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Hairy Root Culture Through *Agrobacterium rhizogenes* for Enhancement of Secondary Metabolites Production in Medicinal Plants: A Review

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Abstract: Plants are a tremendous source for the discovery of new products with medicinal importance in drug development. Several distinct chemicals derived from plants are used in various important ways. Secondary metabolites are economically important as drugs, flavor, dye, pesticides, and food additives. Plants produce the diversity of secondary metabolites which not only plays an important role in adaption according to the environment but also represents an important source of active pharmaceuticals. The possibility of altering the production of bioactive plant metabolites through tissue culture technology is one of the emerging fields of biotechnology to investigate and enhance the production of secondary metabolites. This enhancement through field cultivation has many defects such as slow growth and low and variable yield due to the environmental and biotic factors. Therefore, hairy root culture has been developed as a more efficient alternative biotechnological tool for secondary metabolite synthesis, regardless of environmental, seasonal, and climatic variations. *In vitro* hairy roots formed by genetic transformation have been efficiently utilized for the synthesis of higher levels of flavonoids due to their biochemical and genetic stability as well as their fast growth in media without phytohormones. The focus of the present review is a detailed assessment of research on rhizogenesis in different plants using *Agrobacterium rhizogenes* for the last twelve years particularly for the enhancement of secondary metabolites. The study reveals different techniques involved for rhizogenesis in different plants, compatibility trends of the desired gene, and modifications in the techniques during these years.

Key words: Hairy root cultures, *Agrobacterium rhizogenes,* rhizogenesis, secondary metabolites.

The rationale of the study

Plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavors, and industrially important biochemicals. Accumulation of such metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules. Secondary metabolites play a major role in the adaptation of plants to the environment and in overcoming stress conditions. Environmental factors viz. temperature, humidity, light intensity, the supply of water, minerals, and CO_2 influence the growth of a plant and secondary metabolite production.

The principle advantage of recent technology is that it may provide a continuous and reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be extracted. Plant cell and tissue cultures hold great promise for controlled production of useful secondary metabolites on demand. The current yield and productivity cannot fulfill the commercial goal of plant

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cell-based bioprocess for the production of most of the secondary metabolites. To stretch the boundary, recent advances, new directions, and opportunities in plant cell-based processes are being critically examined. Strategies to improve the production of secondary metabolites must be considered. The productivity of the desired metabolites is limited by the lack of particular precursors. Biotransformation using an exogenous supply of biosynthetic precursors, genetic manipulation, and metabolic engineering may improve the accumulation of compounds.

Agrobacterium rhizogenes (recently revised as *Rhizobium rhizogenes*) [80] is responsible for hairy root disease in a broad range of dicotyledonous plants and some gymnosperms. *A. rhizogenes*, a Gram-negative soil bacterium, infects plants, adventitious roots called 'hairy roots' are induced from the infected site [67]. This event occurs due to the transfer of the particular DNA region called transfer DNA (T-DNA) comprising the loci between the TR and TL regions of the root-inducing (Ri) plasmid of the bacterium into the plant genome. The basic molecular mechanism of T-DNA trimming from the Ri plasmid, transfer to plant cells, and integration into the plant genome is known, although the functions of several genes on the T-DNA have not yet been elucidated. The hairy roots are aseptically cultured *in vitro* without added phytohormones.

Although as in other fields of science, new technology brought drastic changes in techniques and outcomes of plant cell culture. A problem arose during the *in vitro* production of secondary metabolites from plants due to the incompatibility of the plant with a particular technique. Every plant has its definite genetic composition and stability, thus the outcome of applied technique is not always progressive. It needs to be optimized with different conditions and different bacterial strains to get success.

The objective of this study is to get an overview of the application of elicitors and *Agrobacterium rhizogenes* mediated hairy root culture for the production of secondary metabolites in medicinal plants. Various elicitors have been reported to enhance secondary metabolite production in the plant. In Table 1, the work regarding the use of elicitors has been summarized. Data regarding the compatibility of a specific strain of *A. rhizogenes* mediated hairy root culture used for various plants has been recorded for the last twelve years, presented in Table 2. A collective approach has been made to provide the researcher with the information's regarding the outcomes of biotechnological techniques, particularly hairy root culture and application of precursor for the specific plant. This will make easier the path for new researchers.

Trends in enhanced production of secondary metabolites in plants

Plant cell and tissue cultures can be established routinely under sterile conditions from explants, such as plant leaves, stems, roots, and meristems for multiplication and extraction of secondary metabolites [26]. Strain improvement, methods for the selection of high-producing cell lines, and medium optimizations can lead to an enhancement in secondary metabolite production [30]. The capacity for plant cell, tissue, and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology. The strong and growing demand in today's market place for natural, renewable products has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products and has opened the way for new research exploring secondary product expression *in vitro* [26].

Addition of precursor for boosted secondary metabolite production:

Production of secondary metabolites can be enhanced in hairy root cultures by the addition of several types of precursors. Enhanced flavonoid production in hairy root cultures of *Glycyrrhiza uralensis* Fisch by combining the over-expression of Chalcone isomerase gene with the elicitation treatment PEG8000 (2 %) alone, yeast extract (YE) (0.1 %) alone, or both of them, and then the total flavonoids were extracted and measured. The results showed that over a culture period of 3 weeks, the wild-type hairy roots, the untreated transgenic hairy roots, and the double-treated

Table 1. Hairy root culture of different plants used with elicitors for increased metabolites Production (courtesy [61])

Plant	Metabolites	Elicitors	References
Ammi majus	Coumarine,furocouma rine	BION,*Enterobacter sakazaki*	65
Brugmansiacandida	Hyoscyamine alkaloid	JA	63
Datura stramonium	Tropane alkaloid	Methyl jasmonate	81
Echium rauwolfii	Pyrrolizidine alkaloids	Methyl jasmonate Quercetin and Salicylic acid	43
Oxalis tuberose	*Phytosphtora cinnamon*	Harmaline, harmine	2
Psoralea corylifolia L.	Phytoestrogenic isoflavones	Chitosan	38
Panax ginseng	Ginsenoside	Chitosan, MeJA, vanadyl Sulfate	55
Pharbitis nil	Umbelliferone,scopole tin, skimmin	CuSO4, MeJA	79
Salvia miltiorrhiza	Transhinone	Yeast elicitor, Ag	22
Scopolia parviflora	Scopolamine	*Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus*	84
Solanum tuberosum	Sesquiterpine (rishitin, lubimin,phytu berin, phytuberol), lypo-oxygenase	*Rhizoctonia bataticola*, B cyclodextrin, MeJA	33

JA: Jasmonic acid; MeJA: Methyl jasmonate

transgenic hairy roots accumulated 0.842, 1.394, and 2.838 (g/100 g DW) of total flavonoids, respectively [86]. Enhanced morphinan alkaloid production was observed in hairy root cultures of *Papaver bracteatum* by over-expression of salutaridinol 7-o-acetyltransferase gene via *Agrobacterium rhizogenes* mediated transformation [62]. Enhancement of flavone levels was studied through over expression of *Chalcone isomerase* in hairy root cultures of *Scutellaria baicalensis* [52].

Knowledge of all the pathways may pave the way in scaling up of hairy root cultures in fermenter through various combinations of elicitors (Table 1). Therefore, using elicitors to the hairy roots can produce an increased amount of secondary metabolites.

Agrobacterium rhizogenes mediated hairy root culture

Agrobacterium species mostly are pathogenic, generating tumor and hairy roots in the plant by the integration of T-DNA containing pathogenic genes encoding phytohormone and opine synthesis enzymes [8]. Different strains of *A. rhizogenes* showed diverse hairy root induction efficiency. The strains of *A. rhizogenes* that have usually been applied in hairy root induction of medicinal plants include A4, 15834, LBA9402, MAFF03-01724, R-1601, R-1000 and TR105. The *A. rhizogenes* strains differed widely in their ability to induce hairy roots, were reported from *Linum flavum* leaf discs, with the LBA9402 strain being the most efficient [40]. The choice of *A. rhizogenes* strains for hairy root induction is host-dependent. For instance, although the A4 strain was considered highly virulent and was shown to be highly effective in inducing hairy roots of many plant species, it was not effective in inducing hairy roots from *Linum flavum* leaf discs [40].

Ri plasmid of *Agrobacterium rhizogenes*

Virulent strains of *Agrobacterium* contain tumor-inducing (Ti) or Ri plasmids. *A. rhizogenes* contain Ri plasmid (Fig. 1) possessing different gene segments [12]. The transferred DNA (T- DNA) is referred to as the T-region when located on the Ti or Ri plasmid. During infection with *A.*

Table 2. Secondary metabolites produced using hairy root cultures

No.	Name of plant	Name of secondary metabolites	Strain of *Agrobacterium rhizogene*	References
1	*Andrographis paniculata*	Andrographolide	MTCC 532	41
2	*Glycinemax* (L.) Merr	-	K599	13
3	*Nicotiana tabacum cv*	Stilbenes,(t-Pn and t- Pt)	A4 strain	25
4	*Lactuca serriola*	Flavonoids and phenolics	AR15834	46
5	*Perovskia abrotanoides*	Tanshinones	ATCC 15834TR105, and R1000	15
6	*Stevia rebaudiana*	Steviol glycosides	LBA9402 and A4T	48
7	*Mucuna pruriens* (L) DC.	-	MTCC 532 &2364	77
8	*Hyoscyamus reticulatus* L	Hyoscyamine and scopolamine	A7	47
9	*Rauwolfia serpentine* and *Solanum khasianum*	Ajmaline and Ajmalicine	A4 strain	64
10	*Datura stramonium*	Hyoscyamine	A4 strain	4
11	*Artemisia dracunculus* L	Hairy root culture	A4	14
12	*Agastache foeniculum*	Rosmarinic acid	A4 strain	49
13	*Withania somnifera* (L)	Withaferin A and withanolide A,	R1000	68
14	*Berberis aristata* DC	Berberin	MTCC 532 and2364	3
15	*Isatis tinctoria*	Rutin, Neohesperidin, Budd-leoside, Qu-ercetin	LBA9402	21
16	*Psammosilene tunicoide*	Saponin	ATCC 15834	75
17	*Rubia cordifolia*	Anthraquinones	wild culture	88
18	*Picrorhiza kurroa*	Picroliv	LBA 9402	74
19	*Datura metel*	Atropine	A4 strain	58
20	*Platycodon grandiflorum*	Chlorogenic acid (CGA)	strain R1000	71
21	*Capsicum annuum*	Capsinoids, quercetin	ATCC 43056and ATCC 43057	44
22	*Coleus forskohlii*	Forskolin	MTCC 2364	56
23	*Tripterygium wilfordii* Hook. f.	Triptolide and Wilforine	ATCC 15834 and A4	87
24	*Gentiana scabra*	Gentiopicroside	ATCC 15834	24
25	*Fagopyrum tataricum*	Rutin and Quercetin	Wild strain	82
26	*Valeriana officinalis* L	Valerenic acid	A13	70
27	*Tribulus terrestris* L	β-carboline alkaloids	AR15834 and GMI9534	59

table 2. (continued).

No.	Name of plant	Name of secondary metabolites	Strain of *Agrobacterium rhizogene*	References
28	*Arnica Montana* L	Hairy Root Production	LBA 9402.	50
29	*Solanum xanthocarpum*	Solasodine, Carpesterol	MTCC 532	31
30	*Solidago nemoralis*	Hairy Root Production	R1000	23
31	*Phyllanthus amarus*	Genomic and metabolic fingerprinting	ATCC 15834	1
32	*Salvia miltiorrhiza*	Tanshinone	C58C1	45
33	*Decalepis arrayal pathra*	2-hydroxy-4-methoxy benzaldehyde (MBALD)	A4,MTCC 532,TR105,and LBA 5402	66
34	*Salvia miltiorrhiza*	Tanshinone	BCRC15010	20
35	*Bacopa monnieri,*	Bacopa saponins	LBA 9402	42
36	*Scutellaria viscidula*	Flavonoids (Baicalein,wogonoside, wogonin)	Wild culture	39
37	*Plumbago indica*	Plumbagin	ATCC 15834	19
38	*Camptotheca acuminata*	Camptothecin	C58C1	78
39	*Glycyrrhiza nuralensis*	Licochalcone A	ATCC 10060	83
40	*Salvia involucrata*	Apigenin	Hairy root expressing (CaMV) 35SCHI gene	51
41	*Scrophularia buergeriana*	E-p-methoxy cinnamic acid	R1000	53
42	*Linumtauricum*	Ariltetraline lignans	ATCC 15834	28
43	*Hypericum species*	Hairy roots	ATCC 15834and A4	32
44	*Linum tauricum ssp.*	Lignin	TR 105 andATCC 15834	27
45	*Maytenus senegalensis*	Hairy roots	LBA9402 and A4T	29
46	*Arachis Hypogaea* L	Resveratrol	R1601	34
47	*Salvia sclarea*	Salvipisone	LBA 9402	35
48	*Centella asiatica* (L)	Asiaticoside	R1000harboring pCAMBIA1302	36
49	*Vitis amurensis*	Resveratrol	*rolB* gene of*Agro rhizogenes*	73

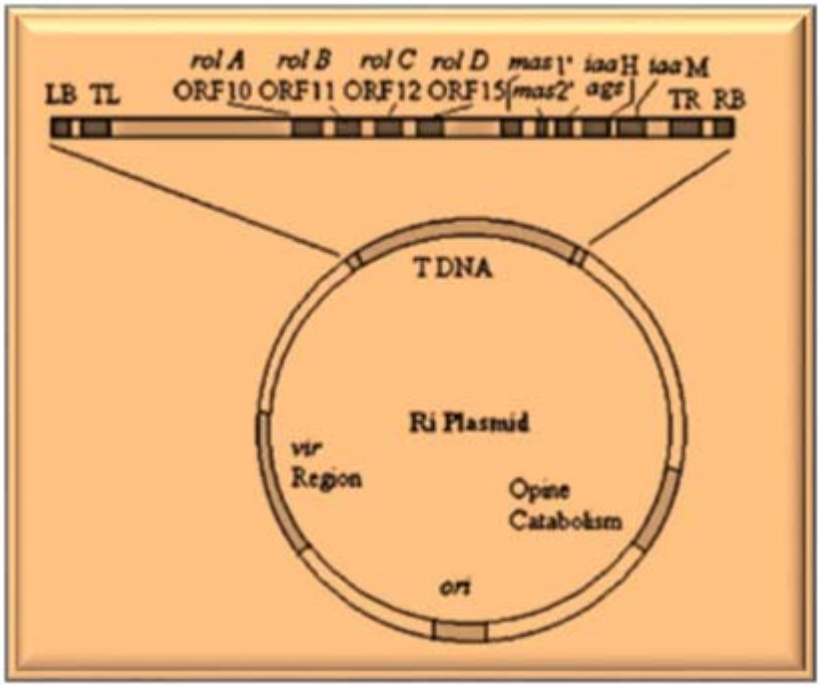

Fig. 1. Schematic representation of Ri plasmid of *A.rhizogenes* (Courtesy [12])

rhizogenes, a piece of DNA is transferred from the bacterium to the plant cell. This piece of DNA is a copy of a segment called T-DNA[10.] T-DNA is part of the approximately 200 kb Ti/Ri plasmid present in *Agrobacterium* which encodes functions for Ti/Ri plasmid conjugation, opine synthesis, and catabolism and the initiation, transfer, and integration of the T-DNA. T-regions on native Ti and Ri plasmids are approximately 10-30 kbp in size. T- regions are defined by T-DNA border sequences. These borders are 25 bp in length and highly homologous in sequence. They flank the T-region in a directly repeated orientation [17].

Ri plasmids can be classified according to the opines produced. In nopaline Ti plasmid, mannopine, and cucumopine Ri plasmid types, a single T-DNA has been found, whereas, in octopine (pTi) and agropine (pRi) types (Fig. 1), two regions (TL-DNA and TR-DNA) have been identified. Two T-DNAs are separated from each other by about 15 kb of non- transferred DNA. In the central, less conserved region of the TL-DNA of agropine T-DNA, the root-inducing (*rol)* genes are located. TR-DNA contains two genes, iaaM, and iaaH, responsible for the biosynthesis of auxins [11] and the genes responsible for the synthesis of the opines mannopine (mas10 and mas20) and agropine (ags) [12].

Both TL-DNA and TR-DNA are transferred and integrated independently into the host plant genome, but the transfer of TL-DNA is essential for induction of the hairy root syndrome, and transfer of TR-DNA does not provoke the formation of roots from transformed cultures. Detailed information regarding the mechanism involved in the genetic transfer can be referred to in literature [7,16,76].

Effect of *rolA, rolB* and *rolC* genes in secondary metabolism

Agrobacterium rhizogenes rolA, rolB and *rolC* oncogenes have always been considered to be modulators of plant growth and cell differentiation. These *rol* genes are potential activators of secondary metabolism in transformed cells from the Solanaceae, Araliaceae, Rubiaceae, Vitaceae, and Rosaceae families [6]. Studies were made on the activity of *rol* genes individually and their combined action on secondary metabolism [60].

In transformed plant cell cultures, the *rolC* gene alone can stimulate the production of tropane alkaloids, pyridine alkaloids, indole alkaloids, ginsenosides. The *rolB* and *rolC* gene activate the production of anthraquinones and stilbenes. The

stimulatory effect of the *rolA* gene on nicotine production was also observed [54]. However, *rolA* and *rolB* failed to stimulate ginsenoside production in transformed ginseng calli, and, similarly, the production of caffeic acid metabolites was reduced in *rolC* transformed callus cultures of *Eritrichium sericeum* and *Lithospermum erythrorhizon* [5].

Studies were also made to know the effect of *rol* genes in secondary metabolites production [7]. In this study, two series of *Atropa belladonna* hairy root lines were obtained: the first transformed via *A. tumefaciens* harboring *rolC* and npt II genes, and the other transformed with *rolABC* and npt II genes. Hyoscyamine and Scopolamine production was measured after 3 and 4 weeks of culture to evaluate the possible role of *rolC* gene in tropane alkaloid formation. The *rolC* gene alone played a significant role (17-fold increase) in the hairy root growth rate. However the *rolABC* genes together led to a much higher (75-fold increase) increase in hairy root growth rate. In contrast, the *rolC* gene alone was as efficient as the *rolABC* genes together (mean value of total alkaloids: 0.36 % dry weight, i.e., 12-fold more than in untransformed roots) to stimulate the biosynthesis of tropane alkaloids in *A. belladonna* hairy root cultures. A correlation exists between the expression of the *rolC* gene and tropane alkaloids, *Catharanthus roseus* alkaloids, and ginsenoside production [7]. Secondary metabolites can be classified based on chemical structure, composition, solubility in various solvents, or the pathway by which they are synthesized. Based on their biosynthetic origins, plant secondary metabolites can be divided into three major groups:

Flavonoids and allied phenolic and polyphenolic compounds

Phenolic compounds confer unique taste, flavor, and health-promoting properties found in vegetables and fruits [69]. Like as phenolic acids, flavonoids are secondary metabolites of plants with polyphenolic structure. They are synthesized by the polypropanoid pathway and the start-up component is phenylalanine molecule. The biological effects of these compounds vary. All flavonoids share the basic C6-C3-C6 structural skeleton, consisting of two aromatic C6 rings (A and B) and a heterocyclic ring (C) that contains one oxygen atom. Flavonoids are well known for their antioxidant activity. An imbalance between antioxidants and free radicals results in oxidative stress, which will/may lead to cellular damage [37].

Terpenoids

These are a large and diverse class of naturally occurring organic chemicals similar to terpenes, derived from five-carbon isoprene units assembled and modified in thousands of ways. Most are multicyclic structures that differ from one another not only in functional groups but also in their basic carbon skeletons. These lipids can be found in all classes of living things, and are the largest group of natural products. About 60 % of known natural products are terpenoids. Terpenoids, such as limonene, myrcene, α-pinene, linalool, β-caryophyllene, caryophyllene oxide, nerolidol, and phytol are flavor and fragrance components common in human diets that have been designated as generally recognized as safe (GRAS) by the US Food and Drug Administration and other regulatory agencies. They display unique therapeutic effects that may contribute meaningfully to the entourage effects of cannabis-based medicinal extracts. Phytocannabinoid-terpenoid interactions could produce synergy concerning the treatment of pain, inflammation, depression, anxiety, addiction, epilepsy, cancer, and fungal and bacterial infections [57].

Nitrogen-containing alkaloids and sulfur-containing compounds

Alkaloids are among the largest groups of secondary metabolites, being extremely diverse in terms of structure and biosynthetic pathways, including more than 20,000 different molecules distributed throughout approximately 20 % of known vascular plants.

Alkaloids are important chemical compounds that serve as a rich reservoir for drug discovery. Several alkaloids like camptothecin [89] and vinblastine isolated from natural herbs exhibit antiproliferation and antimetastasis effects on various types of cancers both *in vitro* and *in vivo*.

Hairy root culture

Hairy root culture or transgenic hairy root cul-

tures have revolutionized the role of plant tissue culture in secondary metabolite production. They are unique in their genetic and biosynthetic stability, faster in growth, and more easily maintained. *Agrobacterium rhizogenes*-mediated transformation has several features desirable for the production of secondary metabolites. The rapid and efficient induction of hairy roots from explant tissues in a wide variety of plant species, including medicinal plants, has been reported [85]. These hairy roots are characterized by a high growth rate and high root branching without added phytohormones. Furthermore, they often produce secondary metabolites for a long period, unlike intact roots. For these reasons, switching from undifferentiated cell culture to hairy root culture is considered an attractive alternative for the production of many valuable secondary metabolites that originally accumulated in root tissues.

Using this methodology a wide range of chemical compounds including some important flavonoids has been synthesized. Hairy root cultures of many plant species have been widely studied for the production of secondary metabolites which are useful as pharmaceuticals, cosmetics, and food additives [18]. Hairy root cultures represent an interesting alternative to dedifferentiated cell cultures for the production of secondary plant products. Because hairy roots originate from a single plant cell infection by *Agrobacterium rhizogenes*, they are usually considered as genetically stable, in contrast with callus lines. Also, in contrast to dedifferentiated cells, the production of secondary metabolites is not repressed during the growth phase of the culture. Therefore, hairy roots usually produce secondary plant compounds without the loss of concentration frequently observed with a callus or cell suspension cultures. Unlike most secondary plant products in cell suspension cultures, it is possible to get a continuous source of secondary metabolites from growing hairy roots [9].

Significance of hairy root culture

• Harvesting roots for extracting secondary metabolites can destroy whole plants. Therefore, interest in producing secondary metabolites by developing hairy root culture has been raised.

• Hairy root culture potentially grows faster without needing an external supply of auxins. In certain cases, they do not need incubation under the light.

• Due to their high genetic stability all hairy root cultures are stable in metabolite production.

• Yield in hairy root cultures can be altered by optimizing various factors such as carbon source and its concentration, ionic concentration of the medium, pH of the medium, light, temperature, and inoculums.

• Also utilization of techniques like precursor feeding, cell immobilization, elicitation, and biotransformation of hairy root culture can improve secondary metabolite production.

The present review is highlighting medicinal plants transformed successfully through *A. rhizogenes* to get a high level of secondary metabolites during the last 12 years.

Conclusion and future prospects

Metabolic engineering and biotechnological approaches are in use as an alternative production system to overcome the limited availability of biologically active, commercially valuable, and medicinally important plant secondary metabolite compounds. To date, rapid success has been obtained in exploring the molecular mechanisms of T-DNA transfer, interaction with host plant proteins, their role in plant defense signaling, and integration to plant genome for stable gene transfer for successful plant genetic transformation. T-DNA and corresponding expression of *rol* genes alter morphology and plant host secondary metabolism. Plant transformation technology has now reached a platform of commercial reality. T-DNA and *rol* genes affect plant secondary metabolism. In the recent past, hairy root technology has been significantly improved in different fields: the engineering of secondary metabolism, the increased accumulation and excretion of metabolites after elicitation, the production of therapeutically recombinant proteins, the trapping of biomolecules released in the medium and the scaling-up of the culture process. Hairy roots are easy to grow and to transform. The genetic and biochemical stability of these differentiated cultures and their efficient productivity offer substantial

advantages over cell suspensions. The immense potential of the hairy root system for the production of metabolites and phytoremediation has begun to attract private companies. Soon, hairy roots will provide biotechnologists with powerful tools to reach the precious underground resources of the plant kingdom.

The technique is much useful in the conservation of the plants, especially for those, having root as an important organ bearing metabolites of medicinal value. Root harvesting a major cause of plant uprooting makes the plant rare. The important medicinal substances can be produced using the technique of rhizogenesis in the bio fermenter. So that the plant products can be synthesized commercially and will be easily available for human society. The data and techniques presented in the review must be useful for the optimization of techniques for rhizogenesis and enhancement of secondary metabolite in other important plants.

References

1. **Abhyankar, G., Khareedu, V. and Vudem, D.R. (2013).** Genomic and metabolomic fingerprinting of *Phyllanthus amarus* (SchummandThonn) hairy root clones. Annals of Phytomedicine. 2(1): 74-88.
2. **Bais, P.H., Vepachedu, R. and Vivanco, J.M. (2003).**Root specific elicitation and exudation of fluorescent β-carbolines in transformed root cultures of Oxalis tuberose. Plant Physiology and Biochemistry. 41(4): 345-353.
3. **Brijwal,L. and Tamta, S. (2015).** *Agrobacterium rhizogenes* mediated hairy root induction in endangered *Berberisaristata*DC. Springer Plus. 4(1): 1-10.
4. **Belabbassi, O.**, **Slaoui, M.K., Zaoui, D., Benyammi, R., Khalfallah, N., Malik, S., Makhzoum, A. and Khelifi., L. (2016).** Synergistic effects of polyploidization and elicitation on biomass and hyoscyamine content in hairy roots of *Datura stramonium*. BASE [Online] 20(3): 408-416 .
5. **Bulgakov, V.P., Veselova, M.V., Tchernoded, G.K., Kiselev, K.V., Fedoreyev, S,A., Zhuravlev, Y.N. (2005).** Inhibitory effect of the *Agrobacterium rhizogenes rolC* gene on rabdosiin and rosmarinic acid production in *Eritrichium sericeum* and *Lithospermum erythrorhizon* transformed cell cultures. Planta. 221(4): 471-478.
6. **Bulgakov, V.P. (2008).** Functions of *rol* genes in plant secondary metabolism. Biotechnol. Adv. 26(4): 318-324.
7. **Bonhomme, V.,Laurain, M.D., Fliniaux, M.A. (2000)** Effects of the *rol*C gene on hairy root: induction development and tropane alkaloid production by *Atropa belladonna*. J. Nat. Prod. 63(9): 1249-1252.
8. **Daspute, A.A., Yunxuan, X., Gu, M., Kobayashi, Y., Wagh, S., Panche, A. and Koyama, H. (2019).** *Agrobacterium rhizogenes*-mediated hairy roots transformation as a tool for exploring aluminum-responsive genes function. Future Science OA, 5(3): FSO364.
9. **Bourgaud, F., Gravot, A., Milesi, S., Gontier, E. (2001).** Production of plant secondary metabolites: a historical perspective. Plant Science. 161: 839-851.
10. **Chilton, M.D., Tepfer, D., Petit, A., David, C., Delbart, F.C. and Tempé, J. (1982).** *Agrobacterium rhizogenes* insert T-DNA into the genome of the host plant root cells. Nature. 295: 432-4.
11. **Cardarelli, M. Spanò, L., De Paolis, A., Mauro, M.L., Vitali, G., Costantino, P. (1985).** Identification of the genetic locus responsible for non-polar root induction by *Agrobacterium rhizogenes* 1855. Plant Mol. Biol. 5(6): 385-391.
12. **Chandra, S. (2012).** Natural plant genetic engineer *Agrobacterium rhizogenes*: role of T-DNA in plant secondary metabolism. Biotechnology Letters. 34(3): 407-415.
13. **Chen, Li., Cai, Y., Liu, X., Guo, C., Sun, S., Wu, C., Jiang, B., Han, T. and Hou, W. (2018).** Soybean hairy roots produced *in vitro* by *Agrobacterium rhizogenes* mediated transformation. The crop Journal. 6(2): 162-171.
14. **Drobot, K. (2016)** Tarragon *(Artemisia dracunculus* L.) "Hairy Root"culture production. Bio-

technologia Acta. 9(2): 55-60.
15. **Ebrahimi, K.S., Zaker, A., Abrishamchi, P., Bahrami, A.R., Ali, M., Ganjeali, and Sodagar, N. (2017).** Hairy root induction and secondary metabolite production in *Perovskia abrotanoides* Karel. J of Plant Process and Function. 6(20): 17-26.
16. **Gelvin, S.B. (2009).** *Agrobacterium* in the Genomics age.Plant Physiol. 150(4): 1665-1676.
17. **Gelvin, S.B. (2003).** Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol Mol. Biol. Rev. 67(1): 16-37.
18. **Georgiev, M.I., Pavlov, A.I., Bley, T. (2007)**. Hairy root type plant *in vitro* systems as sources of bioactive substances. Applied Microbiology and Biotechnology. 74(6): 1175.
19. **Gangopadhyay, M., Dewanjee, S., Chakraborty, D. and Bhattacharya, S. (2011).** Role of exogenous phytohormones on growth and plumbagin accumulation in *Plumbago indica* hairy roots and conservation of elite root clones via synthetic seeds. Ind. Crop Prod. 33(2): 445-450.
20. **Gupta, S.K., Liu, R., Liaw, S., Chan, H.S. and Tsay, H.S. (2011).** Enhanced tanshinone production in hairy roots of *Salvia miltiorrhiza* Bunge under the influence of plant growth regulators in liquid culture. Botanical Studies. 52: 435-443.
21. **Gai**, **Q.Y.**, **Jiao, J., Luo, M., Wei, Z.F., Zu, Y.G., Ma, W., Fu, Y.J. (2015).** Establishment of Hairy Root Cultures by *Agrobacterium rhizogenes* mediated transformation of *Isatis tinctoria* L. for the efficient production of flavonoids and evaluation of antioxidant activities. PLoSOne. 10(3): e0119022.
22. **Ge, X. and Wu, J. (2005)**. Tanshinone production and isoprenoid pathways in *Salvia miltiorrhiza* hairy roots induced by Ag^{+} and yeast elicitor. Plant Science. 168(2): 487-491.
23. **Gunjan, K.S., Lutz, J. and Bushong, A., Rogers, T. and Littleton, J. (2013).** Hairy Root Cultures and Plant Regeneration in *Solidago nemoralis* Transformed with *Agrobacterium rhizogenes*. American J. of Plant Sciences. 4(8): 1675-1678.
24. **Huang, S.H., Lee, T.T., Chan, H.S., Tsay, H.S. (2014).** Establishment of hairy root lines and analysis of iridoids and secoiridoids in the medicinal plant *Gentiana scabra*. Botanical Studies. 55: 17.
25. **Hidalgo, D., Márquez, A.M., Moyano, E., Martínez, R.B., Corchete, P. and Palazon, J. (2017).** Bioconversion of stilbenes in genetically engineered root and cell cultures of tobacco. Sci. Rep. 7: 45331.
26. **Hussain, M.S**., **Fareed, S., Ansari, S.**, **Rahman, M.A., Ahmad, I.Z., Saeed, M. (2012).** Current approaches toward production of secondary plant metabolites. J. of Pharmacy and Bioallied Sciences. 4(1): 10-20.
27. **Ionkova, I. and Fuss**, **E. (2009).** Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and lignan production in *Linum tauricum ssp.* Tauricum. Phcog. Res. 4(17): 14-18.
28. **Ionkova, I. (2009).** Effect of methyl jasmonate on production of ariltetralin lignans in hairy root cultures of *Linum tauricum*.Pharmacognosy Research. 1(3): 102-105.
29. **Jain,N., Light, M.E. and Staden, J.V. (2008).** Antibacterial activity of hairy-root Cultures of *Maytenus senegalensis.* South African Journal of Botany. 74(1): 163-166.
30. **Karuppusamy, S. (2010).** A review on trends in production of secondary metabolites from higher plants by *in vitro* tissue, organ and cell cultures. J. of Medicinal Plants Research. 3(13): 1222-1239.
31. **Khatodia, S., Biswas, K. and Bhatotia, K. (2013).** Induction and Establishment of Hairy Root Culture of *Solanum xanthocarpum* using *Agrobacterium rhizogenes,* Journal of Pharmaceutical and BioSciences. 1: 59-63.
32. **Komarovská, H., Giovannini, A., Kosutha, J. and Cellárovia, E. (2009).** *Agrobacterium rhizogenes*-Mediated Transformation of *Hypericum tomentosum* L. and *Hypericum tetrapterum*

Fries. Z. Naturforsch C. 64(11-12): 864-868.
33. **Komaraiah, P., Reddy, G., Reddy, P.S., Raghavendra, A.S., Ramakrishna, S.V. and Reddanna, P. (2003).** Enhanced production of antimicrobial sesquiterpenes and lipoxygenase metabolites in elicitor-treated hairy root cultures of *Solanum tuberosum*. Biotechnology Letters. 25(8): 593-597.
34. **Kim, J.S., Lee, S.Y. and Park, S.U. (2008).** Resveratrol production in hairy root culture of peanut, *Arachishypogaea* L. transformed with different *Agrobacterium rhizogenes* strain. Afr. J. of Biotechnology: 7(20): 3788-3790.
35. **Kuzma, L., Bruchajzer, E., and Wysokiñska, H. (2008).** Diterpenoid Production in Hairy Root Culture of *Salvia sclarea* L..Zeitschriftfür Natur for schung. C. J. of Biosciences. 63(7-8): 621-624.
36. **Kim, O.T., Bang, K.H., Shin, Y.S., Lee, M.J., Jung, S.J., Hyun, D.Y., Kim, Y.C., Seong, N.S., Cha, S.W., and Hwang, B. (2007).** Enhanced production of asiaticoside from hairy root cultures of *Centella asiatica* (L.) Urban elicited by methyl jasmonate. Plant Cell Rep. 26(11): 1941-49.
37. **Kukic, J., Petrovic, S. and Niketic, M. (2006).** Antioxidant activity of four endemic Stachys taxa. Biol. Pharm. Bull. 29(4): 725-9.
38. **Shinde, A.N., Malpathak, N., Fulzele, D.P. (2009).** Enhanced production of phytoestrogenic isoflavones from hairy root cultures of *Psoralea corylifolia* L. Using elicitation and precursor feeding. Biotechnology and Bioprocess Engineering. 14(3): 288-294.
39. **Lei, W., Shui, X., Zhou, Y., Tang, S. and Sun, M. (2011)**. Effect of praseodymium on flavonoids production and its biochemical mechanism of *Scutellaria viscidula* hairy root *in vitro*. Pak. J. Bot. 43(5): 2387-2390.
40. **Lin, H.W., Kwok, K.H. and Doran, P.M. (2003).** Development of *Linum flavum* hairy root cultures for production of coniferin. Biotechnol. Lett. 25(7): 521-5.
41. **Mahobia, A., Jha, Z. (2018).** Root Cultures: *In vitro* Conservative Method for Metabolite Extraction from *A. paniculata*. Int. J. Curr. Microbiol. App. Sci. 7(03): 2442-2450.
42. **Majumdar, S., Garai, S. and Jha, S.(2011).** Genetic transformation of *Bacopa monnieri* by wild type strains of *Agrobacterium rhizogenes* stimulates production of bacopa saponins in transformed calli and plants. Plant Cell Rep. 30(5): 941-954.
43. **Mawla, A.E. (2010).** Effect of certain elicitors on production of pyrrolizidine alkaloids in hairy root cultures of *Echium rauwolfii*. Pharmazie. 65(3): 224-226.
44. **Md. Setamam, Sidik, N.J., Rahman, Z.A., and Mohd Zain, C.R.C. (2014).** Induction of hairy roots by various strains of *Agrobacterium rhizogenes* in different types of Capsicum species explants. BMC Research Notes. 7: 414.
45. **Ming, Q. (2013).** Elicitors from the endophytic fungus *Trichoderma atroviride* promote *Salvia miltiorrhiza* hairy root growth and tanshinone biosynthesis. Journal of Experimental Botany. 64(18): 5687-5694
46. **El-Esawi, MA., Elkelish, A., Elansary, H.O., Ali, H.M., Elshikh, M., Witczak, J. and Ahmad, M. (2017).** Genetic Transformation and Hairy Root Induction Enhance the Antioxidant Potential of *Lactuca serriola* L. Oxidative Medicine and Cellular Longevity. Article ID 5604746, 8 pages.
47. **Moharrami, F., Hosseini, B., Sharafi, A. and Farjaminezhad, M. (2017).** Enhanced production of hyoscyamine and scopolamine from genetically transformed root culture of *Hyoscyamus reticulatus* L. elicited by iron oxide nanoparticles. *In Vitro* Cell. Dev. Biol.-Plant: 53(2): 104-111.
48. **Mthembu, Z. (2017).** Development of a hairy root bioreactor from *Stevia rebaudiana* to produce steviol glycosides. Thesis (MScAgric) Stellenbosch University**.** scholar.sun.ac.za.
49. **Nourozi, E., Hosseini, B. and Hassani, A. (2016).** Influences of various factors on hairy root induction in *Agastache foeniculum* (Pursh) Kuntze. Acta Agriculturae Slovenica. 107(1): 45-54.
50. **Petrova, M., Zayova1, E. and Vlahova, M. (2013).** Induction of hairy roots in *Arnica montana*

L. by *Agrobacterium rhizogenes*. De Gruyter. 8(5): 45-54.

51. **Pistelli, L. Giovannini, A., Ruffoni, B., Bertoli, A. and Pistelli, L. (2010).** Hairy Root Cultures for Secondary Metabolites Production. Bio Farms for Nutraceuticals: Advances in Experimental Medicine and Biology. 698: 167-84.
52. **Park, N.I., Xu, H., Li, X., Kim, S.J., Park, S.U.(2011).** Enhancement of flavone levels through over expression *of Chalcone isomerase* in hairy root cultures of *Scutellaria baicalensis.* Funct. Integr. Genomics. 11(3): 491-496.
53. **Park, S.U., Li, X., Hyun, E.S., Yeol, L.C., Young, L.S. (2010).** E-p-methoxycinnamic acid production in hairy root cultures of scrophularia buergeriana miquel. Arch. Biol. Sci., Belgrade. 62(3): 649-652.
54. **Palazon, J., Cusido, R.M., Roig, C. and Pinol, M.T. (1998)** Expression of the *rol*C gene and nicotine production in transgenic roots and their regenerated plants. Plant Cell Rep 17: 384-390.
55. **Palazón, J., Cusidó, R.M., Bonfill, M., Mallol, A., Moyano, E., Morales, C., TeresaPiñol, M. (2003).** Elicitation of different *Panax ginseng* transformed root phenotypes for an improved ginsenoside production. Plant Physiology and Biochemistry. 41(11): 1019-1025**.**
56. **Pandey, R., Krishnasamy, V., Kumaravadivel, N. and Rajaman, K. (2014).** Establishment of hairy root culture and production of Secondary metabolites in Coleus (*Coleus forskohlii).* J. of Medicinal Plants Research. 8(1): 58-62.
57. **Russo, E.B. (2011).** Taming THC: potential cannabis synergy and phytocannabinoid- terpenoid entourage effects. Br. J. Pharmacol. 163(7): 1344-1364.
58. **Shakeran, Z., Keyhanfar, M., Asghari, G., Ghanadian, M. (2015).** Improvement of atropine production by different biotic and abiotic elicitors in hairy root cultures of *Datura metel*. Turkish J. of Biology. 39: 111-118.
59. **Sharifi**, **S., Sattari**, **T.N., Zebarjadi**, **A., Ahmad, M. and Hamidreza, G. (2014).** The influence of *Agrobacterium rhizogenes* on induction of hairy roots and β-carboline alkaloids production in *Tribulus terrestris* L. Physiol. Mol. Biol. Plants. 20(1): 69-80.
60. **Shkryl, Y.N., Veremeichik, G.N., Bulgakov, V.P., Tchernoded, G.K., Mischenko, N.P., Fedoreyev, S.A., and Zhuravlev, Y.N. (2008).** Individual and combined effects of the *rolA, B*, and *C* genes on anthraquinone production in *Rubia cordifolia* transformed calli. Biotechnol. Bioeng. 100(1): 118-125.
61. **Sharma, M., Sharma, A., Kumar, A. and Kumar, S.B. (2011).** Enhancement of secondary metabolites in cultured plant cells through stress stimulus. American Journal of Plant Physiology. 6(2): 50-71.
62. **Sharafi, A., Hashemi Sohi, H., Mousavi, A., Azadi, P., Dehsara, B. and Hosseini, K.B. (2013).** Enhanced morphinan alkaloid production in hairy root cultures of *Papaver bracteatum* by overexpression of salutaridinol 7-o-acetyltransferase gene via *Agrobacterium rhizogenes* mediated transformation. World J. of Microbiology and Biotechnology. 29(11): 2125-31
63. **Spollansky, T.C., Pitta-Alvarez, S.I., Giulietti, A.M.,(2000).** Effect of jasmonic acid and aluminium on production of tropane alkaloids in hairy root cultures of *Brugmansia candida*. Electronic J. of Biotechnology. 3(1): 72-75.
64. **Srivastava, M., Sharma, S. and Misra, P. (2016).** Elicitation Based Enhancement of Secondary Metabolites in *Rauwolfi aserpentina* and *Solanum khasianum* Hairy Root Cultures. Pharmacognosy Magazine. 12(3): S315-20.
65. **Staniszewska, I., Królicka, A., Maliñski, E., Lojkowska, E., and Szafranek, J. (2003).** Elicitation of secondary metabolites in *in vitro* cultures of *Ammi majus* L. Enzyme and Microbial. Technology. 33(5): 565-568.
66. **Charantharayil, S., Sherina, T.V., Anu Anand, V.P., Varghese, R., Pillai, P. and Vasudevan, S. (2013).** *Agrobacterium rhizogenes* mediated transformation of the medicinal plant *Decalepis*

arayalpathra and production of 2-hydroxy-4-methoxy benzaldehyde.Plant Cell Tiss. Organ Cult. 112(2): 217-226.

67. **Su, W.W. and Lee, K.T. (2007).** Plant Cell and Hairy Root Cultures-Process Characteristics, Products, and Applications. Bioprocessing for Value-Added Products from Renewable Resources. 10: 263-292.
68. **Thilip, C., Soundar, R.C., Varutharaju, K., Aslam, A. and Shajahan, A. (2015).** Improved *Agrobacterium rhizogenes*-mediated hairy root culture system of *Withania somnifera* (L.) Dunal using sonication and heat treatment. Biotech. 5(6): 949-956.
69. **Tomás-Barberán, F.A. and Espín, J.C. (2001).** Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. J. of the Science of Food and Agriculture. 81(9): 853-876.
70. **Torkamani, M., Abbaspour, N., Jafari, M. and Samadi, A., (2014).** Enhanced production of valerenic acid in hairy root culture of *Valeriana officinalis* by elicitation. Open Life Sciences. 9(9): 853-863
71. **Tuan, P., Kwon, D.Y., Lee, S., Arasu, M.V., Al-Dhabi, N.A., Park, N. and Park, S.U. (2014).** Enhancement of Chlorogenic Acid Production in Hairy Roots of *Platycodon grandiflorum* by Over-Expression of An *Arabidopsis thaliana* Transcription Factor AtPAP1. International Journal of Molecular Sciences. 15(8): 14743-14752.
72. **Tzfira, T., Vaidya, M. and Citovsky, V. (2004).** Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*. Nature. 431(7004): 87-92.
73. **Kiselev, K.V., Dubrovina, A.S., Veselova, M.V., Bulgakov, V.P., Fedoreyev, S.A. and Zhuravlev, Y.N. (2007).** The rolB gene-induced overproduction of resveratrol in *Vitis amurensis* transformed cells. J. Biotechnol. 128(3): 681-92.
74. **Verma, P. C., Singh, H., Negi, A.S., Saxena, G., Rahman, L.U. and Banerjee, S. (2015).** Yield enhancement strategies for the production of picroliv from hairy root culture of *Picrorhiza kurroa* Royle ex Benth. Plant Signalling and Behaviour. 10(5): e1023976.
75. **Wang, H.,Gao, S., Teixeira da Silva, J.A., Ma, G. (2015).** *Agrobacterium rhizogenes*- mediated genetic transformation of *Psammosilenet unicoides* and identification of high saponin-yielding clones. Environmental and Experimental Biology. 13: 19-23.
76. **White, F.F., Taylor, B.H., Huffman, G.A., Gordon, M.P. and Nester, E.W. (1985).** Molecular and genetic analysis of the transferred DNA of the root inducing plasmid of *Agrobacterium rhizogenes*. J. of Bacteriology. 164(1): 33-44.
77. **Vishwakarma, K.S., Mohammed, S.I., Chaudhari, A.R., Salunkhe, N.S. and Maheshwari, V.L. (2017).** Micropropagation and *Agrobacterium rhizogenes* mediated transformation studies in *Mucuna pruriens* (L)DC. Indian J. of Natural Products and Resources. 8(2): 172-178.
78. **Ni, Xiaoling., Wen, S., Wang, X., Wang, W., Xu, H., Kai, Guoyin. (2011).** Enhancement of camptothecin production in *Camptotheca acuminata* hairy roots by overexpressing ORCA3 gene. J. of Applied Pharmaceutical Science. 01(08): 85-88.
79. **Yaoya, S., Kanho, H., Mikami, Y., Itani, T., Umehara, K., Kuroyanagi, M. (2004).** Umbelliferone Released from Hairy Root Cultures of *Pharbitis* nil Treated with Copper Sulfate and Its Subsequent Glucosylation. Bioscience Biotechnology and Biochemistry. 68(9): 1837-41.
80. **Young, J.M., Kuykendall, L.D., Martínez-Romero, E., Kerr, A., Sawada, H. (2001).** A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium* undicola de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes, R. rubi, R. undicola* and *R. vitis* .International J. of Systematic and Evolutionary Microbiology. 51(1): 89-103.
81. **Zabetakisa, I., Edwards,R., Hagan, D.O. (1999).** Elicitation of tropane alkaloid biosynthesis in transformed root cultures of *Datura stramonium*. Phytochemistry. 50(1): 53-56.

82. **Zhao, J. L., Zou, L., Zhang, C., Li, Y., Peng, L., Xiang, D., Zhao, G. (2014).** Efficient production of flavonoids in *Fagopyrum tataricum* hairy root cultures with yeast polysaccharide elicitation and medium renewal process. Pharmacognosy Magazine. 10(39): 234-40.
83. **Zhang, H.C., Liu, J.M., Chen, H.M., Gao, C.C., Lu, H.Y., Zhou, H., Li, Y., Gao, S.L. (2011).** Up-regulation of licochalcone A biosynthesis and secretion by tween 80 in hairy root cultures of *Glycyrrhiza uralensis* Fisch. Mol. Biotechnol. 47(1): 50-56.
84. **Jung, H.Y, Kang, S.M., Kang, Y.M., Kang, M.J., Yun, D.J., Bahk,J.D., Yang, J.K., Choi, M.S. (2003).** Enhanced production of scopolamine by bacterial elicitors in adventitious hairy root cultures of *Scopolia parviflora*. Enzyme and Microbial Technology. 33(7): 987-990.
85. **Zhang, M., Gao, W., Wang, X.J. (2014).** Medicinal plant hairy roots generating and their applications. China Journal of Chinese Materia Medica. 39(11): 1956-60.
86. **Zhang, H.C., Liu, J.M., Lu, H.Y., Gao, S.L. (2009).** Enhanced flavonoid production in hairy root cultures of *Glycyrrhiza uralensis* Fisch by combining the over-expression of chalcone isomerase gene with the elicitation treatment. Plant Cell Reports. 28(8): 1205-13.
87. **Zhu, C., Miao, G., Guo, J., Huo, Y., Zhang, X., Xie, J., Feng, J. (2014).** Establishment of *Tripterygium wilfordii Hook. f.* Hairy root culture and optimization of Its culture conditions for the production of triptolide and wilforine. J. Microbiol. Biotechnol. 24(6): 823-834.
88. **Siva, R., Sairam, V., Saleel, Y., Promit, B., Abhishek, A., Sarang, H., Abhishek, K., Alifiya, P., Anshul, G., Siddharth, D.(2015).** *Agrobacterium* mediated transformation in *Rubia cordifolia* for obtaining hairy root producing Anthraquinone dye. J. of Microbiology and Biotechnology. 4(2): 16-17.
89. **Ramesha, B.T., Zuehlke, S., Vijaya, R.C., Priti, V., Ravikanth, G., Ganeshaiah, K.N., Spiteller, M., Shaanker, R.U. (2011).** Sequestration of Camptothecin, an Anticancer Alkaloid, by Chrysomelid Beetles. J. of Chemical Ecology. 37(5): 533-6.