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Enhancement of Secondary Metabolites in Cultured Plant Cells Through Stress Stimulus

[1]Manorma Sharma, [1]Archana Sharma, [2]Ashwani Kumar and [3]Saikat Kumar Basu
[1]Department of Botany, Vedic PG Girls College, University of Rajasthan Jaipur 302004, India
[2]Department of Botany, University of Rajasthan, Jaipur 302004, India
[3]Department of Biological Sciences, University of Lethbridge, Alberta, T1K 3M4, Canada

Corresponding Author: Ashwani Kumar, Department of Botany, University of Rajasthan, Jaipur 302004, India

ABSTRACT

Planttissue culturesare exposed to stresses and stress combinations that they may not have encountered in nature in their long evolution. It is a remarkable reflection on the plasticity of the plant genome that it can decipher and respond to novel *in vitro* stresses. Today various tissue culture techniques are used to enhance yield of secondary metabolites by trigger stress response like using Elicitors, Precursors and Biotransformation, change in environmental conditions, change in medium constituents etc. The focus of the study is to enhancement of secondary metabolites by triggerthe expression of stress marker genes. This would help in conservation of this species and provide pharmaceutical component in less time and cheap cost.

Key words: Stress response signaling, jasmonic acid, salicylic acid, hairy root culture, octadecanoid pathway, reactive oxygen species, elicitors

INTRODUCTION

Plants, as sessile organisms face many stress situations and their growth and productivity are adversely affected by nature's wrath in the form of various abiotic and biotic stress factors. All these stress factors are a menace for plants and prevent them from reaching their full genetic potential and limit the crop productivity worldwide (Mahajan and Tuteja, 2005). To cope up with these stresses plants posses immune system which are of two type, first system uses transmembrane Pattern Recognition Receptors (PRRs) that respond to slowly evolving microbial-or pathogen-associated molecular patterns (MAMPS or PAMPs), such as flagellin (Zipfel and Felix, 2005) and second one acts largely inside the cell, using the polymorphic NB-LRR protein products encoded by most*R*genes (Jones and Dang, 2006). Hence, the plant immune system mostly rely on proteomic plasticity to remodel themselves during periods of developmental change, to endure varying environmental conditions and to respond to biotic and abiotic stresses (Timperio *et al.*, 2008; Badea and Basu, 2009). Besides these plants have evolved a number of inducible defense mechanisms to respond both biotic and abiotic stress. Proteomics (Joseph and Jini, 2010) and metabolomics could help to reveal these mechanisms by looking at the changes in protein and metabolite abundance, respectively (Badea and Basu, 2009, 2010).

The resistance occurs on infection site known as Localized Acquired Resistance (LAR) while infection appears in the distant uninfected parts of the plant named as Systemic Acquired Resistance (SAR) (Hammerschmidt, 2009). The plant defense system stimulates when it received extracellular or intracellular signal by the receptor present on plasma membrane. Binding of receptor and signal compound activate signal transduction cascade that leads to activation or de novo biosynthesis of transcription factors which regulate the expression of biosynthetic genes

involved in plant secondary metabolism (Zhao *et al.*, 2005). Stress responses frequently involve reversible phosphorylation, ion fluxes (Ca2þ, Kþ, Hþ), Salicylic Acid (SA) or oxylipins such as Jasmonic Acid (JA), Ethylene, Reactive Oxygen Species (ROS), transcription factors and promoter elements (Stratmann, 2003).

Extensive research into various aspects of primary metabolic (Bender and Kumar, 2001) and secondary metabolic processes pave the way for the intensive study of proteomics and genomics of plant world. Comprehensive Structural researches carried out in photosynthesis system (Kumar and Neumann, 1999; Kumar *et al.*, 1987; Kumar *et al.*, 1983a,b, 1984; Bender *et al.*, 1981; Kumar, 1974a, b) and plant tissue culture (Kumar and Shekhawat, 2009; Kumar *et al.*, 1990, 1989, 1983a) form the basis for enrichment of secondary metabolites by manipulating various pathways. Most prominent research area in present scenario is to develop stress resistant plant. In Lieu of all the pitfall of defense responses, an important benefit of stress response is accumulation of secondary metabolites in plants. These metabolites are released due to defense responses which are triggered and activated by elicitors, the signal compound of plant defense responses (Zhao *et al.*, 2005). The induction of secondary metabolism gene expression by wounding, herbivore-derived molecules, pathogen elicitors and oxidative stress caused by heat, drought, flooding, UV light, or temperature extremes is often mediated by integrating signaling molecules such as jasmonate, salicylic acid and their derivatives (Nascimento and Fett-Neto, 2010).

Primary role of secondary metabolites which are released due to defense responses is to protect plant but due to its medicinal value it attracts attention of researchers. The major obstacles in obtaining these metabolites include environmental and geopolitical instabilities, limited knowledge of biosynthesis and signal transduction pathways of metabolites, slow growing nature of some species, low yields found in nature and unpredictable variability in accumulation are some of the factors which have increased the challenges for supply of pharmaceutical active principles (Karl-Hermann *et al.*, 2009). Moreover, it is also necessary to ensure the quality of herbal medicine. At this situation Plant cell culture provides a renewable, easily scalable source of plant metabolites. Several strategies such as manipulating the nutrient, optimizing the culture conditions, feeding of precursor and elicitation can be applied in order to substantially increase the yields of secondary metabolites in plant cell cultures (Kumar and Sopory, 2010, 2008). Post-green revolution advances made in biotechnology paved the way of cultivating the high-yielding, stress and disease resistant Genetically Modified (GM) varieties of wheat, rice, maize cotton and several other crops (Basu *et al.*, 2010). Hence in present scenario *in vitro* process is a promising Ecofriendly tool for the rapid multiplication of endangered medicinal species in cheap cost and less time. However, it will take a long way to convert small culture vessel in large bioreactors. The main focus of this review is to highlight the major elicitors and their impact on enhance secondary metabolites synthesis and stress response signaling.

CLASSIFICATION OF ELICITORS

Elicitors are molecules that stimulate defense or stress-induced responses in plants (Radman *et al.*, 2004). Broader definition of elicitors includes both substances of pathogen origin and compounds released from plants by the action of the pathogen (endogenous elicitors). On the basis of nature elicitors can be divided into two types biotic and abiotic. The biotic elicitors have biological origin, derived from the pathogen or from the plant itself while abiotic elicitors have not a biological origin and are grouped in physical factors and chemical compounds (Kumar and Shekhawat, 2009). Further on the basis of plant-elicitor interaction it may be classified into race specific and general elicitors (Staskawicz *et al.*, 1995; Vasconsuelo and Boland, 2007). Major metabolites elicited through elicitors are described in Table 1.

Table 1: Examples of elicitors used to enhance secondary metabolites

Plant	Product (s)	Elicitor (s)	Reference(s)
Arabidopsis thaliana	Indole glucosinolates, Camalexin	*Erwinia carotovora.*	Brader *et al.* (2001)
Cupressus lusitanica	Beta-thujaplicin	Fungal elicitor	Zhao *et al.* (2001)
Medicago truncatula	Beta-amyrin	Yeast elicitor	Broeckling *et al.* (2005)
Salvia miltiorrhiza	Diterpenoid tanshinones	Yeast elicitor	Yan *et al.* (2005)
Silybum marianum	Silymarin	Yeast extract	Sanchez-Sampedro *et al.* (2005)
Coleus forskolin	Methyl jasmonate (MeJA)	Forskolin	Babu (2000)
Panax ginseng	Saponins	Low-energy ultrasound	Wu and Lin (2002)
Taxus chinensis	Trifluoroethyl salicylate (TFESA)	Taxuyunnanine C (Tc)	Qian *et al.* (2006)
Datura stramonium	Alkaloids (tropane)	*Phytopthora megasperma*	Kurosaki *et al.* (2001)
Azadirachta indica	Azadirachtin	Jasmonic acid, salicylic acid	Satdive *et al.* (2007)
Nicotiana tabacum	Capsidiol, debneyol, scopoletin, nicotine	Cryptogein	Taguchi *et al.* (2001)
Nicotiana plumbaginifolia	Nicotine	Cellulase, MeJA	Lecourieux *et al.* (2002)
Salvia miltiorrhiza	Diterpenoid tanshinones	Yeast elicitor	Qiong *et al.* (2005)
Glycine max	Glyceollins, apigenin, genistein, luteolin	Glucan, MeJA	Modolo *et al.* (2002)
Dacus carota	Methoxymellein, 4-hydroxybenzoic acid	Fungal elicitor	Petersen and Simmonds (2003)
Ocimum basilicum	Rosmarinic acid	*Aspergillus niger*	Bais *et al.* (2002)
Rhodiola sachalinensi	Salidrosides	*Aspergillus niger*, *Coriolus versicolor*, *Ganoderma lucidum*	Zhou *et al.* (2007)
Panax ginseng	Saponin	Oligogalacturonic acid Low energy ultra sound	Hu *et al.* (2003a, b)
Lycopersicon esculentum	Scopoletin	Fungal elicitor, MeJA	Thoma *et al.* (2003)
Ammi majus	Scopoletin	*Enterobacter sakazaki*	Staskawicz *et al.* (1995)
Glycyrrhiza glabra	Soyasaponin,5-deoxyflavonoid	MeJA	Hayashi *et al.* (2003)
Vitis vinifera	Stilbene, resveratrol, anthocyanins	MeJA, ethylene	Aziz *et al.* (2003)
Salvia miltiorrhiza	Tanshinone	Hyperosmotic stress, yeast elicitor	Shi *et al.* (2006)
Taxus chinensis	Taxol	Fungal elicitation	Wang *et al.* (2001)
Brugmansia suaveolens	Tropane alkaloids	*Spodoptera frugiperda* Methyl jasmonate	Alves *et al.* (2007)
Catharanthus roseus	5'-phosphodiesterase (Pdase)	*Alteromonas acleodii*, alginate oligomers	Aogyagi *et al.* (2006)
Rubia tinctorum	Anthraquinone derivatives	Fungal polysaccharides, SA, JA	Orban *et al.* (2008)
Pinellia ternata	Alkaloids	*Pseudomonas* sp. and *Enterobacter* sp.	Liu *et al.* (2009)
Vanilla planifolia	Vanillin	Acetone dried red (beetperoxidase)	Sreedhar *et al.* (2009)
Abrus precatorius	Glycyrrhizin	*Aspergillus niger* and *Rhizopus stolonifer*	Karwasara *et al.* (2010)
Calendula officinalis	Oleanolic acid	Trichoderma viride, pectin,chitosen	Wiktorowska *et al.* (2010)
Pueraria candollei var. *candollei* and *P. candollei* var. *mirifica*	Isoflavonoid	Laminarin, Chitosan, oligosaccharide, MeJA	Korsangruang *et al.* (2010)
Carthamus tinctorius	α-tocopherol and pigment	*Trametes versicolor*, *Mucor* sp., *Penicillium notatum*, *Rhizopus* stolonifer and *Fusarium oxysporum* and abiotic (NaCl, $MgSO_4$, $FeSO_4$, $ZnSO_4$, and $FeCl_3$)	Chavan *et al.* (2010)

Table 1: Continued

Plant	Product (s)	Elicitor (s)	Reference(s)
Taxus chinensis	Taxol	Nitric oxide	Wang and Wu (2005)
Ginkgo biloba	Bilobalide and ginkgolides	Biotic	Jung *et al*. (2003)
Ganoderma lucidum	Ganoderic acid and Ganoderma polysaccharides	Fungal	Zhu *et al*. (2008)
Vaccinium corymbosum	Non-volatile phenolic compounds	UV-B radiation	Eichholz *et al*. (2011)
Drosera burmanii	pPlumbagin	Methyl jasmonate, yeast Extract and chitosan	Putalun *et al*. (2010)
Arabidopsis	Indole glucosinolates, camalexin	Thaliana Fungal elicitor, MeJA, SA; JA-independent	Brader *et al*. (2001)
Coleus blumei	Rosmarinic acid	Yeast elicitor	Szabo *et al*. (1999), Petersen and Simmonds (2003)
Eschscholtzia californica	Benzophenanthridines, sanguinarine	MeJA, Fungal elicitor *Puccinia*	Thoma *et al*. (2003)
Avena sativa	Avenanthramides	*cornoata* phytotoxin Victorin	Lee *et al*. (2004), Yang *et al*. (2004)
Catharanthus roseus	Ajmalicine, catharanthine, vindoline	Yeast elicitor, MeJA	Menke *et al*. (1999), Zhao *et al*. (2001b)
Eschscholtzia californica	Benzophenanthridines, sanguinarine	MeJA, Fungal elicitor	Thoma *et al*. (2003)
Glycine max	Glyceollins, apigenin, daidzein, genistein, luteolin	h-glucan, MeJA, 12-oxo-PDA; Glutathione	Modolo *et al*. (2002)
Cupressus lusitanica	β-thujaplicin	Oligosaccharide, MeJA	Zhao *et al*. (2001), Zhao *et al*. (2005)
Daucus carota	Fungal elicitor from *Pythium aphanidermatum*	6-methoxymellein; 4-hydroxybenzoic acid	Kurosaki *et al*. (2001)
Glycyrrhiza glabra	Soyasaponin 5-deoxyflavonoid	MeJA	Hayashi *et al*. (2003)
Lithospermum erythrorthizon	Shikonin, rosmarinic acid	Polysaccharides, fungal elicitor, MeJA	Mizukami *et al*. (1993), Yazaki *et al*. (2002)
Hyoscyamus muticus	Solavetivone, rishitin, lubimin, scopolamine	SA	Kanga *et al*. (2004)
Oryza sativa	Momilactones, sakuranetin, phytocassans	N-acetylachitoheptaose, MeJA, sphingolipid cerebrosides	Suharsono *et al*. (2002), Umemura *et al*. (2002)
Hypericum perforatum	Hypericin, pseudohypericin, hyperforin	Mannan, Colletotrichum gloeosporioides infection	Kirakosyan *et al*. (2000), Walker *et al*. (2002)

Types of elicitors

Biotic elicitors:

- Polysaccharides derived from plant cell walls (pectin or cellulose), microorganisms (chitin or glucans) and glycoproteins
- low-molecular-weight organic acids
- low-molecular-weight phytochemicals produced by plants in response to physical damage, rodents, herbivores, insects, fungi, virus or bacteria attack
- proteinkinases

Abiotic elicitors:

- Chemicals like inorganic salts, heavy metals, some chemicals that disturb membrane integrity. Chemicals and pollutants (heavy metals, pesticides and aerosols), Excess water (Xooding), nutrient deprivation in soil
- Physical factors like mechanical wounding, ultraviolet irradiation, high salinity, high or low osmolarity, extreme temperature wind (sand and dust particles in wind), drought, ozone or high pressure

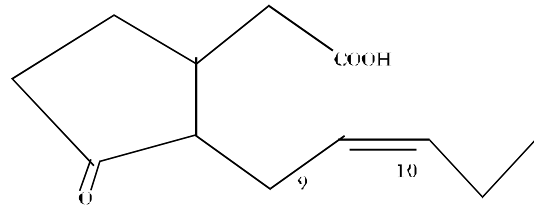

Fig. 1: Molecular structure of JA

Exogenously supplied methyl jasmonate activates a multitude of jasmonate induced proteins, some of which are probably associated with accumulation of secondary metabolites. Through different elicitation process the JA is induced to activate biosynthesis of economically important secondary metabolites. Treatment with jasmonate elicits the accumulation of several classes of alkaloids and terpenoids Molecular structure of jasmonic acid is shown in Fig. 1.

Jasmonic Acid (JA) and its volatile equivalent, methyl jasmonate (MeJA), are plant hormones involved in chemical and physiological defense responses (Fritz *et al*., 2010). They are oxylipins (oxygenated fatty acids) that originate from linolenic acid released from chloroplast membranes by lipase enzymes and subsequently oxygenated by lipoxygenases (LOXs) to hydroperoxide derivatives (Balbi and Devoto, 2008). The oxylipin Jasmonic Acid (JA) and Its metabolites, metabolite formation. Elicitation or stress stimulus leads to a rapid release of α-linolenicacid from the lipid pool of the plant cell which through an intracellular signal cascade elicits secondary metabolite production important for plant defense (Memelink *et al*., 2001). α-Linolenic acid is converted by a lipoxygenase, an Allene Oxide Synthase (AOS) and an Allene Oxide Cyclase (AOC) into the intermediate 12-oxo-phytodienoic acid. This compound is converted into JA through the action of a reductase and three rounds of β-oxidation (Menke *et al*., 1999; Mueller, 1997).

In unstressed cells containing low JA levels, transcription activator MYC2/JIN1, a bHLH that promote expression of JA-responsive genes are repressed by members of the Jasmonate ZIM-domain (JAZ) protein family (Chini *et al*., 2007; Thines *et al*., 2007; Yan *et al*., 2007) turn the collectivelyknown as jasmonates, are important plant signaling moleculesthat mediate biotic and abiotic stress responses as well asaspects of growth and development (Santner and Estelle, 2007). JA actives stress response in cell by two ways (1) JA produced at the wound site serves as a mobile signal to activate responses in systemic tissues. (2) wound-induced production of a mobile signal other than JA activates synthesis of the hormone in systemic tissues (Abraham and Howe, 2009). Elicitors of JA responses include chitin, oligogalacturonides and cell wall-degrading enzymes (Norman-Setterblad *et al*., 2000).

Jasmonate modulates gene expression at the level of translation, RNA processing and transcription. It works as a transcription transducer or mediator for elicitor signaling between the elicitor-receptor complex and the gene activation process responsible for the induction of enzyme synthesis that leads to the formation of low molecular weight defense compounds phytoalexins such as flavanoids, alkaloids, terpenoids, thionins, phenylpropenoid and polypeptides (Kumar, 2010). In comparison of exogenous supply of MeJA and indirectly induction by other elicitors it was found that induction of JA through other elicitors found to be more effective to produce secondary metabolites rather than provide alone. In one of the researches in *Pueraria* tuberose cell culture, it has been demonstrated (Goyal and Ramawat, 2008) that elicitation through yeast extract increase isoflavonoids production ~20% higher over the yields at optimal concentrations of MeJA and SA.

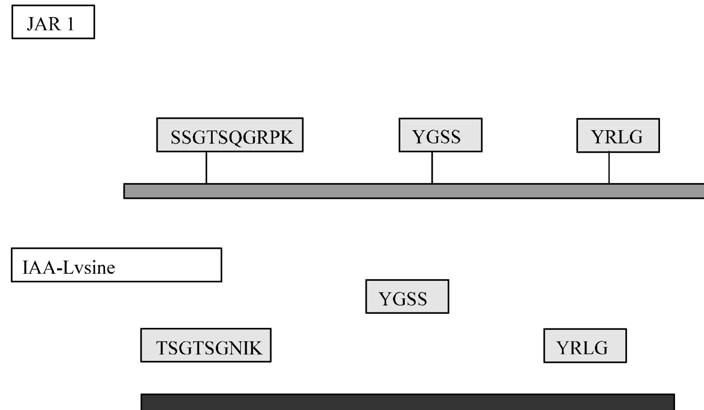

Fig. 2: Genetic configuration of JAR1 and IAA-Lysine synthetase

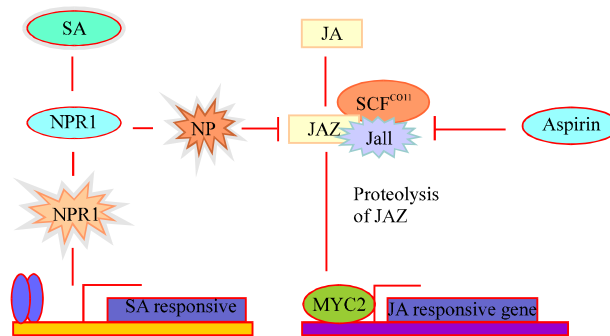

Fig. 3: Transcriptional regulation of JA biosynthesis and its inhibitors

The α-Linolenicacid and 12-oxophytodienoicacid are known to be distant precursors of jasmonic acid (Mueller *et al*., 1997) induced transcriptional activation of genes involved in secondary pathway on. In stressed cell JAR1 (Jasmonate resistant 1; genetic configuration describe in Fig. 2) encodes a enzyme jasmonate-amido synthetase (it is a member of the GH3 family of proteins) that conjugates JA to JA-Ile (Jasmonic acid-isoleucin) which in turn promotes the interaction between Coronatine insensitive 1(COI1) and the JAZ repressors. COI1 is the F-box protein component of the E3 ubiquitin ligase SCFCOI1 (Skp/Cullin/F-box) (Balbi and Devoto, 2008; Abraham and Howe, 2009). It leads to degradation of JAZ repressors by ubiquitin/26S proteasome degradation pathway, releasing MYC2 that binds to G-box (CACGTG) or the T/Gbox (AACGTG) in the promoters of JA-regulated genes (Dombrecht, 2007). Binding of MYC2 induces transcription of early JA-responsive genes including the JAZs. De novo synthesis of JAZ proteins restores MYC2 repression and turns the pathway of (Fig. 3).

The other positive regulators or transcription factors of JA responses to wounding (stress response) are WRKY18 (Xu *et al*., 2006), AtERF2, AtERF4, MYB21, MYB24 wRKY70 At1g74930 and At3g53600 (Wang *et al*., 2001; Pre *et al*., 2008; Wasternack, 2003) which also dependent on COI1.

BIOSYNTHESIS OF JA

Oxylipins are classes of biologically active compounds that are generated by oxidative catabolism of polyunsaturated fatty acids (adding oxygen to the 9 or 13 position of the C18 chain

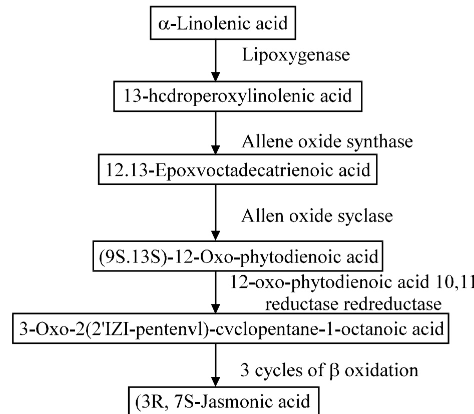

Fig. 4: Biosynthesis of JA

of linoleic and linolenic acids) by the coordinated action of lipases, lipoxygenase and a group of cytochromes P450 (CYP74 family), including Allene Oxide Synthase (AOS) and hydroperoxide lyase (HPL) (Zhao *et al.*, 2005). Most of the oxylipins including JA,MeJA, are synthesized by AOS dependent octadecanoid pathway (Creelman and Mullet,1997). α-LeA released by lipase activity on chloroplast membranes is the primary substratefor this pathway (kumar *et al.*, 1999). The biosynthetic pathway is well established and at least five key enzymes (lipase, lipoxygenase, allene oxide synthase,allene oxide cyclase and OPDA reductase) are known to be involved in this process (Schaller, 2001). α-LeA along with lipoxygenases (LOXs) formed two hydroperoxides 13S-hydroperoxyoctadecatrienoic acid (13-HPOT) or (9S)-hydroperoxyoctadecatrienoic acid (9-HPOT). Further 13-HPOT leads to JA pathway. In the first step of JA biosynthesis 13-HPOT along with 13-AOS converted into 12,13EOT an unstable allene oxide. AOSs belong to the family of CYP74A enzymes which are independent from molecular oxygen and NADPH, exhibit low affinity to CO and use the hydroperoxide group as a source for reducing equivalents and oxygen (Feussner and Wasternack, 2002). The ensuing enzyme AOC (allene oxide cyclase) converts 12,13EOT into cis (+) -12-oxophytodienoic acid (OPDA). Non-enzymatic side-reactions in the absence of AOC are the formation of racemic OPDA and the cleavage to α- and γ-ketol (Wasternack, 2003). All the steps till now have been completed in plastid after OPDA formation, OPDA and β-oxidation proteins are targeted to peroxisomes (Reumann *et al.*, 2004; Kumar *et al.*, 1999). Which is the main compartment of plant cells in which fatty acid β-oxidation occurs (Baker *et al.*, 2006) via the ABC transporter PXA1/CTS/PED3 acting in fatty acid and IBA import (Footitt *et al.*, 2002; Hayashi *et al.*, 2002; Theodoulou *et al.*, 2005) and is activated by a peroxisomal OPDA: CoA ligase (Schneider *et al.*, 2005).

Later on OPDA's cyclopentenone ring or enone structure is reduced by peroxisomal OPDA reductase (OPR) and form 3-oxo-2(2'|Z|-pentenyl)-cyclopentane-1-octanoic acid. In the last step of JA biosynthesis would require three enzymes of b-oxidation viz acyl-CoA oxidase (ACX1),a multifunctional protein (AIM1/MFP2) and a L-3-ketoacyl CoA thiolase(KAT) those evidences are genetically have been genetically proved in Tomato and Arabidopsis (Li *et al.*, 2005; Wasternack *et al.*, 2006). SA and asparin are well known inhibitors of JA synthesis which inhibit the reaction at transcriptional level by its binding to JAZ as describe in Fig. 3. In designing

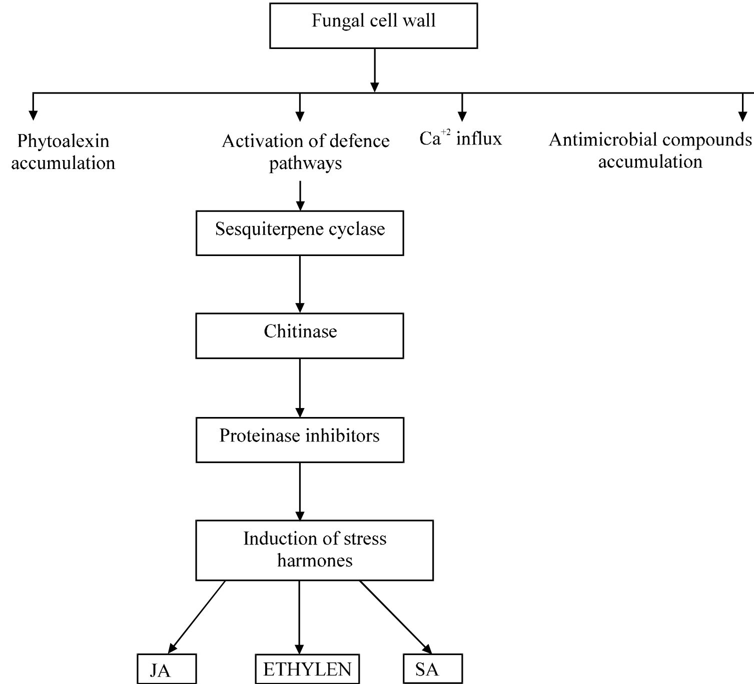

Fig. 5: Possible induced pathways by fungal cell wall elicitation

experiment for *in vitro* elicitation through JA we have to consider the biosynthesis pathway of JA as well as its inhibitors and promoters. JA biosynthesis study is of great importance as it induces many other stress factors directly or indirectly.

Fungal cell wall: Elicitation of cell cultures with fungal cell wall components has long been known to result in the synthesis of low molecular weight compounds. Fungal cell wall works as polysaccharide elicitor which induces calcium concentration in cell and activates various defense responsive pathways leads to accumulation of phytoalexin and low molecular weight antimicrobial compounds (Cordell, 1997). Fungal cell wall acts as a chemical messenger with specific regulatory properties. Possible induced pathways by fungal cell wall are described in Fig. 5.

Upon elicitation with fungal cell wall elicitors from *Phytophthora cinnamoni*, the production and exudation of harmine/harmaline was enhanced in both transformed and non-transformed roots (Bais *et al*., 2003). Diosgenin accumulation in cell suspension cultures of *Dioscorea zingiberensis* C.H. Wright has been reported to be enhanced by treatment with saccharide elicitors from its endophytic fungus *Fusarium oxysporum* isolate Dzf17 (Zhang *et al*., 2009; Zhao *et al*., 2011). In this research oligosaccharide was prepared by partial acid hydrolysis of the isolated Dzf17 fungal cell wall fragments works as an elicitor to increase JA.

Spectacular increase in yield of anthraquinone content along with high lucidin primveroside, ruberithic acid and pseudopurpurin production were measured in the cell suspensions of *Rubia tinctorum* elicited by fungal polysaccharides (Orban *et al*., 2008). Elicitation response of fungal cell wall varies to its concentration and strain used. In *Xanthophyllomyces dendrorhous* among Six fungal elicitors prepared from *Rhodotorula rubra, Rhodotorula glutinis, Panus conchatus, Coriolus versicolor, Mucor mucedo, Mortieralla alpina M-2,3, M. mucedo* elicitor

concentration of 30 mg L^{-1} promoted the biomass and total carotenoids yield most remarkably, resulting in 69.81±6.00% and 78.87±4.15% higher than the control, respectively (Wang *et al.*, 2001).

HAIRY ROOT CULTURE

In the Rhizosphere, plants may suffer from wounds by soil pathogens or other sources. This leads to the secretion of phenolic compounds like acetosyringone which have chemotactic effects that attract the bacteria. Among these soil pathogens *Agrobacterium* is well known name in the field of biotechnology. *Agrobacterium* is a genus of Gram-negative bacteria discovered by Nicholas Davidson from Glasgow that uses horizontal gene transfer to cause tumors in plants. *Agrobacterium* species are phytopathogens that cause a variety of neoplastic diseases, including crown gall (*Agrobacterium tumefaciens* and *Agrobacterium vitis*), hairy root (*Agrobacterium rhizogenes*) and cane gall (*Agrobacterium rubi*).

Neoplastic hairy root culture obtained by infection of explants with *A. rhizogenes*, a gram-negative soil bacterium, offers an efficient system for secondary metabolite production. Hairy root culture, also called transformed root culture, is a type of plant tissue culture that is used to study plant metabolic processes or to produce valuable secondary metabolites. Hairy root cultures resulting from the infection of plant materials by *Agrobacterium rhizogenes* produce the same secondary metabolites as those usually synthesized in intact parent plant roots with similar or higher yields (Zehra *et al.*, 1999). It is a promising tool in biotechnology to generate valuable secondary metabolites. The fast growing nature of hairy roots, low doubling time, biosynthetic stability, ease of maintenance, high yield of secondary metabolites and no need of growth hormones offers an additional advantage. Moreover, transformed roots are able to regenerate whole viable plants and maintain their genetic stability during further sub-culturing and plant regeneration (Saito *et al.*, 1992). Insertion of specific secondary metabolite precursor or enzyme gene along with the Ri T-DNA genes can alter metabolic pathways and production of useful metabolites or compounds of interest through that catalyze certain hydroxylation, methylation and glycosylation reactions (Giri and Narasu, 2000).

The main aim to produce hairy root culture is to find out the efficient parameters for commercial production. The researches which have been carried out in this field create enormous combinations of hairy root culture and elicitors *viz* MeJA, JA, SA, yeast extract, metal ions etc. to produce high yield of secondary metabolites (Kumar and Roy, 2006; Ismail *et al.*, 2009). Treatment of hairy root cultures with elicitors increase production of various secondary metabolites many fold. Among many biotic and abiotic elicitors MJ and salicialic acid (Ansari and Misra, 2007) proved to be more efficient to enhance yield of secondary metabolites viz hyoscyamine alkaloid ~1200% in *Brugmansia candida* (Tatiana C. Spollansky *et al.*, 2000), Pyrrolizidine alkaloids 19-fold in *Echium rauwolfii* (Abd El-Mawla, 2010), pyridine alkaloids, mainly nicotine in *Nicotiana tabacum* (Zayed and Wink, 2010), Alkamide production in *Echinacea purpurea*, *Echinacea pallida* and *Echinacea angustifolia* (Romero *et al.*, 2009), phytoestrogenic isoflavones in *Psoralea corylifolia* L. (Shinde *et al.*, 2009) valepotriate 50- and 12-fold in *Valerianella locusta* (Kittipongpatana *et al.*, 2002), Ginsenoside in *Panax ginseng* (Yu *et al.*, 2000; Palazon *et al.*, 2003) etc. In one of the researches carried out by (Zabetakisa *et al.*, 1999) elicitation through MJ increased tropane alkaloid from *Datura stramonium* more in comparison with fungal elicitor and oligogalacturonide.

Plants equally react to wound in the tissues directly damaged (local response) as well as in the non-wounded areas (systemic response) (Szczegielniak, 2007). Mechanical wounding or infection

of plants with bacteria, insects and herbivorous caused an accumulation of secondary metabolites in plants. Molecules involved in the wound response include jasmonic acid (Farmer *et al.*, 1992), abscisic acid (Pena-Cortes *et al.*, 1996), ethylene (O'Donnell *et al.*, 1996; O'Donnell *et al.*, 2001), oligosaccharides (Walker-Simmons *et al.*, 1984; Darvill *et al.*, 1992) and system in (Ryan and Pearce 1998). Plant hormones abscisic acid and jasmonic acid (JA) works as key components of the wound signal transduction pathway. Systemin leads to an increase of both phytohormones (ABA and JA) only in wild-type but not in ABA-deficient plants (Pena-Cortes *et al.*, 1996).

Wound-induced resistance (WIR) involves direct activation of many genes, including those encoding protease inhibitors. Wound response is highly complex process includes crosslink of various pathways. Most of the pathways play around endogenous mediator of the octadecanoid pathway as the salicylate and ethylene pathways (Reymond and Farmer, 1998; Turner *et al.*, 2002) rest which are independent of JA 2004 (Reymond *et al.*, 2000; Leon *et al.*, 2001; LeBrasseur *et al.*, 2002; Harman *et al.*, 2004) may be indirectly affect the process. In the initial researches it was assumed that wound in plants cure by harmone traumatin (Zimmerman and Coudron, 1979) Systemin, is the first wound-signaling 18 amino acid (Corrado *et al.*, 2007) peptide for which a wound-signaling function has been demonstrated (Ryan and Pearce, 1998). Perception of system in by the membrane-bound receptor SR160 results in activation of MAPKs, synthesis of Jasmonic Acid (JA) and expression of defense genes (Kandoth *et al.*, 2007; Nadarajah and Sidek, 2010; Nadarajah *et al.*, 2009). Therefore systemin locally contributes to the biosynthesis of JA which regulates the production of, or acts as, a mobile wound signal that is transported throughout the plant to trigger the systemic wound response (Li *et al.*, 2002, 2005).

During wounding, systemin precursor protein prosystemin (200 amino acid) carboxy-terminal end cleaved into 18 amino acid peptide systemin (McGurl *et al.*, 1992). The systemin gene has 10 introns and 11 exons, ten of which are organized as five homologous pairs with an unrelated sequence in the eleventh (McGurl *et al.*, 1992). After proteolitic cleavage from prosystemin, systemin bind to receptor SR160. This binding activates series of events to activate a PLA2 to release linolenic acid precursor of JA from membranes which include a depolarization of the plasma membrane, the opening of ion channels, an increase in the concentration of intracellular Ca^{+2} (Dombrowski and Bergey, 2007) might activate a NADPH-oxidase-like enzyme in the plasma membrane, resulting in the production of H_2O_2, a Reactive Oxygen Species (ROS), the inactivation of a plasma membrane proton ATPase resulting in a net influx of protons into the cytosol the activation of a MAP kinase (Schaller and Oecking, 1999) the synthesis of calmodulin (Bergey and Ryan, 1999). A net proton influx leads to apoplastic alkalinization (AR), a prerequisite for activation of cell wall peroxidase (CWPox) (Stratmann, 2003) and the activation of a PLA2. After releasing from plasma membrane linolenic acid form JA through octadecanoid pathway. Other signals such abscissic acid (ABA), ethylene, H_2O_2, UV light, oligogalacturonides (OGAs) and fatty acidamino acidconjugates (FACs) activate JA formation where as salicylate (SA), its derivative acetyl-SA (aspirin) and NOcan repress JA formation (Wasternack *et al.*, 2006). The JA-responsive (JR) genes JR1, JR2 and JR3 are strongly induced by wounding and by JA while the wound-responsive (WR) genes WR3 and acyl CoA oxidase (ACO) are induced by wounding only (Leon *et al.*, 2001). An important factor in positive regulation of JA biosynthesis upon wounding is the wound-inducible protein kinase (WIPK), a member of the class of mitogen-activated protein kinases (Van verk *et al.*, 2009).

Table 2: HRC of different plants used with elicitors for increased metabolites production

Plant	Metabolites	Elicitors	References
Ammi majus	Coumarine, furocoumarine	BION®, *Enterobacter sakazaki*	Staniszewska *et al*. (2003)
Brugmansia candida	Hyoscyamine alkaloid	JA	Spollansky *et al*. (2000)
Datura stramonium	tropane alkaloid	MJ	Zabetakisa *et al*. (1999)
Echium rauwolfii	Pyrrolizidine alkaloids	MJ, quercetin and SA	Abd El-Mawla (2010)
Oxalis tuberosa	*Phytosphtora cinnamoni*	Harmaline, harmine	Bais *et al*. (2003)
Psoralea corylifolia L.	Phytoestrogenic isoflavones		Shinde *et al*. (2009)
Panax ginseng	Ginsenoside	Chitosan, MeJA, vanadyl sulfate	Palazon *et al*. (2003)
Pharbitis nil	Umbelliferone, scopoletin, skimmin	$CuSO_4$, MeJA	Yaoya *et al*. (2004)
Salvia miltiorrhiza	Transhinone	Yeast elicitor, Ag,	Ge and Wu (2005)
Scopolia parviflora	Scopolamine	*Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus*	Jung *et al*. (2003)
Solanum tuberosum	Sesquiterpene (rishitin, lubimin, phytuberin, phytuberol), lypooxygenase	*Rhizoctonia bataticola*, B cyclodextrin, MeJA	Komaraiah *et al*. (2003)

JA is also known as wound harmone, it is lipid derived secondary messenger use in the wound-signal transduction pathway and is a potent inducer of proteinase inhibitor II. mRNA levels of the gene for proteinase inhibitor II is generally suppressed in the same wounded plants, probably due to SA overproduction (Sano *et al*., 1994), Differential Role of proteinase inhibitor I and II genes in bacterial infection has been reported by Pautot *et al*. (1991), Farmer *et al*. (1992), Malone and Alarcon (1995), Turner *et al*. (2002) and Ryan and Pearce (1998) in tomato. According to this study Proteinase inhibitor I accumulated more in disease susceptible plant while Proteinase II in disease resistant plant (Valueva *et al*., 2003). Besides these genes two plant species, potato (*Solanum tuberosum* cv. Superior) and a nontuberizing potato species (*Solanum brevidens*) were tested for MeJA induction of the potato cathepsin Dinhibitor at the RNA level using a leaf-petiole cutting systemin. This research revealed that cathepsin Dinhibitor proteins are also related to wound response whose transcript concentration is tremendously increased after JA treatment.

Knowledge of all these pathways may pave way in scaling up of hairy root cultures in fermenter through various combinations of elicitors. Therefore, using elicitors or by performing metabolic engineering to the hairy roots can produce increased amount of secondary metabolites (Table 2).

UV radiation: Ultraviolet (UV) light is electromagnetic radiation with a wavelength shorter than that of visible light but longer than X-rays, in the range 10 to 400 nm and energies from 3 eV to 124 eV. The name means beyond violet (from Latin *ultra*, beyond), violet being the color of the shortest wavelengths of visible light. UV light (10-400 nm) has a shorter wavelength than violet light. The sun emits ultraviolet radiation in the UVA (320-400nm), UVB (280-320) and UVC (200-280) bands.

UV radiations cause various damage in the plant at molecular level includes formation of cyclobutane-pyrimidine dimmers in DNA, Inactivation of photosystem II (PSII), Decreased thylakoid membrane integrity, Decreased levels of chlorophyll and carotenoids, Down-regulation of photosynthetic genes, Changes in chloroplast ultrastructure,, Peroxidation of lipids, Photooxidation of IAA, Increased activity superoxide dismutase and glutathione reductase, Rise in levels of glutathione and ascorbate etc. lieu of all these damages associated with UV radiation activates genes of the phenylpropanoid pathway and flavonoid pathways leading to accumulation

of flavonoid, anthocyanins, alkaloids waxes and polyamines (Treutter, 2005; Koltermann *et al.*, 2007; Schreiner *et al.*, 2009; Aziz *et al.*, 2003). The induction of secondary compounds in the flavonoid pathway is multi-step regulated by environmental factors, UV-radiation and nutrients (Lavola *et al.,* 2002) which may also effect the accumulation of secondary metabolites.

Radiations show band specific stress responses, UV-B activates the genes similar to the genes induced by pathogen and wounding leads to activation of secondary metabolites genes and reactive oxygen species while UV-C irradiation relate to Protease inhibitor genes and wound responsive genes. Similarity between wound and UV induced responsive genes have been identified in *Nicotiana attenuate* and about 250 genes found to be similar to wounding response (Izaguirre *et al.*, 2003).

The activity of the antioxidant enzymes catalase, glutathione dehydrogenase and guaiacol peroxidase increased by UV-B radiation in sunflower cotyledons (Costa *et al.*, 2002). Secondary metabolite analysis showed increases in (+) -catechin, quercetin, cinnamic acid derivative, apigenin and pentagalloylglucose in birch leaves under enhanced UV-B radiation (Elena *et al.*, 2001). Beside UV induced wound response similarities some of researchers have found the correlation between UV radiation and ROS. In sunflower cotyledons the activity of the antioxidant enzymes catalase, glutathione dehydrogenase and guaiacol peroxidase increased by UV-B radiation (Costa *et al.*, 2002). However, little information is available on the exact stress responses elicited by UV radiation. That is ROS signaling can be elicited by more than one pathway and hard to define through which pathway UV induced ROS signaling.

CONCLUSION

Plants are well known source of pharmaceuticals but due to its low yield it is not economically feasible. On the other hand sacrifice endangered plants for secondary metabolites may distinct these valuable species. To combat these obstacles *in vitro* tissue culture is a promising tool to get these metabolites. Even in *in vitro* culture applying other strategies viz. like media manipulation, phytohormone regulation, precursor feeding, plant cell immobilization, biotransformation and bioconversion, hairy root cultures and genetically modified cell etc can be used to obtain good yield of secondary metabolites in tissue culture. Among all these elicitation of tissue culture has found to be more economical beneficial.

During elicitation more than one pathways and reactions start working in which some are cross linked while some inhibit the stress response. Due to complexity of these stress responsive pathways and its crosslink with other pathway exact mechanism of elicitor responsive pathways is not understood. To get know potential elicitation process we have to look into all the aspects of microbiology, Phyto-chemistry, Biochemistry, Plant science, Pharmacognosy, Molecular biology and Fermentation technology associated with stress response.

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