3.9 mg/g)	[52]
Bioreactor cultivation			
Low shear setric impeller	Suspension	50 mg/L (3.22 mg/g)	[50]
Centrifugal impeller	Suspension	71 mg/L (3.74 mg/g)	[50]
Bioreactor+ elicitation	Suspension	161.1 mg/L (11.5 mg/g)	[47]
Integrated	Suspension	751 mg/L (12.2 mg/g)	[48]
Modified bioreactor + mesh	Hairy root	20.23 mg/L	[59]
Modified bioreactor + PUF	Hairy root	28.52 mg/L	[59]
Mist bioreactor	Hairy root	27.24 mg/L	[60]
Integrated	Hairy root	97.28 mg/L	[54]
Content in native plant			
	Seeds	2.6 mg/g DW	[42, 53–56, 59, 60]
	Normal roots	0.00032% DW	[41]
	Seeds	0.4301 μ g/g DW	[5]
	Seeds	12.88 mg/gm DW	[46]
	Seeds	5.13 mg/g DW	[43, 47, 48, 51, 52]

of solvent) values [48]. Kuruvilla et al. [49] reported enhanced secretion and biosynthesis of azadirachtin in suspension cultures on exposure to DMSO, Chitosan, and Triton X-100. The exudation of the bioactive compound was maximum at 7.5% DMSO resulting in 4.2 mg/L azadirachtin. Exposure to higher concentrations inhibited the cell growth. On the other hand, Triton X-100 was effective only at the highest concentration (150 ppm). Chitosan caused a fourfold enhancement in azadirachtin secretion in the external medium. Prakash and Srivastava [48] selected three solvents depending on the log *P* values namely, *n*-hexadecane (with log *P* = 8.8), di-*n*-butyl phthalate (with log *P* = 5.4), and 1-decanol (with log *P* = 4.0). Only *n*-hexadecane (2 and 5% concentrations) could release up to 21%

azadirachtin in the medium (10 mg/L, extracellular) with minimum impact on cell viability, possibly due to a higher log *P* value [48].

A little data are available regarding bioreactor design and operation for suspension/hairy root cultures for azadirachtin synthesis. Prakash and Srivastava [50] compared the metabolite synthesis in a stirred tank bioreactor with a low shear setric impeller and a novel centrifugal impeller (specifications: impeller outer diameter (*D*) = 82 mm, draft tube inner diameter (*d*) = 27 mm, blade width (*h*) = 14 mm, length of the draft tube = 105 mm, blade number = 6, blade angle (β) = 60°). Suspension cultures grown in bioreactor with modified centrifugal impeller demonstrated higher azadirachtin accumulation of

71 mg/L (3.74 mg/g) as opposed to 50 mg/L (3.22 mg/g) with the use of a setric impeller. The results have been attributed to the fact that the centrifugal blades increase the residence time of the gas bubbles that allowed for sufficient oxygen transfer and hence higher oxygen transfer rates. This was verified by the authors from the higher $K_L a$ values in the presence of the centrifugal impeller. The elicitor combination mentioned previously when replicated in a 3 L bioreactor with setric impeller a threefold enhancement in azadirachtin synthesis (161.1 mg/L; 11.5 mg/g) as opposed to control bioreactor (50 mg/L; 3.2 mg/g) was reported. The volumetric productivity was also significantly higher in elicited bioreactor studies (16.1 mg/(L d)) [46].

Till date there has been only one effort on modeling the bioprocess for *A. indica* cell cultivation. The developed mathematical model could describe the batch kinetics with reasonable efficiency and was then extrapolated to guide nutrient feeding in fed-batch mode after incorporating suitable dilution factors. The experimental implementation of the fed-batch cultivation resulted in 82 mg/L (4.1 mg/g DW) of azadirachtin with a volumetric productivity of 2.41 mg/(L h) in a 3 L bioreactor [51]. Eventually, the extrapolation of the model to continuous cultivation and its implementation in bioreactor along with cell retention led to an overall azadirachtin production of 280 mg/L (3.9 mg/g DW) [52]. Cell retention was of added advantage as higher dilution rates could be achieved for a rather slow growing plant cell system preventing washout conditions to develop [52]. In spite of drastic variations in the azadirachtin concentrations the yields in the above cases were nearly same because the cell mass was also promoted. Finally, these authors benefited by coupling of various strategies, viz. mathematical model guided continuous cultivation with elicitor addition, precursor feeding, and permeabilization yielding 751 mg/L (12.2 mg/g DW) of azadirachtin along with a volumetric productivity of 7.25 mg/(L h), the highest reported from suspension culture of *A. indica* so far [48]. The various strategies discussed above have been summarized in Table 1.

3.2 Biosynthesis from hairy root culture

Cell suspension cultures being dedifferentiated, there is lack of storage tissue and the product thus released in the culture media is prone to degradation by the enzymes released into the medium. Development of a certain degree of differentiation can make a significant contribution to the synthesis of phytochemicals. Satdive et al. [41] based on their evaluation of different basal media namely, Ohyama and Nitsch (ON), Gamborg (B5) medium, and MS medium, found ON medium the most desirable for increasing the metabolite production in hairy root cultures (0.0166% DW as opposed to 0.00032% DW in normal roots). It was suggested that since ON medium had higher inorganic salt concentration it might have favored both growth and production. Srivastava and Srivastava on the other hand used statistical protocols for a more rapid optimization of nutrient concentrations for hairy root cultures of *A. indica*. The authors could attain 73.84 mg/L (5.2 mg/g DW) of total azadirachtin, a 68% increase than the unoptimized control [53].

Phytochemical production can be enhanced further via biotic and abiotic stimulations, either singly or in different

combinations. A fivefold increase in azadirachtin (0.074% DW) on exposure to *Claviceps purpurea* filtrate and a sixfold (0.095% DW) and ninefold (0.14% DW) enhancement, respectively, on treatment with signal compounds, jasmonic acid and salicylic acid, were observed by Satdive et al. [41] in Ohyama and Nitsch's medium. For the signaling molecules, maximum azadirachtin accumulation and leaching in the external medium was facilitated at the lowest concentration (100 mM). Another study carried out on hairy root cultures claimed salicylic acid and *Curvularia lunata* fungal filtrate (1% v/v) as the most potent elicitors. *A. indica* is known to function against the plant pathogen *C. lunata* that possibly triggered the azadirachtin biosynthesis (7.1 mg/g DW) sufficiently higher than in the seed kernels [54]. The same authors on supplementation of cholesterol as an indirect precursor in hairy root cultures showed the azadirachtin production of 70.42 mg/L (3.1 mg/g DW), again comparable to the content in the seed (2–6 mg/g DW) [55]. As per these investigators, the sterol biosynthetic pathway generally diverts the precursor from the azadirachtin production. However, this could be avoided on exogenous additions owing to feedback inhibition of the sterol biosynthesis.

Oxygen, though one of the most important nutrients in the aerobic tissue culture is most scarcely available. This situation is more pronounced in hairy roots owing to their peculiar characteristics [56]. A lone study on the effect of modifying the air flow rate/oxygen enrichment in hairy root cultures in context with azadirachtin productivity has been reported by Srivastava and Srivastava [56]. First method implemented was the variation in aeration rate wherein azadirachtin productivity of 1.84 mg/(L d) (3.5 mg/g DW) could be attained for aeration volume of 0.8 vvm. Another means of oxygen enhancement attempted by the authors was the supplementation of pure oxygen in the inlet air. This indeed proved to be effective with a threefold increase (4.43 mg/(L d), 5.9 mg/g DW) on exposure to 100% oxygen, however, at the cost of increased production expenditure. Since azadirachtin is a highly oxygenated molecule, higher levels of oxygen in the medium could have promoted its accumulation. Biomass concentration on the other hand decreased beyond 60% v/v of oxygen in air. This was attributed to high oxidative stress leading to cytotoxicity lowering the overall mass of the hairy roots. To avoid this interesting method of addition of specific chemicals called the oxygen vectors was tested. The results however were not encouraging and further optimization of the concentrations was suggested by the authors.

The control of the bulk oxygen concentration depending on the demand of the vigorously growing hairy root especially that in the root core could be successfully achieved by a mathematical model described by Srivastava et al. [57].

One of the biggest challenges for commercial exploitation of hairy root cultures is the production bottlenecks. The peculiar morphology limits the use of conventional bioreactor set ups and some specific designs have been developed suiting the culture characteristics and requirements [58] particularly by a single group. Different liquid bioreactor configurations, viz. stirred tank, bubble column, modified bubble column with floating mesh, and modified bubble column with polypropylene foam support (PUF) have been tested to facilitate high-density hairy cultures of *A. indica* [59]. A severe shearing and necrosis of the roots was observed on cultivation in a conventional stirred tank

bioreactor in spite of being equipped with a low shear setric impeller. Similar results were obtained in bubble column bioreactor as well. According to the authors since the hairy roots are shear sensitive and immobilizing in nature these configurations were not suitable for scale up. Moreover, the completely dispersed roots became a victim of a morphological malfunction, hyperhydricity, hence could not proliferate. Eventually, the authors dealt with these issues by placing the roots onto different supports using two modified stirred tank bioreactors, one incorporating a floating polypropylene mesh and another with polyurethane foam. Azadirachtin volumetric productivity of 1.14 mg/(L d) could be attained in the latter [59]. The authors thereafter attempted to implement an integration of growth regulators, permeabilizers, elicitor, and precursor in shake flask resulting in 113 mg/L of azadirachtin. The same when scaled up to a modified stirred tank bioreactor with PUF foam support matrix yielded 97.28 mg/L (6.4 mg/g DW) of the compound [54].

To overcome the mass transfer problems associated with the submerged type of bioreactors gas phase bioreactors were developed by the authors for *A. indica* hairy root cultivation. The nutrients were made available by dispensing the liquid either in spray or mist form. Not only the reduction in the boundary layer thickness and hyperhydricity but the higher solubility of oxygen in air than aqueous medium also played a positive role. Of these, the nutrient mist design promoted the highest growth and yielded 2.8 mg/g DW azadirachtin (27.4 mg/L), which translated into a volumetric productivity of 1.09 mg/(L d). Owing to the smaller droplet size, this configuration of generating mist caused less liquid entrapment than in nutrient spray system. Furthermore, the mist could easily percolate to the inaccessible areas such as the root center [60].

4 Antifeedant activity of cultured extracts

Different groups have reported the biopesticidal activity of the extracts from cultured calli and hairy roots of *A. indica*. The effect of cell suspension *in vitro* callus extracts and micropropagated plants has been reported against the desert locust by Allan and co-workers [35]. Rafiq et al. [61] recently studied the efficacy of the suspension and callus extracts of AZRL with a 100% mortality rate against jassids (*Amrasca biguttula* Ishida), thrips (*Thrips tabaci*), and whiteflies (*Bemisia tabaci* (Gennadius)) using a dilution of 1:10 and 1:100 v/v extract in distilled water. A promising effect of these suspension and callus cultures have also been reported against *Pectinophora gossypiella*, *Helicoverpa armigera*, and *Spodoptera litura* [61]. These researches earlier demonstrated a significant reduction in the feeding rate of lepidopteron insects [62].

Satdive et al. [41] observed the hairy roots extracts (containing 0.0016% DW, total azadirachtin mixture) to have superior antifeedant activity as opposed to leaves against the *S. litura* larvae. The dilutions, 1:1 and 1:5, of the hairy root extracts showed 100% antifeedency. On the other hand, a much lower activity of 90 and 86%, respectively, was obtained for the same dilution ratios of the leaf extract. Srivastava and Srivastava [59] by independent experiments with hairy root powder (containing 5.2 mg/g DW azadirachtin) and its solvent extract demonstrated a high antifeedant index against desert locust *Schistocerca greg-*

aria. While the hairy root powder in ethanol showed an antifeedant index of 97% the value for crude extract obtained from the roots was at 83.5%, both lower than the standard azadirachtin solution (35%) obtained from Indian Agricultural Research Institute, New Delhi, India.

5 Patents and potent research areas

As per the SCOPUS database, there are about 139 patents for the key word azadirachtin. First was an European patent (EP 0605139, also published as: CA2111630A1, CN1092106A, EP0605139A2, US5698423) on azadirachtin biosynthesis in cell suspension culture (source: SCOPUS and Google Patents) [63]. This invention discussed the method of azadirachtin synthesis from *A. indica* callus via *A. tumefaciens* and eventually developing liquid cell culture such that azadirachtin is produced in sufficient amounts therein, followed by recovery of the compound. Another patent discussed the production of peroxidases using *A. indica* suspensions wherein an extraordinary enzymatic activity was recorded (US 7,390,641 B2, EP 1758992 A2) [64]. The peroxidase enzyme was extracted only from certain plant parts mainly from the roots. This invention particularly dealt with the development of compact callus cultures and enzyme recovery via novel methods resulting in minimum loss of the enzyme activity. Another aspect of the patent was development of optimized hormone combination along with undefined supplement (coconut water) for optimized callus growth and higher enzyme activity. The cost of production was much lower as compared to the commercially available counterparts. As per the authors, a rough cost of enzyme from the plants was around \$2 per 2 lakh units of the enzyme whereas it was \$450 for the same amount commercially. Although callus induction was achieved the patent does not directly deal with azadirachtin production. With regard to mass scale propagation, there is a single Indian patent dealing with azadirachtin biosynthesis from hairy root culture (Application No: 148/Del/2010) [65] in tailor-made mist bioreactor. Only patents relevant to tissue culture are discussed here.

In spite of several phytochemical and pharmacological studies carried out limited information is available regarding the biosynthetic pathway and related genes involved. Bhambhani et al. [66] recently cloned and characterized the genes, *AiH-MGR1* and *AiHMGR2*, which encode for 3-hydroxy-3-methylglutaryl coenzyme A reductase responsible for catalyzing the rate limiting step in the isoprenoid biosynthesis. Of the two genes, *AiHMGR2*, showed a direct correlation with azadirachtin biosynthesis in the fruit tissue. No correlation was observed between the *AiHMGR1* expression and accumulation of bioactive molecules. More such studies and eventually use of these explants as a source of callus/hairy root induction would serve as a wealthy source of the compound, thereby, making the process more viable.

6 Concluding remarks

A better understanding of the underlying biological mechanisms controlling the metabolic pathways with genetic manipulations and engineered hairy roots/calli harboring desirable foreign gene sequences could lead to competitive bioactive

compound yields [67]. Moreover, whole plants can be regenerated from these tailored cultures. The other obstacle requiring thorough research is the photosensitivity of azadirachtin especially Azadirachtin A. For it to be effective over a longer period of time several UV absorbers have been tested, which either capture the UV rays or prevent the photoexcitation of this biopesticide [20]. Level of persistence of azadirachtin in food products is not yet clear. As is evident from mass cultivation studies, the suitability of different bioreactors and scale up remains to be addressed in detail for mass scale production to cater to the market requirement.

Practical application

The review summarizes the progress made in the application of tissue culture techniques for biosynthesis of azadirachtin especially the mass production in different bioreactor scales and configurations. The aspect of mathematical modeling for growth and biosynthesis has been highlighted. Recent research and patents in this area have been discussed. The review is of particular importance for scientists, researchers, and/or students working in the area of bioreactor scale *in vitro* cultivation of *A. indica* and overproduction of azadirachtin. The different strategies discussed could serve as a guide for a wide range of tissue/organ cultures.

7 References

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