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Production of Azadirachtin from Plant Tissue Culture: State of the Art and Future Prospects

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Abstract With increasing awareness towards environment-friendly and non-toxic pesticide azadirachtin obtained from neem tree (*Azadirachta indica*) is gaining more and more importance. Its broad-spectrum activity, peculiar mode of action. eco-friendly and non-toxic action towards beneficial organisms has offered many advantages over chemical pesticides. All currently use commercial formulations based on azadirachtin contains azadirachtin extracted from seeds of naturally grown whole plants which is labour intensive process depending upon many uncontrollable geographical and climatic factors. Plant tissue culture can be a potential process for the production, offering consistent, stable and controlled supply of this bioactive compound, However the research on tissue culture aspects of production are in preliminary stage and requires culture and process optimization for the development of a commercially viable process. This review states the present status and future challenges of plant tissue culture for azadirachtin production.

Keywords: azadirachtin, callus culture, suspension culture, bioreactor

INTRODUCTION AND PERSPECTIVES

Azadirachtin obtained from neem tree (*Azadirachta indica*. A. Juss. (family: Meliaceae)) is one of the most important biopesticide currently in use. The broad-spectrum activity of azadirachtin at very low concentration coupled with the unique mode of action and non-toxicity to mammals make azadirachtin an ideal candidate for insecticidal use. Since the advent of DDT chemical pesticides have been controlling the pest problem in some of the crop system very efficiently but due to their extreme persistent, bioaccumulation, toxicity towards non-target beneficial organism, tendency to cause malignancy and increasing development of insecticidal resistance has created the serious threat to crop protection program all over the world, hence in recent years instead of the use of neurotoxic, broad spectrum, synthetic pesticides much attention is being paid towards more specific, bioactive, biodegradable environmental friendly plant or microbial based biopesticide [1] and in this regard azadirachtin shows the great potential [2]. Azadirachtin is peculiar as a biopesticide: it actively attacks the insect feeding, reproductive cycle and growth regulatory effects. A wide range of phytophagous insects, stored product pests and aquatic arthropods has been reported to be sensitive towards azadirachtin [3]. Apart from its action against insects it is also claimed to have antifungal, antibacterial and antiprotozoan activity [3]. Azadirachtin only effects the insects that consume it thus other friendly insects, predators and parasites and species which may help in pollination and other plant functions are not harmed, it quickly biodegrades by sunlight hence does not accumulate in nature, further the possibilities for insects to develop the resistant is less likely because of its varying mode of action and the abundance of active compounds similar to azadirachtin present in formulations. The first commercial neem insecticide, Margosan-O was registered by the environmental protection agency (EPA) in 1985 for use on non-foods crop [1] since then various other products based on azadirachtin are being formulated and sold by a large number of companies. Azadirachtin is tetranorterpenoid ($C_{35}H_{44}O_{16}$) and was isolated as one of the first active ingredients of neem in 1968 [4]. In small amount azadirachtin is present in all parts of tree but its highest concentration (0.2-0.6%) is present in mature seeds [5]. All commercial neem insecticidal formulations and other product based on azadirachtin contains azadirachtin which is extracted from the seeds of naturally grown whole plant [6,7], this approach has the disadvantages of heterogeneity in azadirachtin content depending upon the plant genotype and environment. Seasonal variation in azadirachtin in seeds of *Azadirachta indica* has been reported [8]. Neem tree grows only in limited regions of world of arid zones of Asian and African subcontinents, as the tree is susceptible to excessive frost [9,10] *i.e.* the geographical distribution of neem tree is limited, also the seeds does not have longer shelf life and azadirachtin percentage dropped up to 32% within four months of

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storage [11]. Azadirachtin is a high value chemical and as the awareness towards more safe, environmental friendly pesticides are increasing, its demand is also increasing continuously [2]. Current supply of bioactive compound from neem tree will not meet the increasing demand if the extraction from seeds remain the only source hence there is need for the development of commercially viable alternatives for its production. Chemically it is a complex molecule [12-14] and this complexity precludes a synthetic production system. Keeping in consideration of all these factors, plant cell culture can be seen as a potential alternative production system. With the cell culture methods, production can be more controllably ensured in terms of the product quality and quantity, independent of geographical and climatic barriers. In cell culture the culture conditions and process variable can be more easily optimized and cell culture can also offer better selectivity and yield of the desired bioactive compound. Therefore production of azadirachtin from plant cell culture can be an area of active research and development, however most of the data available on azadirachtin is regarding its chemistry, biological effects and extraction and analysis from field growing plants, no comprehensive knowledge is available on tissue culture aspects of azadirachtin production. This review deals with the current state of the art and future challenges of plant cell culture technology for azadirachtin production.

CHEMISTRY AND MODE OF ACTION OF AZADIRACHTIN

Chemically azadirachtin is a tetranorterpenoid having molecular formula $C_{35}H_{44}O_{16}$. The full chemistry of azadirachtin is reviewed by Ley *et al.* [14]. Azadirachtin is a highly oxidized limonoid with many reactive functional groups in close proximity to each other. Azadirachtin A is the major component of neem seeds. Although azadirachtin B to azadirachtin I are also present, but in only trace amounts. The chemical synthesis of azadirachtin is now almost complete [13,15,16] and research on the synthesis of simpler model compounds of azadirachtin that contains only part of the functionality of azadirachtin but displaying similar biological activity and synthesis of derivatives of azadirachtin is ongoing [17]. Ley group has also described a potential relay route for the synthesis of azadirachtin in which the natural product was converted to an advanced intermediate in a sequence of six reactions and was returned to primary compounds using seven steps [18]. In the same report many new compounds for biological testing have been claimed. The report on the transformation of azadirachtin using synthetic reagents and biocatalyst containing useful reporter group for development of binding assay have been reported [13,19,20].

Ever since the discovery of pesticidal activity of neem [21] and isolation of azadirachtin as the primary chemical responsible for the pesticidal activity, the investigation on this compound is continuously increasing. Presently nine types of azadirachtin have been isolated from neem seed extract and their structure has been elucidated [22]. Most of the data available on azadirachtin is related to its biological effect that is action against number of pests of numerous species and at stages of insect development of many order (three international neem conferences)[21,23-29]. The mode of action of azadirachtin have been reviewed across the pest species [3]. The possible mode of action of azadirachtin has been reported as antifeedancy, effect on ecdysteroid and juvenile hormone and direct effect on most of the tissues known to be controlled by developmental hormone that is it acts as antifeedant, reproductive and growth regulator. Apart from its action against insects, fungi, viruses and protozoa that account the major enemy of crops are also reported to be sensitive to azadirachtin [3]. Besides its properties as biopesticides the new potential of azadirachtin are being explored including these are *in vitro* inhibition of sexual development of malarial parasite [30] and as a mosquito larvicide [31] and inhibitory potential on dengue virus type-2 replication [32]. Neem leaf extract and neem seed oil is being explored for treatment of whole number of disease although the principal component has not being identified in such studies but possibilities of involvement of azadirachtin are wide open.

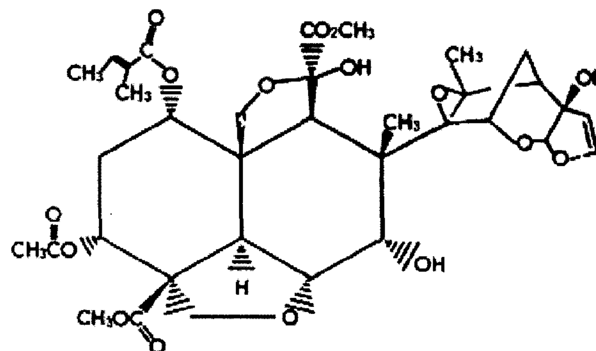

Fig. 1. Chemical structure of azadirachtin.

PLANT TISSUE CULTURE FOR AZADIRACHTIN PRODUCTION

Callus, Suspension and Hairy Root Culture

Undifferentiated callus and suspension culture containing azadirachtin and other secondary metabolites have been established by different authors by using different explants. The presence of azadirachtin in callus culture of leaf and bark explants [33], from leaf explant [34,35] from leaf and flower explant [36] have been reported. In these studies the presence of azadirachtin was confirmed by using thin layer chromatography and supercritical fluid chromatography or reversed phase high performance liquid chromatography (RP-HPLC) while the azadirachtin could not be detected in callus

Table 1. Azadirachtin content (per gram DW) in different tissue cultured systems

Culture system	Explants	Medium composition	Azadirachtin content	Reference
Callus	Leaves	MS+IBA (4 mg/L) + BA (2 mg/L)	7 μg/g DW	[34]
Callus/ Suspension/ Shoot culture	Leaves	MS+IBA (4 mg/L)+ BA (2 mg/L)	n.d.	[37]
In vitro roots	Embryo	MS +NAA (0.1 mg/L) +BA (0.5 mg/L)	4 μg/gDW	[38]
In vitro shoots	Embryo	1/2MS+IBA (0.5 mg/L)	8 μg/gDW	
Callus	Leaves Flowers	MS +2,4-D (1 mg/L) + Kn (0.5 mg/L) + glycin 90.3 g/L	0.0268 g/gDW 0.0246 g/gDW	[36]
Callus	Leaves Bark	White+ IAA (0.2 mg/L) + BA (1 mg/L)	64 μg/gDW 44 μg/gDW	[33]
Callus/Suspension culture	Leaves	MS+IBA (4 mg/L) +BA (2 mg/L)	*	[35]
Hairy Roots	Leaf Stem	MS without growth hormone	3.6 μg/gDW 2.7 μg/gDw	[90]

n.d.- Not detected, * 5.36 mg/L

and in vitro propagated shoots [37-39]. Production data of azadirachtin in different tissue culture system is shown in Table 1. Seeds obtained from micropropagated neem plants also have been reported to contain azadirachtin [40]. Hairy roots from *Azadirachta indica* cells have also shown azadirachtin content among them, which peaked at 6th weeks. The study report the development of two lines, one derived from stem of a micropropagated shoot culture (S) and other from leaf of aseptically grown whole plant (L), with both cell line having same growth rate and doubling time but differing in azadirachtin concentration, further azadirachtin synthesis was shown to be non growth associated and shows the highest concentration on the onset of stationary phase which started from 6th week and rapid reduction in azadirachtin by 8th week when maximum biomass being achieved [41]. All these early studies dealt with basic culture techniques of callus induction and culture maintenance. There have been numerous reports available on callus formation from different explants in last decade but these studies were aimed on the in vitro plant regeneration and azadirachtin content was not analyzed [42-47]. All these studies showed variations in the callus induction response depending upon the age of explant, size of explants and cultural conditions employed among diverse neem genotype.

Media Compositions and Culture Conditions

In vitro culture of neem for azadirachtin production is commonly initiated in MS media [48] supplemented with 20-30 g/L sucrose and one or two auxin and cytokinin. The concentration of auxins varies from 4 mg/L to 8 mg/mL for indole butyric acid (IBA); 0.2 mg/L for indole acetic acid (IAA), and 1 mg/L for 2,4-dichlorophenoxyacetic acid (2,4-D). Cytokinin used were benzyl adenine (BA) and kinetin (Kn) in concentration range of 0.1-4.0 mg/L and 0.5 mg/L respectively (Table 1). However Wewetzer [33] observed the higher azadirachtin concentration in White medium [49] as compared to MS media from the same cell lines, variation in azadirachtin content was observed derived from leaf and bark explants from two cell lines and affected by different nutrient media and carbohydrate source applied. Nicaraguan cell line (from leaves) shows the highest azadirachtin content (<0.5-64 μg/g DW) than those of tongo cell line (<0.5-11 μg/g DW) on White media while the azadirachtin content was found to be three times higher on media supplemented with 15 g/L sucrose for Nicaraguan bark (<0.5-64 μg/g DW) as compared with those having 30 g/L sucrose (<0.5-22 μg/L) suggesting the effect of genetical and culture condition based azadirachtin production [33].

There is little knowledge about effects of environmental conditions like temperature, pH and light intensity on cell growth and azadirachtin concentration. In most of the study, cultures were grown at 25-28°C in 16/8 h light/dark regime. Allan *et al.* [34] has reported callus growth in dark conditions with 0.0007% azadirachtin yield in MS media. In most of the studies, the pH of growth media was maintained at 5.8. This is probably due to most stability of azadirachtin in mild acidic pH [50].

EXTRACTION AND ANALYSIS METHODS

A great deal of work with azadirachtin is focused on its extraction and analysis from neem seeds and leaf. Although numerous reports are available on the isolation of this compound [4,6,51,52] its isolation in a pure state remains a tedious and time-consuming affair involving repeated partioning between solvents and extensive chromatography. Recently a simple method for enrichment of azadirachtin has been reported describing the different % recovery of azadirachtin in different solvents and suggesting the selection of solvent depending on the putity requirement in different formulations [53]. Purification of azadirachtin (>99.9%) using preparative HPLC [7,54] and by combination of flash chromatography and HPLC [6] has been established. The analysis is mostly done by chromatographic methods using UV detection [7,55-59] however time to time different strategies by different authors have been reported [59,60]. Colorimetric determination of azadirachtin related limonoids is reported recently using acidified vanillin solution [61], same group of authors had subsequently reported a multivariate calibration technique for the estimation of azadirachtin related limonoids as well as the simple terpenoids in different parts of neem tree [62]. This colorimetric procedure allows direct and rapid measurement of azadirachtin related limonoids as

well as simple terpenoids in the crude extract, although not mentioned but this colurimetric procedure can easily be implemented for azadirachtin measurement in cell culture studies thus allowing a rapid screening system for selection of high azadirachtin yielding explants and cell lines. Implementation of solid phase extraction technique for analysis of secondary metabolites in samples from tissue culture has been described [63]. Extraction from cell culture involves the same steps as partioning between solvents with the omission of defatting stage, which is quite cumbersome, adding another advantage of azadirachtin production from plant cell culture system.

POSSIBILITIES AND CHALLENGES

The first commercial neem insecticide designated as Margosan-O was registered by the environmental protection agency (EPA) for use on non foods crop in United States as early in July, 1985 [1] and till now four neem products viz. "Green Gold^{R} neem extract", "Align ^{TM}", "Azatin R" and "Turplex ^{TM}" have been registered [64]. Various other products based on azadirachtin are being formulated and sold by a large number of companies, these are Neemix 90EC (azadirachtin concentration, 90 g/liter-1), Neemmzid, Trilogy 90EC, Bio-neem, Turplex and Bollwship (All Thermo Trilogy), Fortune Aza and Fortune biotech (Fortune), Neem Surakasha, Proneem, Neem wave and Aza technical (All Karapur Agro), Neem Azal (Trifolio-M), Kayneem (Krishi Rasyan), Neemolin (Rallis), Surfire and Neemachtin (Consep) and Nimbecidine (T stanes) [65]. Till today the only route to obtain the desired product is seed extraction. The reproductive phase in *Azadirachta indica* begins after 5-6 years but economic yields are obtained at the age of 10-15 years [66,67]. Moreover, availability of seeds is seasonal and substantially reduced fraction of seeds are collected due to operational problems and quality considerations. The seeds lose viability very soon and percentage of azadirachtin decreases considerably during storage [11]. Above all, the plants environment influences the plant productivity and secondary metabolite content and season-to-season variations in azadirachtin content of seeds from same geographical regions. Pathogens present on seed surface may produce and accumulate toxic metabolite such as aflatoxin and be extracted with azadirachtin during processing thus the consistent supply of azadirachtin and absence of any toxic metabolite are the major concern for any azadirachtin based product. Plant cell culture system provides the advantage over both the concern and can ensure the consistent supply of azadirachtin, free from any toxic metabolite. Further there are numerous examples of cell culture that produce natural compounds in amounts equal to or higher than the whole plant. Along with the example of commercial success of shikonin from *Lithospermum erythrorhizon* [68], berberin from *Coptis japonica* [69] and ginseng from *Panax ginseng* [70,71] however the production of this commercial important compound is in infancy only and several process parameters affecting the biosynthetic potential in cell culture system need to be optimized. There are both biological and technological parameters that decide the productivity of a particular compounds in cell suspension culture and bioreactor and that are discussed with reference to *Azadirachta indica* cell culture system.

Culture Considerations

Azadirachtin content is known to vary according to genotype and environment conditions [8,33,72-76]. For the development of efficient culture system for azadirachtin production screening and establishment of productive cell line is a prerequisite. Cell line screening is needed to be performed by comparative studies of different cell lines on the growth and azadirachtin yield i.e. derivations of cell strain with inherent capacity to produce increased yield and further selection for better yielding cell lines. The colurimetric procedure [60] can offer a potential, efficient and fast screening system for determination of azadirachtin content from different cell lines. Azadirachtin callus or cell lines may also be induced from the crown gall of different parts of plant infected with *Agrobacterium tumefaciens*. The formation of transformed callus indicating the potential of use of this process on *Azadirachta indica* plant has been described [77] but no data on azadirachtin production was recorded. Therefore, results can not be compared for azadirachtin content with non-transformed callus.

The productivity of cell line is greatly influenced by the culture conditions of which the culture medium is most important. No extensive study is performed to analyze the effect of different carbon source and their concentration, nitrogen and phosphorus concentration, NH_3: NO_3^- ratio and plant growth regulators on biomass yield and azadirachtin production. There is only a single report [33] available on effect of different medium and varying sucrose concentration. These preliminary studies on azadirachtin production in different medium strongly suggest the need of optimization of different media for enhanced growth and product response. MS and White's media differ mainly in nitrogen and phosphorus concentration. White's contains nitrogen in the form of nitrate only whereas the MS in the form of both nitrate and ammonium ions, thus the differences in these two different media seemed to be due to the difference in nitrogen and phosphorus source and the concentration, suggesting the necessity of study of effects of major medium components for optimization of azadirachtin production. The increased production of secondary metabolite using nitrate as a sole N source was described in *Panax ginseng* cell cultures [78]. Very low yield of azadirachtin in cell culture (Table 1) may be attributed due to the lack of optimization of medium components therefore the optimization of media components and cultural conditions holds a good promise for attempting enhancement of azadirachtin production in plant cell culture. At early stage of optimization of medium for growth and secondary metabolite

production in cell culture, Plackett-Berman design [79-82] and response surface methodology [83] are promising tools for optimization of media, it helps to eliminate the less significant parameter and in studying large number of factors with only a few number of experiments with combined effect of all the factors involved which is not possible in conventional single variable optimization. Initiation and establishment of a stable homogenous suspension culture is prerequisite for mass propagation of any compounds from cell culture. In some culture systems the initiation and maintenance of suspension culture proved to be difficult, several strategies have been stated which ensures the development of near homogenous suspension culture [84,85].

There exists the fairly good possibility of increasing the azadirachtin yield of *Azadirachta indica* tissue culture through metabolic regulation *i.e.* with the use of elicitors and addition of precursors of azadirachtin synthesis in culture medium. Different biotic and abiotic compounds are known to trigger the synthesis and accumulation of secondary metabolite in many cell culture [86,87]. Extract of micro flora of neem seeds and other plant parts can be very well tested for their influence on azadirachtin synthesis. Precursor feeding study may not find its full application unless and until the biosynthetic pathway of this compound is not established, however the research in this area has started unrevealing the compounds involved in the biosynthesis and most probably proceeds through mevalonate, squalene, apo-tirucallol and from that through a series of oxidations, ring cleavage, and degradation [13]. These intermediates of azadirachtin synthesis may be incorporated into suspension culture medium and their effect can be analyzed for optimum concentration, time of inoculation and toxicity if any on biomass yield and azadirachtin production. Mevalonic acid (MVA) had already been reported to significantly enhance the saponin yield in ginseng cell culture [88,89]. Immobilization is yet another approach that holds a good promise for increasing product yield and reutilization of cells for a comparatively larger period than free cells provided metabolites does not accumulate within the cells and are secreted into the culture medium. Azadirachtin is known to present intracellularly thus limiting the use of cell immobilization technology. The study describing the release of azadirachtin in medium after treatment of plant cell culture with permeabilizing agents [35] can provide a good ground for seeing the possibilities of combination of immobilization and permeabilization for the adoption of immobilization cell technology to *Azadirachta indica* cell culture. Tissue culture of transformed hairy roots may be a good alternative for production of azadirachtin as hairy roots are very fast growing, provide stable and high level production of secondary metabolite and can be propagated in phytohormone free medium with very ease. Development of hairy roots containing azadirachtin and one of its derivatives has already been described [41] albeit the azadirachtin concentration is quite low (maximum to 0.0036%) emphasizing to establish higher yields of azadirachtin by further manipulations. Plant cell suspension culture and hairy root culture investigations can go parallaly to ensure a consistent and homogenous supply of azadirachtin for increasing number of azadirachtin formulations.

Extraction, analysis and purification of azadirachtin from tissue-cultured sample are also an area of research that needs to be investigated thoroughly. The extraction procedures are rather exhaustive involving the partitioning in different solvents and thus needs to be optimized there by permitting the stability and minimum loss of compound during extraction procedure. The non detection of azadirachtin in some callus culture [37,39] may be attributed to the difficulties in extraction procedure. In the study, the method of Govindchari *et al.* [7] was followed for extraction, which involves the extraction with ethanol and in the study conducted by Chaturvedi [39] methanol was used for extraction. However, the partitioning in aqueous and organic layer seems necessary to remove the polar compounds like fatty acids, oil sugar and proteins therefore the enrichment of azadirachtin from crude alcoholic extract can be considered as a prerequisite step during extraction.

Instability of azadirachtin under sunlight is major concern. The sensitivity to sunlight may limits its use as for acceptable population reduction, the insecticide should remain intact on the foliage of a while giving the insect time to ingest the material and cause the mortality. The study of photo stabilization of azadirachtin by UV absorber [90] can be explored for more stable final product for field application.

Engineering Considerations

Scale-up of suspension culture in bioreactor that provides the best conditions possible for growth and product formation is necessary for mass propagation of any secondary metabolites. Engineering challenge for mass propagation lies in the scale-up of azadirachtin production process. Extensive literature survey reveals the non-availability of any report on the large-scale production of azadirachtin under controlled conditions in bioreactor. It would be interesting to study the cultivation of *Azadirachta indica* in bioreactor to enhance the production of azadirachtin.

Different reactor configurations have been reviewed for plant cell cultures [91,92]. The suitability of particular reactor type, impeller type and choice between batch, fed-batch or continuous operation condition needed investigation based on the dynamics of culture of *Azadirachta indica* in suspension culture. Increase in product yield by batch operation in different kind of bioreactor has been reported for many plant cell culture [93,94]. Based on the nutrient limitation data derived from batch studies, various fed-batch operational strategies could be designed and by controlled addition of a limiting nutrient, kinetics of cells can be studied. The fed-batch process is a simple and often very effective approach to improve the productivity of batch culture; the data on increase in productivity is already

documented in some of the studies [95-97]. Application of continuous culture with a constant withdrawal of culture medium is well documented and cells could be well adopted to overcome the limitations of batch and fed-batch process and for improvement of volumetric product yield. Increase specific biosynthetic rate have been reported for variety of secondary metabolites in bioreactor. A continuous flow reactor with cell recycle or cell retention by using spin filter bioreactor (SFB) is a flexible cultivation system and can overcome the disadvantage of limited cell-cell contact, by using spin filter device, the system approaches immobilized cell culture leading to higher biomass and better cell to cell contact therefore a overall enrichment in product yield. The control of parameters that affects cell growth and product synthesis is an essential aspect of bioreactor operation. Overall output of bioreactor can be controlled using various control strategies as on line measurement of process parameters such as temperature, pH, dissolved oxygen, carbon dioxide and other gases. The mass culturing of plant cells in bioreactor is challenged with technological barriers of mixing, oxygen and aeration effects, complex rheological properties of plant cell like cell aggregation and adhesion and shear sensitivity. All these technological barriers need to be carefully optimized at bioreactor level for evaluation of characteristics parameters for design of an economic viable, potent large-scale production of azadirachtin process.

CONCLUDING REMARKS

As awareness towards environmental friendly and non-toxic pesticide is growing continuously the azadirachtin is gaining more and more attention all over the world due to its effectiveness among key pests, minimal to no impact on non-target organisms and compatibility with other biological control agents. The production of azadirachtin from seeds is a labour intensive and expensive process and to fulfill the increasing demand other alternatives need to be investigated. Although the research into azadirachtin cell tissue culture is in very initial state and there is long way to go before the establishment of a economical viable process, in the lights of improvement in culture technology by optimization of all physiological, environmental and bioengineering aspects discussed above plant tissue culture could be developed as a promising alternative for azadirachtin production.

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