IN-VITRO MULTIPLICATION AND PRODUCTION OF HYPERCIN FROM *HYPERICUM PERFORATUM*

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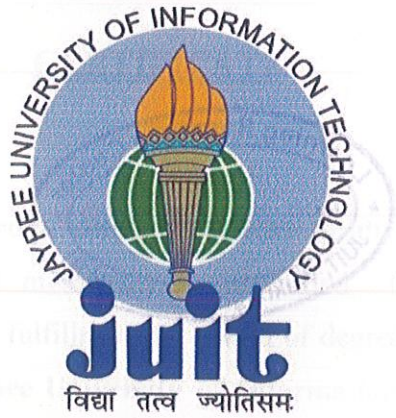

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WAKNAGHAT

SOLAN, HIMACHAL PRADESH

Date:

CERTIFICATE

This is to certify that the work entitled "***In-vitro* production of *Hypericum perforatum* and production of secondary metabolites"** submitted by **GAURAV BHATT and SALONEE DATTA** in partial fulfillment for award of degree of Bachelor of Technology (BIOTECHNOLOGY) **of Jaypee University of Information Technology** has been carried out in my supervision. This work has not been submitted partially or wholly to any other university or institution for award of this or any other degree programme.

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SUMMARY

St. John's wort (*Hypericum perforatum*) is a perennial herb distributed in temperate and subtropical regions of the world. It proved to be a useful medicinal herb for its anti-depressant (for mild to moderate depression), anti-inflammatory, anti-viral properties .The key chemical components in the plant are hypericin and Hyperforin .However, opinions differ as to active constituents attributable to anti-depressant properties. The present investigation aims at *in-vitro* establishment and long term maintenance of callus and regeneration of *Hypericum perforatum*, for eventual commercial exploitation of its phyto-pharmaceuticals.

Successful induction of callus has been achieved in M.S. media. The studies showed that fully defined MS media in combination with Kinetin, IBA and 2, 4-D was effective for both initiation and sustained growth of callus tissue. The process of subsequent sub-culturing of calli led to the complete regeneration of plant showing a rapid and vigorous growth. Plants with vigorous leaf and root growth were obtained after they were sub cultured into MS media with growth hormones, IBA(1mg/lt.) and kinetin(3mg/lt.). In vitro root induction obtained best on media having NAA (2mg/l) along with IBA (1mg/l). *In vitro* grown plantlets were transfer to glass house for hardening and final field transfer. *In vitro* grown shoots were cultured in same shooting medium having elicitors biotic and abiotic, which did not affect the growth but the amount of hypercin is accumulated in good amounts in shoot cultured in methyl jasmonate containing medium. So this study would be used as potential alternative for year round production of hypercin through cell culture technology.

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CHAPTER 1:
INTRODUCTION

1.1 *Hypericum perforatum:*

Hypericum perforatum L., commonly known as St. John's wort, is a traditional medicinal plant that has for centuries been utilized in folk medicine for a range of purposes . The plant has attracted attention because of its wound-healing, anti-inflammatory, diuretic and sedative properties and it has been used to treat neurological disorders and traumas.

Today, *H. perforatum* is one of the most prescribed and clinically investigated medicinal plant species: alcoholic extracts of the aerial parts of flowering plants are widely utilized for the treatment of mild and moderately severe depression . Several clinical studies have demonstrated that St. John's wort extracts are as efficient as conventional synthetic antidepressants but have fewer side-effects. The side-effects of *H. perforatum* preparations mainly concern photosensitivity when administrated at high doses and interaction with some other pharmaceuticals. The plant is also under intensive investigation for its anti-inflammatory, antiviral, antibacterial and antitumoral effects and has been suggested for the treatment of substance dependence .

H. perforatum belongs to the Clusiaceae family. It is currently known that the genus *Hypericum* comprises over 450 species of herbaceous perennials, evergreen and deciduous shrubs as well as trees . The herbaceous perennial *H. perforatum* is native to Europe, Asia and North Africa but has been introduced into many temperate regions of the world, including North and South America, South Africa, Australia and New Zealand . In these regions, the plant has often become an aggressive weed. The plant typically grows on sunny hillsides, dry meadows and fields, forest clearings, along roadsides and other similar habitats .The erect stem of St. John's wort reaches a height of up to 100 cm. The plant has opposite, sessile leaves and five-petalled bright yellow flowers that are arranged in terminal corymbs. The majority of the small, brown seeds are developed apomictically. *H. perforatum* is a highly variable species. At least three subspecies are recognized based on the morphology of the leaves and, although the plant is usually a tetraploid ($2n = 4x = 32$), differences in chromosome number have also been detected . Originally, *H. perforatum* may have evolved from *H. maculatum* (or their common ancestor) via auto polyploidization .

The presence of different types of secretory structures, including dark glands, translucent glands and various secretory canals, is characteristic to *H. perforatum*. The dark glands appear as black or dark-red multicellular nodules that are visible in leaf margins, stems, flower petals and stamens. They

consist of a core of large cells surrounded by a sheath of one to three flat cell layers . In some parts of the flowers, the dark glands also form elongated dark canals . The translucent glands cause leaves to look perforated. The structures are schizogenous cavities delimited by an inner layer of flattened secretory cells and an outer layer of large parenchymatous cells . The translucent glands are found in sepals and petals of flowers as well as in leaves, where they are scattered throughout the leaf lamina. Additionally, secretory canals of three different types have been described for *H. perforatum* tissues .

1.2 Secondary metabolites of *H. perforatum*:

NAPTHODIANTRONES:

H. perforatum contains a wide range of biologically active secondary metabolites that belong to different chemical groups. Naphthodianthrones (hypericins), phloroglucinols (hyperforins), xanthones, essential oils, phenolic acids and a broad array of flavonoids, including flavonols, bi-flavones and pro-cyanidins, have been identified in extracts of the plant .Particularly, naphthodianthrones, phloroglucinols and flavonoids are present in *H. Perforatum* tissues. Several pharmacological studies have confirmed that these compounds are the main contributors to the antidepressant activity of the species through various mechanisms of action . Additionally, Indian St. John's wort plants have been found to contain unusually high quantities of xanthones exhibiting antidepressant properties .

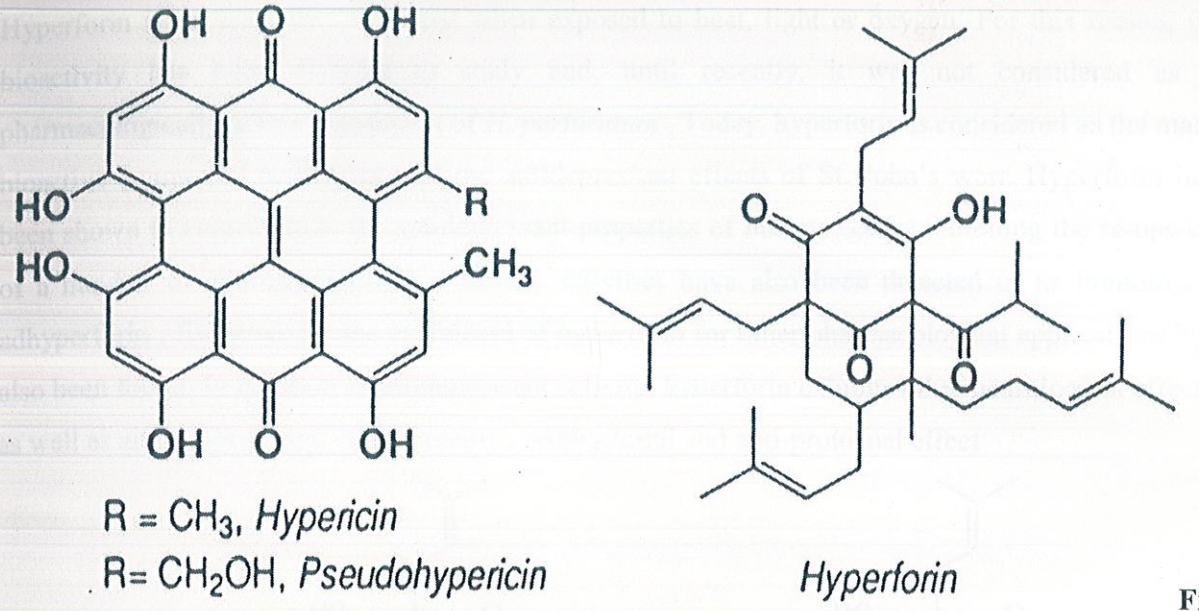


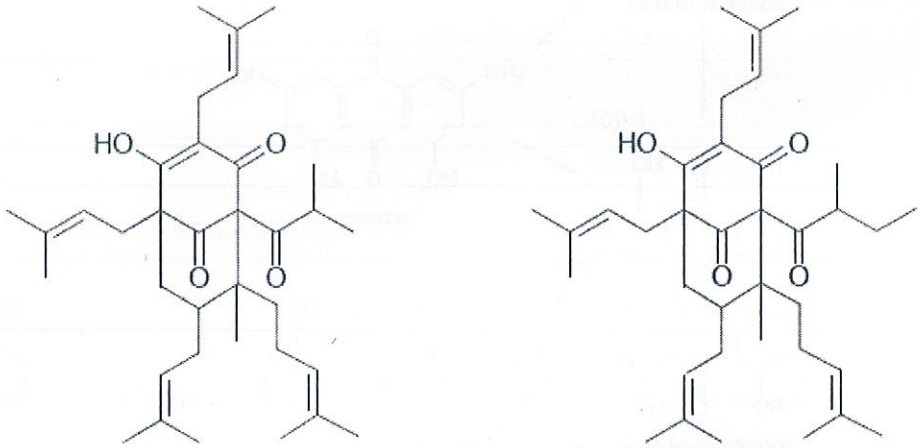
Fig. 1. **The main naphthodianthrones of *H. Perforatum*:**

1.2.2 Phloroglucinols:

Different types of phloroglucinol derivatives are widely distributed in the genus *Hypericum*. The major phloroglucinols found in *H. perforatum* are polyprenylated acylphloroglucinol hyperforin and its homologue adhyperforin (Fig. 2). Hyperforin generally exists in a plant in two- to ten-fold higher amounts than adhyperforin . Their biosynthetic intermediates hyperfirin and adhyperfirin as well as their homologues secohyperforin and secoadhyperforin can also be detected as minor constituents in *H. perforatum* . Additionally, trace amounts of various oxygenated analogues of hyperforin have been found in extracts of the species . At present, it is not known whether the analogues are natural compounds or degradation products of hyperforin that are generated during the extraction procedure .

Hyperforins occur most abundantly in *H. perforatum* flowers, especially in pistils and subsequently forming fruits, but they are also highly present in leaves . A hyperforin content of 6.9 percent in flower buds, 8.5 percent in unripe fruits and 1.5 percent in leaves has been reported . Hyperforins have been shown to accumulate in the translucent glands of *H. perforatum* . It has also been suggested that hyperforins would be biosynthesized in the parenchymatous secretory cells delimiting the cavity of translucent glands .The benefit of hyperforins to the plant still remains unclear, but they may serve as a defensive compound against herbivores and other pests .

Hyperforin is an unstable compound when exposed to heat, light or oxygen. For this reason, its bioactivity has been difficult to study and, until recently, it was not considered as a pharmacologically active constituent of *H. perforatum* . Today, hyperforin is considered as the main bioactive compound responsible for the antidepressant effects of St. John's wort. Hyperforin has been shown to contribute to the antidepressant properties of the species by inhibiting the re-uptake of a number of neurotransmitters . Similar activities have also been detected in its homologue adhyperforin . Evidence for the usefulness of hyperforin for other pharmacological applications has also been found. In addition to antidepressant activity, hyperforin exhibits other neurological effects as well as antitumoral , anti-inflammatory , antibacterial and anti-protozoal effect



hyperforin adhyperforin

Fig. 2. The main phloroglucinols of *H. perforatum*.

1.3 Biosynthetic pathways of hypericins and hyperforins:

The biosynthetic routes leading to Hypericin and Hyperforin are still poorly understood, but both of the compound groups are presumed to be biosynthesized via a polyketide pathway in which type III polyketide synthases (PKSs) act as key enzymes .

The biosynthetic pathway leading to hypericins most likely starts with the condensation of one molecule of acetyl coenzyme A (CoA) with seven molecules of malonyl- CoA to form a linear octaketide chain that subsequently undergoes specific cyclization to form emodin anthrone. The reaction is carried out by a type III PKS having octaketide synthase hyperforin adhyperforin (OKS) activity. Emodin anthrone is further oxidized to emodin probably by the activity of emodinanthrone oxygenase . Condensation of emodin anthrone and emodin followed by dehydration yields emodin dianthrone, which subsequently undergoes phenolic oxidation that leads to the formation of protohypericin . All these reactions have been suggested to be catalyzed by a phenolic coupling protein called Hyp-1 . Oxidation of the methyl group of protohypericin has been presumed to yield

protopseudohypericin . Conversion of the protoforms to hypericin and pseudohypericin can take place under the influence of light, but Hyp-1 has also been suggested to be involved in these reactions . The chemical synthesis of hypericin has been achieved by following the pattern of the biosynthesis, using emodin anthrone as a precursor .

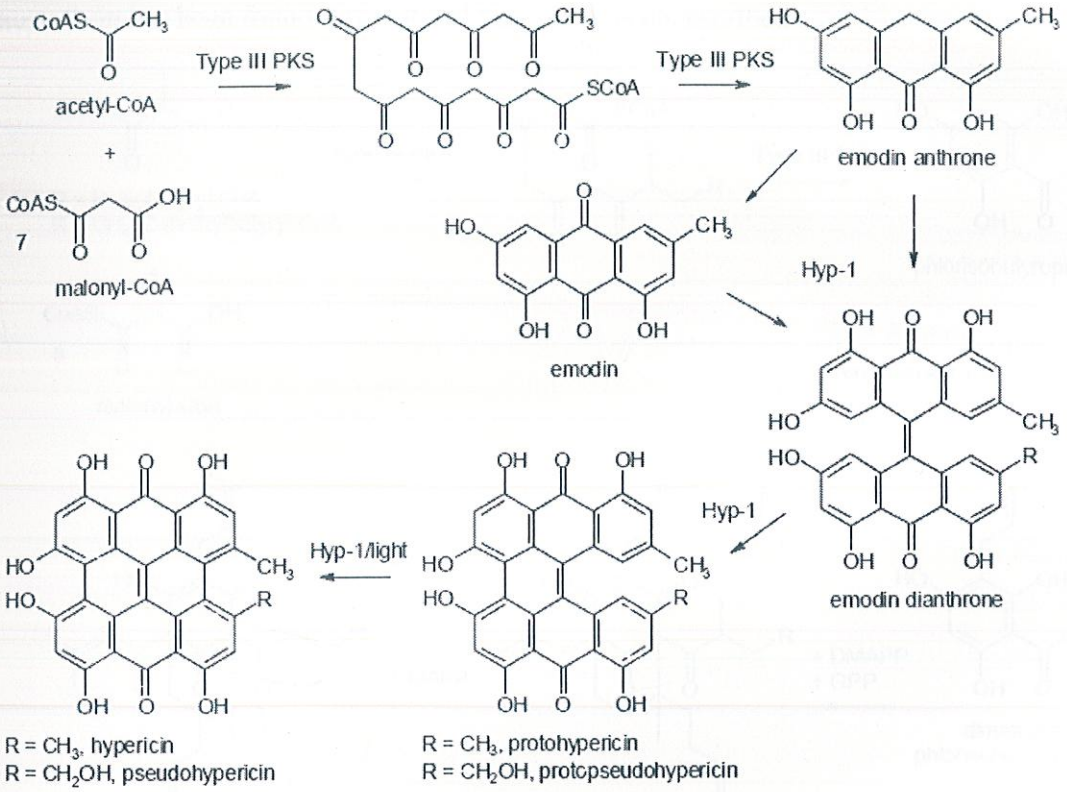


Fig. 3. Proposed biosynthetic pathway of hypericins.

The biosynthesis of hyperforins is divided into two sections that comprise the formation of the acylphloroglucinol nucleus and the subsequent attachment of prenyl side chains to it. The nucleus of hyperforin derives from the condensation of one molecule of isobutyryl-CoA with three molecules of malonyl-CoA to yield a linear tetraketide intermediate that is further cyclised, leading to the formation of phlorisobutyrophenone . A type III PKS called isobutyrophenone synthase (BUS) is responsible for the catalyzation of the reaction . In the biosynthesis of adhyperforin, 2-methylbutyryl-CoA instead of isobutyryl-CoA is utilized as a starter substrate (Klingauf *et al.* 2005, Charchoglyan *et al.* 2007). The nucleus of hyperforins is subsequently prenylated stepwise, using isoprenoid units, derived via a non-mevalonate pathway, as prenyl donors . The first prenylation

step is catalyzed by a prenyltransferase using dimethylallyl diphosphate (DMAPP) as a prenyl donor . Furthermore, two molecules of DMAPP and one molecule of geranyl diphosphate (GPP) are finally joined to the acylphloroglucinol nucleus to yield hyperforin and adhyperforin. For the biosynthesis of secohyperforins, only DMAPP has been suggested to be utilized as a prenyl donor (Charchoglyan *et al.*2007). An overview of the biosynthesis of the main hyperforins found in *H. perforatum*, based on current knowledge, is presented in Fig. 4. The chemical synthesis of hyperforin has been found difficult and thus remains undescribed

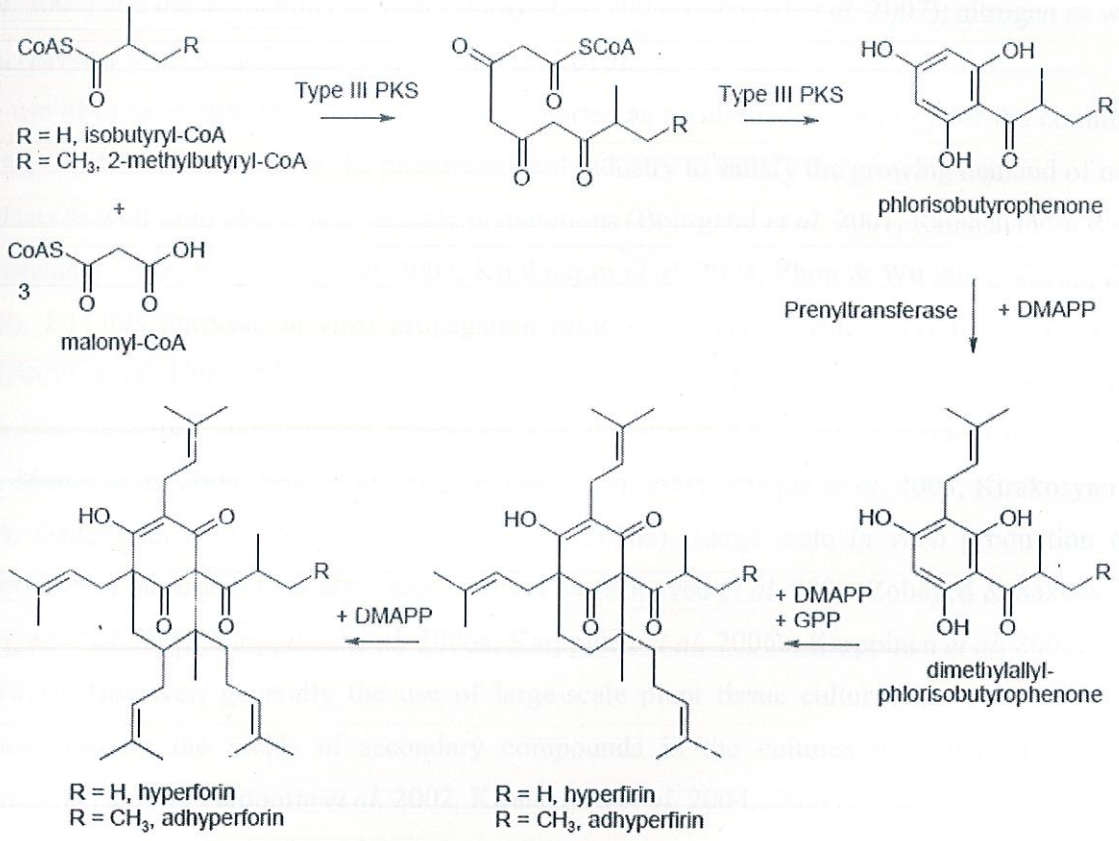


Fig. 4. Proposed biosynthetic pathway of hyperforins.

1.4 Production of secondary metabolites in plant tissue cultures:

Commercial St. John's wort preparations show inconsistency in their contents of bioactive compounds, which has raised doubts concerning the safety and efficacy of the products (Wurglics *et al.* 2001, Kirakosyan *et al.* 2004, Couceiro *et al.*2006, Bruni & Sacchetti 2009). The amounts of

hypericins and hyperforins in extracts prepared from field- or greenhouse-grown plants are greatly influenced by many factors, such as the time of harvest (Southwell & Bourke 2001, Walker *et al.* 2001, Gray *et al.* 2003, Couceiro *et al.* 2006), geographical origin of the plants (Walker *et al.* 2001) as well as the presence of biotic or abiotic contaminants (Sirvent & Gibson 2002, Murch *et al.* 2003, Bruni *et al.* 2005, Tirillini *et al.* 2006). The levels of the compounds have also been shown to vary between genetic variants (Couceiro *et al.* 2006) and be affected by several environmental factors, such as CO2 concentration (Mosaleeyanon *et al.* 2005), temperature (Zobayed *et al.* 2005, Couceiro *et al.* 2006) and the availability of water (Gray *et al.* 2003, Zobayed *et al.* 2007), nitrogen as well as light (Briskin & Gawienowski 2001, Germ *et al.* 2010).

The use of *in vitro* culture systems has been reported as an alternative strategy for the commercial production of plant material in the pharmaceutical industry to satisfy the growing demand of natural products as well as to obtain less variable preparations (Bourgaud *et al.* 2001, Ramachandra Rao & Ravishankar 2002, Verpoorte *et al.* 2002, Kirakosyan *et al.* 2004, Zhou & Wu 2006, Karuppusamy 2009). For this purpose, *in vitro* propagation protocols were described also for *H. perforatum* (Čellárová *et al.* 1992, Zdunek and Alfermann 1992). Since the first reports, numerous protocols have been described and levels of hypericins and hyperforins have been monitored in the cultures (*e.g.* Murch *et al.* 2000, Bais *et al.* 2002, Košuth *et al.* 2003, Pasqua *et al.* 2003, Kirakosyan *et al* 2004, Gadzovska *et al.* 2005, Karppinen *et al.* 2006a). Large-scale *in vitro* production of *H. perforatum* in bioreactors has also been reported on (Zobayed *et al.* 2003, Zobayed & Saxena 2003, Zobayed *et al.* 2004, Karppinen *et al.* 2006a, Karppinen *et al.* 2006b, Karppinen *et al.* 2006c, Cui *et al.* 2010). However, generally the use of large-scale plant tissue cultures has only had limited success because the yields of secondary compounds in the cultures have been too low for commercialization (Verpoorte *et al.* 2002, Kirakosyan *et al.* 2004, Zhou & Wu 2006).

In addition to the optimization of growth conditions and the selection of high producing cell lines, elicitation, biotransformation of precursors and genetic engineering have been employed as strategies to improve the biosynthesis of secondary compounds in plant tissue cultures (Bourgaud *et al.* 2001, Ramachandra Rao & Ravishankar 2002, Verpoorte *et al.* 2002, Zhou & Wu 2006, Karuppusamy 2009). For this purpose, an array of investigations has been conducted to enhance the levels of hypericins and hyperforins in tissue cultures of *H. perforatum* through the addition of elicitors (*e.g.* Kirakosyan *et al.* 2000, Walker *et al.* 2002, Kirakosyan *et al.* 2004, Gadzovska *et al.* 2007, Liu *et al.* 2007b, Pavlíc *et al.* 2007) and potential precursors (Liu *et al.* 2007c) to the cultures. The development of an efficient transformation method has also been under investigation for certain *Hypericum* species in order to improve the biosynthesis of therapeutically useful compounds through genetic engineering (Di Guardo *et al.* 2003, Vinterhalter *et al.* 2006, Franklin *et al.* 2007, Komarovská *et al.* 2009). Among the species, *H. perforatum* still remains highly recalcitrant

towards *Agrobacterium*-mediated transformation methods (Franklin *et al.* 2007, Franklin *et al.* 2008). Recently, the importance of understanding the biosynthetic pathways and their regulation has been emphasized as a prerequisite for the efficient manipulation of desired secondary metabolites in *in vitro* cultures (Verpoorte *et al.* 2002, Kirakosyan *et al.* 2004, Zhou & Wu 2006, Bruni & Sacchetti 2009, Karuppusamy 2009). Over the past decade, medicinal plants have received considerable interest for their phytomedicinal chemical compounds. Among them, *Hypericum perforatum* L. has been considered according to its biochemical characteristics and secondary metabolite production (Gadzovska *et al.,* 2005). St. John's wort (*Hypericum* perforatum L.), a perennial herb that is native to Europe and West Asia, has been used as a medicinal plant for the treatments of mild to moderate depression, inflammation and wound healing (Brolis *et al.,* 1998; Stevinson & Ernst, 1999). In recent studies, St. John's wort has been reported as a potential source for anticancer, antimicrobial and antiviral medicines (Pasqua *et al.,* 2003; Schempp *et al.,* 2002).

These features are obviously attributed to the presence of a broad spectrum of secondary metabolites, mainly naphthodianthrons, phloroglucinols, (Greeson *et al.*, 2001), xanthones (Hostettmann and Hostettmann, 1989), flavonoids, procyanidins, tannins, essential oils, amino acids, phenylpropanoids and other water-soluble components (Greeson *et al.,* 2001). Among them, naphthodianthrone hypericin and pseudohypericin along with the Phloroglucinol derivative hyperforin are thought to be the active components (Umek *et al.,* 1999; Tatsis *et al.,* 2007; Schwob *et al.,* 2002). Therefore, all these features manifest the economic importance of the genus Hypericum and in particular of Hypericum perforatum L. This phenomenon is further evidenced by the fact that the market for Hypericum perforatum L. has exceeded $210 million in the USA and $570 million worldwide annually (Sirvent and Walker, 2002). *Hypericum* sp. is considered to be an important source for therapeutic agents, according to hypericin and hyperforin contents in extracts. Phytopharmaceutical preparations of *Hypericum* are usually produced from field-grown plants. Unfortunately, quality of these products may be affected by different environmental conditions, pollutants and fungi, bacteria, viruses, and insects which can alter the concentration of medicinal metabolites (Murch *et al.,* 2000). Seventeen-fold difference in hypericin- and 13-fold difference in pseudohypericin-levels can be found in different preparations (Greeson *et al.,* 2001). Southwell and Bourke, 2001 recently reported that the concentration of hypericin and pseudohypericin in leaves of St. John's wort varied up to 50-fold in summer and winter grown plants. Recently, Murch *et al.*, reported the complete loss of capacity to produce hyperforin and the reduced accumulations of hypericin and pseudohypericin in St. John's wort seedlings grown in nickel supplemented growing medium. Recently, several studies have reported that environmental factors such as light intensity and CO_2 concentration can significantly alter the metabolite concentrations. Briskin *et al.,* 2001 reported that growing St. John's wort plants at high light intensity (photosynthetic photon flux

(PPF) of 400 mmol m^{-2} s^{-1}) significantly increased the hypericin concentration. The authors hypothesized that the high photosynthetic activity under high light intensity resulted in increased amount of carbon assimilation and enhanced the hypericin concentration in the leaf tissues. In contrast, some researchers have obtained the high rate of metabolite production in non-photosynthetic tissues by enhancing the light intensity, for instance, Kurata *et al.,* 1997 found that the high light irradiation enhanced purine alkaloid (caffeine and theobromine) production in Coffea arabica cell suspension cultures due to the physiological changes in cell growth, sugar and oxygen uptake rates. Zhong *et al.,* 1991 successfully increased anthocyanin production in cell culture of Perilla frutescens (shiso) by increasing light intensity.

Therefore, growing plants under a controlled environment can be considered as an alternative way for medicinal plant production to ensure safety and efficacy. When plants are grown under a controlled environment with artificial light, it is possible to maximize plant biomass production with minimum use of resources and minimum or no pollutants released to environment. Under a controlled environment, uniform growth can be expected, and production planning and scheduling can be possible and contamination by diseases, insects, metals and other harmful subjects can be reduced or eliminated (Kozai *et al.,* 2001). In phytopharmaceutical industry, one solution could be the production of micropropagated plants, in sterile and standardized conditions. Many investigations have been conducted to establish and to enhance hypericin and pseudohypericin production (Kirakosyan *et al.,* 2000; Kirakosyan *et al.,* 2001; Sirvent *et al.,* 2002; Walker *et al.,* 2002). In vitro systems have been reported as an effective tool for obtaining genetically uniform plants, which can be a source of variable pharmaceutical preparations (Santarem and Astarita, 2003). Plant regeneration of H. perforatum L. has been achieved using as explants whole seedlings or their excised parts (Cellarova *et al.,* 1992; Brutovska *et al.,* 1994), hypocotyl sections (Murch *et al.,* 2000a; Santarem and Astarita, 2003; Zobayed *et al.,* 2004) and leaves (Pretto and Santarem, 2000; Bezo and Stefunova, 2001) using various types and concentrations of auxins and cytokinins.

Production of secondary metabolites via plant cell and tissue cultures yields various advantages, including standardisation and quality. These criteria are also valid for the main economically important chemicals in St. John's wort, namely hypericin, pseudohypericin and hyperforin (Zobayed *et al.,* 2004). Hypericum perforatum L. is a unique species that undergoes multiple forms of reproduction in the wild. Several reproductive processes have been identified in wild harvested Hypericum perforatum L. (Matzk *et al.,* 2001). This reproductive flexibility has led to high chemical variability in field grown plants. Therefore, the application of in vitro techniques provides an approach for the production of standardized plant material (Murch *et al.,* 2000b). Hypericin is generally extracted from flowers of wild or cultivated Hypericum perforatum L. plants. Both the limited area of occurrence of this plant and seasonal harvesting necessitate a search for alternative

methods for production of these compounds. In addition, plant tissue and cell cultures are important tools which allow extensive manipulation of the biosynthesis of the secondary compounds and yield a higher productivity compared to that of intact plants (Kirakosyan *et al.,* 2001).

The limited area of occurrence of this plant, seasonal harvesting, loss of biodiversity, variability in quality, and contamination issues, trigger to search alternative methods for hypericin and hyperforin production. Optimization of culture conditions could allow significant change in the level of bioactive metabolites in *Hypericum* species.

In the light of the above cited information, it would be desirable to evaluate the effects of different incubation conditions on shoot and callus cultures of Hypericum and thereby to investigate the effect on biosynthesis and accumulation of hypericin and hyperforin contents in the tissue cultures established from Hypericum perforatum L. and then develop an antidepressant/ stress-reliever formulation in India. So the studies carried out with following objectives:

1. To carry out in vitro multiplication, callus induction and regenertation of *Hypericum perforatum*.
2. In vitro production of hypercin by using elicitors and growth hormones through cell cultures technology.

CHAPTER 2:

LITERATURE REVIEW

ST. JOHNS WORT *Hypericum perforatum*

2.1 Life History/Identification:

St. Johnswort is a perennial herb that can grow to be two to five feet tall. It is also known as amber, goatweed, klamathweed, rosin rose, and tipton weed. The roots of the plant are an abbreviated taproot system, meaning that some roots bore deeply into the earth while some of the roots remain shallow. Taproots can penetrate as far as five feet into the soil. The roots that are shallow produce vegetative buds from which new crowns develop. St. Johnswort has numerous erect stems that appear to have a rust color. The stems are also woody and relatively smooth to the touch. The leaves of this plant are oblong and/or elliptic and attach directly to the stem. St. Johnswort has transparent glands in its leaves that produce an oil that is toxic to animals. From May to September, this weed has an extensive flowering period. The flowers are anywhere from one to two centimeters in diameter and they develop in clusters. Flowers are yellow, have five petals, and have transparent black dots around the edges of each petal. The flowers also contain numerous amounts of stamens. The seed capsules of this plant are rounded at the end and are small (about 3/16 to 7/16 of an inch long). They have a rusty color and each capsule contains numerous seeds. Seeds are dark brown and are about 3/64 of an inch long. Each seed contains a germination inhibitor, but the germination increases over time during rainy periods because the inhibitor is washed off. St. Johnswort reproduces through seeds and roots, and can be spread by the wind, wildlife, and through human activity. The plant is an aggressive non-native that is considered noxious in many states. It is typically found in old meadows, pastures, along roadsides, on rangeland, and in waste areas, usually on dry, gravelly, or sandy soils. St. Johnswort thrives in direct sunshine and has proven to have the ability to push out native plants in areas where it grows. St. Johnswort is native to Europe, North Africa, and parts of Asia, including India, China, and Japan. The major reasons for its introduction into the United

States was its ornamental value or its cultivation for medicinal purposes. By 1945, the weed had infested about 2.5 million acres in California alone.

Flagstaff Localities:

This species was found in one location on the Kaibab National Forest during a 1997 survey. The location was near a place of human habitation and near a spring. Some of the species of herbs including mint (Mentha piperita) were present. The plants probably persisted after human abandonment of the site. Other locations around human habitation and former occupancy may exist.

Economic Impact:

St. Johnswort enjoys a long and colorful history. Dioscorides, Pliny, and Hippocrates all used it during ancient Greek times. It was believed to possess magical properties to dispel evil spirits. There was a common medieval belief that sleeping with a piece under your pillow on St. John's Eve would protect one during the following year. Today, St. Johnswort extracts have been administered for the treatment of mania, hysteria, depression, dysentery, jaundice, and a host of other afflictions. Unfortunately, outside of its native range it has the potential to do as much harm as good. St. Johnswort can be a serious problem in rangeland and pastures where dense stands, through their displacement of valued forage and indigenous plant species, can greatly depreciate livestock and wildlife carrying capacities, and endanger the biological diversity of these grazing lands. When livestock (cattle, sheep, goats, horses, and swine) eat a sufficient quantity this plant, they become poisoned by a compound called hypericin. Hypericin is absorbed from the intestinal tract and goes into the circulatory system, causing cellular damage and potentially severe sunburn. The affected skin first becomes swollen and tender, then reddened. The skin can be burned to the point where large areas of skin peel off. This is extremely painful for the animal and predisposes it to infection. Affected animals are reluctant to have the areas examined, and may act abnormally or not want to eat due to the discomfort.

2.2 Control:

Preventing the introduction and further spread of this plant to uninfected areas is much easier, more environmentally desirable, and more cost-effective than is the subsequent management of large-scale infestations. An integrated combination of control methods will be needed to successfully manage St. Johnswort.

2.2.1 Cultural Control:

Practices that encourage residual competitive species or that establish competitive, well-adapted, palatable grasses or legumes at a St. Johnswort-infested site should be a part of any management effort. Research has shown that a program of cultivation, fertilization, and reseeding with a perennial grass controlled St. Johnswort populations in pastures and rangeland over a two to five year period. The choice of plant species to be seeded should reflect site conditions, management, and future use.

2.2.2 Mechanical Control:

Hand pulling and grubbing of young, isolated plants can often be effective. Be sure to remove and destroy pulled plants to prevent possible vegetative re-growth and/or seed dissemination. For larger infestations, mowing can diminish the spread of the plant if it is performed prior to seed formation. Two or more cuttings may be necessary during the growing season. Burning has actually increased the density and vigor of St. Johnswort stands and is not recommended as a control method.

2.2.3 Chemical Control:

(Noted here are chemical control techniques that have been used in other areas. Always check with weed specialists or chemical suppliers before treatment to ensure correct dosage and application. Mention of these products does not imply endorsement by the Northern Arizona Weed Council or The Nature Conservancy.):

1. Escort□ (chemical name: metsulfuron) applied at 1 ounce of product per acre can provide for effective control if it is sprayed after the plants have emerged and are actively growing. Apply with a nonionic surfactant at 0.25% v/v.
2. 2, 4-D can be used before St. Johnswort blooms, preferably on new seedlings. However, 2,4-D is not very effective in completely killing the plant in a single application, so repeat applications are necessary.
3. Tordon 22K□ (chemical name: picloram) is a restricted use herbicide, but can be applied on the early growth stages of the weed. One to two quarts per acre for spot treatments and one

to two pints per acre tank-mixed with 1 quart of 2,4-D as a broadcast treatment is recommended.

2.2.4 Biological Control:

(No exotic species should be introduced into an ecosystem without extensive research into the long-term effects. Mention of the species below does not imply appropriateness for use in Northern Arizona.):

There are several biological control agents approved for use in the control of St. Johnswort. These include three beetle species – Agrilus hyperici, Chysolina hyperici and Chysolina quadrigemina. A moth Aplocera plagiata is also available. More information on these agents is available through the USDA, Animal and Health Inspection Service. These agents would probably not be effective control methods in Northern Arizona, due to the small size of known populations of the plants. For effective control, biological agents generally need large enough populations to sustain their own populations through several generations.

Note: No single control method, or any one-year treatment program, will ever achieve effective control of an area contaminated by St. Johnswort. The fast growth, deep root system, and prolific seed production of this plant require long-term cooperative integrated management programs and planning to prevent, contain, and reduce St. Johnswort infestations.

2.3 DISTRIBUTION:

Hypericum perforatum grows naturally in temperate and subtropical regions of Europe,North America, Russia, India ,and China .In India it grows wild in the western Himalayas-at places in Jammu and Kashmir and Himachal Pradesh at altitudes above 1500 meters.

2.4 PLANT DESCRIPTION:

St. John Wort is a perennial herb with rhizomatous roots and clusters of yellow flowers. Flower bloom in late summer .Leaves exhibit prominent oil glands and the whole appear perforated when held up against the light.

2.5 CHEMICAL COMPOSITION:

Contains a high concentration of Melatonin. Herb and flowers contain various polyphenols: flavonoids (rutin,hyperoside, isoquercetin, quercitrin, quercetin, amentoflavone, astilbin, miquelianin), phenolic acids (chlorogenic acid, 3-O-coumaroylquinic acid), and various naphtodianthrones: (hypericin, pseudohypericin, protohypericin, protopseudohypericin), phloroglucinols (hyperforin, adhyperforin). The naphthodianthrones hypericin and pseudohypericin along with the phloroglucinol derivative hyperforin are thought to be the active components. It also contains essential oils composed mainly of sesquiterpenes.

PHARMACOLOGY:

The exact mechanism by which St. John's Wort functions is unclear and subject to conjecture. The St John's Wort mechanism is believed to involve inhibition of serotonin (5-HT) reuptake, much like the conventional selective serotonin reuptake inhibitor (SSRI) antidepressants.[35] The major active antidepressive constituents in St. John's Wort are thought to be hyperforin and hypericin, although other biologically active constituents present, for example, flavonoids and tannins, may also be involved.

Some believe that hyperforin is the major constituent responsible for antidepressant activity, and it has been shown to inhibit the uptake of 5-HT, dopamine, and noradrenaline. Hyperforin also has affinity for GABA and glutamate receptors. On the other hand, a hyperforin-free extract of St John's Wort may still have significant antidepressive effects.

MECHANISM OF ACTION:

The apparent broad spectrum mechanism of action of St.John Wort is not fully understood,yet biologically active constituents may include **hyperforin** and adhyperforin, **hypericin** and psuedohypericin, flavonoids, xanthones, oligomerics, procyanidines, and amino acids. Its antidepressant activity may be mediated by serotonergic (5-HT), noradrenergic and dopaminergic system as well as by means of gamma-aminobutyric acid(GABA) and glutamate amino acid nuerotransmitters.The action of St.John's Wort may be diffent from that of standard antidepressants.

2.6 USE AS ANTI-DEPRESSANT:

Today, St.John's Wort is most widely known as a herbal remedy for depression in US and GERMANY. In some countries, such as GERMANY it is commonly prescribed for mild depression ,especially in children and adolscents. It is also found affective as a natural remedy for treating seasonal affective disorder or SAD (a form of depression that occurs during the winter because of lack of sunlight).

Standardized products of the plants are generally over the counter in USA.

Extracts are usually sold in tablets or capsule form and also in tea bags and tincture.

CLINICAL EVIDENCE:

Clinical studies of St. John's Wort preprations have mainly focused efficacy of the herb in clinical depression. Several studies and meta-analysis have found it to be effective in the treatment of mild to moderate depression, with few side effects than many conventional antidepressants.

EVIDENCE FOR EFFICACY:

A comprehensive meta study indicated that extracts of *Hypericum perforatum* may be significantly more efficacious than placebo for the treatment of mild to moderate depression.

This study, which covered the results from 23 smaller, earlier studies is most often cited by manufactures and supporters of St. John wort.

An updated review of the above study based on new inclusion of data revealed that Hypericum preparations were significantly superior to placebo and similarly effective as standard antidepressants.

A further meta-analysis confirmed that Hypericum was more efficacious than placebo and similarly as efficacious as tricyclic antidepressants, with little side effects.

2.7 OTHER MEDICAL USES:

Alcoholism:

It is hypothesized that because of SJW's ability to relieve depressive symptoms, people tend to lose out on alcohol addiction.

Hyperforin, a major constituent, has also been found to have antibacterial properties ;in ultrapurified form a concentration of 0.1mg/ml kills methicillin-resistant Staphylococcus aureus.

Since hyperforin can stimulate the release of the neurotransmitter norepinephrine, It is presumed that the drug might alleviate the symptoms of attention-deficit hyperactivity disorder(ADHD). However, a randomized controlled trial of St.John's wort found no significant difference between the herbal extract and placebo in the management of ADHD symptoms over eight weeks.

Keeping in view of its therapeutical potential, we intented to carry out the present investigation with a primary objective for in-vitro culture of the plants for the purpose of conservation as well as commercial exploitation of its active components which are medicinally useful.

CHAPTER 3

MATERIALS AND METHODS

3.1 Collection of plant material

Plants stocks collected from NBPGR, regional station, Shimla had been used for the present study. These plants were planted in the JUIT GREENHOUSE and are being maintained there. The leaves, stems and roots were used for further experiments as explants.

3.2 INITIAL ESTABLISHMENT OF ASEPTIC CULTURES:

The young shoots were surface sterilized with 70% (v/v) ethyl alcohol for 30 s and 0.1% (w/v) HgCl2 for 3 min followed by repeated rinsing with sterile distilled water.

Initially, aseptic culture were established by culturing 2–3 cm long excised shoot explants with 1–2 node(s) containing axillary buds on MS (Murashige and Skoog 1962) medium supplemented with Kn and 2,4-D alone or in combinations (0.0, 0.25, 0.5, 1.0, 2.0 and 5.0 mg l-1). For all the experiments pH of the medium was adjusted to 5.6 ± 0.02 prior to autoclaving at 15 lbs. at 121°C for 15 min. The culture conditions (temperature 25 ± 2°C, 14 h photo-period using cool white florescent light, and light intensity 20 lmol m-2 s-1) were maintained throughout the course of study. Minimum six replicates were maintained for each treatment. Shoot multiplication and callus initiation response was examined after 8 weeks of culture.

3.3 METHODOLOGY-1

3.3.1 PREPARATION OF CULTURE MEDIA:

Stocks were made for the preparation of MS media were mixed together and then table sugar (30mg/lt.) was added to it.

Growth hormones kinetin:IBA (3:1.), 2,4-D(3mg/ltr.) were then added. The pH was adjusted to 5.5 to 5.6.

Agar (9gm) was finally added to the mixture.

The tap water was then added to adjust the final volume to 1 litre.

Media was then heated till boiling and poured into flask.

Flask were then autoclaved at 121° and 15 psi.

3.3.2 PREPARATION OF EXPLANTS FOR INOCULATION AND INCUBATION:

Explant, mainly consisting of leaves and stem also some portions of root were taken and thoroughly washed under tap water for 15 minutes, which was followed by washing with detergent Labolline. The treated plant materials were then subjected to sterilisation using Bavistin (0.5 gm/100ml) for 60 seconds and Mercuric chloride(0.1 gm/100ml) for 45 seconds, followed by repeated washing with autoclaved water. Inoculation of the explant was done into autoclaved flasks containing M.S. media in Laminar Air Flow. The inoculated flasks were then incubated in the culture room at 25± 2□□under fluorescent light. Callus was induced using M.S. media supplemented with different growth hormones, viz ,Kinetin and 2,4-D.

3.4 METHODOLOGY 2:

Plants with vigorous leaf and root growth were obtained after continuous subculturing of calli in advanced stages of development. These were then subcultured into MS media with growth hormones, 2,4-D, Kinetin(3 mg/lt) and IBA(1mg/lt)

3.5 METHODOLOGY 3

METHODOLOGY for HPLC

The sample to be separated and analyzed is introduced, in a discrete small volume, into the stream of mobile phase percolating through the column. The components of the sample move through the column at different velocities, which are functions of specific physical or chemical interactions with the stationary phase. The velocity of each component depends on its chemical nature, on the nature of the stationary phase (column) and on the composition of the mobile phase. The time at which a specific analyte elutes (emerges from the column) is called the retention time. The retention time measured under particular conditions is considered an identifying characteristic of a given analyte. The use of smaller particle size packing materials require the use of higher operational pressure ("backpressure") and typically improves chromatographic resolution (i.e. the degree of separation between consecutive analytes emerging from the column). Common mobile phases used include any miscible combination of water with various organic solvents (the most common are acetonitrile and methanol). Some HPLC techniques use water free mobile phases (see Normal Phase HPLC below).

The aqueous component of the mobile phase may contain buffers, acids (such as formic, phosphoric or trifluoroacetic acid) or salts to assist in the separation of the sample components. The composition of the mobile phase may be kept constant ("isocratic elution mode") or varied ("gradient elution mode") during the chromatographic analysis. Isocratic elution is typically effective in the separation of sample components that are not very dissimilar in their affinity for the stationary phase.

In gradient elution the composition of the mobile phase is varied typically from low to high eluting strength. The eluting strength of the mobile phase is reflected by analyte retention times with high eluting strength producing fast elution (=short retention times). A typical gradient profile in reversed phase chromatography might start at 5% acetonitrile (in water or aqueous buffer) and progress linearly to 95% acetonitrile over 5–25 minutes. Period of constant mobile phase composition may be part of any gradeint profile. For example, the mobile phase composition may be kept constant at 5% acetonitrile for 1–3 min, followed by a linear change up to 95% acetonitrile.

The composition of the mobile phase depends on the intensity of interactions between analytes and stationary phase (e.g. hydrophobic interactions in reversed-phase HPLC). Depending on their affinity for the stationary and mobile phases analytes partition between the two during the separation process taking place in the column. This partitioning process is similar to that which occurs during a liquid-liquid extraction but is continuous, not step-wise. In this example, using a water/acetonitrile gradient, more hydrophobic components will elute (come off the column) late, once the mobile phase gets more concentrated in acetonitrile (i.e. in a mobile phase of higher eluting strength).

The choice of mobile phase components, additives (such as salts or acids) and gradient conditions depend on the nature of the column and sample components. Often a series of trial runs are performed with the sample in order to find the HPLC method which gives the best separation.

Safety Precautions: Consult the Material Safety Data Sheet (MSDS) for any chemical used that is unfamiliar. All chemicals should be considered hazardous - avoid direct physical contact. For more safety information, see http://hazard.com/msds/.

Principle: Samples are extracted in methanol using an ultrasonic bath. After sample preparation, the sample is analyzed using high-performance liquid chromatography (HPLC) and quantified by comparison to external hyperforin standard. The HPLC column is MetaChem Polaris™ C18-A, 5µm, 4.6 x 250 mm, the mobile phase is isocratic acetonitrile/pH 2.5 ammonium phosphate buffer at 1.5 mL/min and UV detection at 270 nm.

Standards: Hyperforin, Addipharma, Hamburg, Germany, PN RHF 00298

Note: Hyperforin reference standards may contain approximately 10% adhyperforin impurity.

Note: This is not an all-inclusive listing of sources for reference standards. World wide there are many reputable suppliers of botanical reference standards whose product may be used. However

quality control protocol and/or government regulation may mandate further qualification of the materials for proper identity and purity.

Standard Preparation:

Hyperforin Standard

Accurately weigh approximately 5 ±0.01mg of hyperforin reference standard into a low actinic 5-mL volumetric flask. Add 3 mL of methanol and dissolve with the aid of sonication. If hyperforin does not readily go into solution, add a few drops of DMSO. Dilute to volume with methanol for a stock concentration of 1.0 mg/mL.

Dilute the stock standard using methanol and low actinic glassware to create calibration curve standards of 0.5 mg/mL, 0.1 mg/mL, and 0.01 mg/mL.

Note: Further dilutions may be made for additional datum points.

Notice: Store standard solutions in the dark at 0 - 10°C when not in use. Standard will be stable for 2-3 weeks under these conditions.

Apparatus:

- Calibrated analytical balance accurate to ±0.1 mg or ±0.01 mg
- Sonicator, VWR Scientific Aquasonic Model 250D or equivalent centrifuge tubes
- Column, HPLC, MetaChem Polaris™ C18-A, 5µm, 4.6 x 250 mm
- High-Performance Liquid Chromatography System as described in USP chapter <621>. Verify and document that apparatus, software, and subsystems meet performance requirements for Installation Qualification/Operation Qualification (IQ/OQ).
- Filter, 0.45 µm, nylon, disposable
- Syringe, 3-cc, plastic, disposable with Luer-Lok tip Amber vials, 2-mL, HPLC auto-sampler compatible, with crimp top
- Crimper for HPLC vial cap

Reagents:

- Water, HPLC grade or Nanopure
- Ethanol, OmniSolve AX0445-1 or equivalent
- Acetonitrile, HPLC grade

- Phosphoric acid, 85%, ACS grade
- Ammoniumdihydrogenphosphate, ACS grade
- Ice

Solutions:

Prepare a 0.01M ammoniumdihydrogenphosphate buffer by dissolving 1.15(.57gm) g ammoniumdihydrogenphosphate into 1 L(500ml) water and adjust the pH to 2.5 ±0.1 with phosphoric acid.

Sample Preparation:

Plant Material:

Accurately weigh approximately 500 ±0.1 mg of ground plant material and place into a low actinic 100-mL volumetric flask. Add about 80 mL of methanol and sonicate at room temperature for 30 minutes. Dilute to volume with methanol and mix. Filter or centrifuge, place solution into an amber HPLC vial and cap. Inject immediately into the HPLC. If the sample is not to be immediately injected into the HPLC, refrigerate at 0°C. Remove the sample from refrigeration immediately prior to injection into the HPLC. DO NOT ALLOW THE SAMPLE TO SIT AT ROOM TEMPERATURE. Alternately, place the vial into a chilled autosampler rack to await injection.

Notice: For efficient extraction and reproducible results, plant materials must be finely ground and passed through a 40-mesh screen (Tyler Equivalent 35 mesh or 0.0165 inch.)

Extract Material:

Accurately weigh approximately 500 ±0.1 mg of powdered extract and place into a low actinic 100-mL volumetric flask. Add about 80 mL of methanol and sonicate in cold water (0°C to 5°C) for 30 minutes. Dilute to volume with methanol and mix. Filter or centrifuge and place solution into an amber HPLC vial and cap. Inject immediately into the HPLC. If the sample is not to be immediately injected into the HPLC, refrigerate at 0°C. Remove the sample from refrigeration immediately prior to injection into the HPLC. DO NOT ALLOW THE SAMPLE TO SIT AT ROOM TEMPERATURE. Alternately, place the vial into a chilled autosampler rack to await injection.

Chromatographic Conditions:

Column: MetaChem Polaris™ C18-A, 5µm, 4.6 x 150 mm

Column Temperature: 25°C

Mobile Phase: isocratic: ACN:pH 2.5 buffer (85:15)

Injection Volume: 20 µL

Flow Rate: 1.5 mL/min

Detection: 270 nm

Run Time: 30 minutes

Equivalent Column: Phenomenex Luna™ C18(2), 5µm, 4.6 x 250 mm

PROCEDURE:

Prepare reference standard solutions and sample preparations as directed.

Prepare an extraction solvent blank.

Make a single injection of the blank.

Make single injections of the reference standard preparations.

Prepare a linearity curve for hypericin, with the origin ignored, using peak area vs. concentration (mg/mL). Perform linear regression analysis on the data. The r^2 must be ≥ 0.999.

Make single injections of sample preparations.

Calculate the percent hypercin in the samples.

Quality Assurance:

A duplicate sample preparation and standard preparation should be analyzed with each set of 20 or less samples.To monitor method variance, a St. John's Wort control sample of known composition should be assayed with each batch and the assay results control charted.

Calculations:

Quantify hyperforin against hyperforin using the following equation.

$$\% \text{ w/w hyperforin} = \frac{(C)\,(FV)\,(D)\,(100\%)}{(W)}$$

Where:

C = The sample's hyperforin concentration (mg/mL) from linear regression analysis.

FV = The final volume of the sample preparation (normally 100 mL).

D = The dilution factor of the sample preparation (if needed).

W = The sample weight (mg).

3.6 REGENERATION OF *Hypericum perforatum*

The process of continuous subculturing of calli, yielded vigorous leaf and root growth in aprrox 30 flasks. Plants with vigorous leaf and root growth were obtained for they were subcultured into M.S media with growth hormones, 2,4-D(3mg/lt.) and kinetin(3mg/lt.):IBA(1mg/lt.).
The regeneration of complete plant grown about 2 inches long was achieved in about 75 days from the time of inoculation.

3.7 HARDENING

Once the initial formation of roots could be seen, the rooted shoots were gently removed from the culture vessels, washed under running tap water, treated with 0.5% Bavistin and transferred to pots containing different combination and concentrations of sand, soil, vermiculite, perlite and coco peat in the glasshouse conditions for acclimatization and hardening.

CHAPTER 4

RESULTS AND DISCUSSIONS

a) Establishment of axenic cultures of *H. perforatum*

4.1 Selection of plant material and establishment of *in vitro* shoot cultures

Axillary shoot tips (0.5-1.5 cm long) excised from pot grown plants were washed in 2% (v/v) detergent solution Teepol (Qualigen, India) and surface sterilized for 2-3 min in 0.5% (w/v) Bavistin (BASF, India, Ltd.) and for 30 seconds in 0.1% (w/v) Mercuric Chloride (Merck, india) followed by 4-5 washings in sterile distilled water. The sterile shoot apices were cultured on MS medium supplemented with different concentrations and combinations of auxins and cytokinins (purchased from Sigma Aldrich).

4.2 Preparation of media and incubation of cultures for rapid multiplication

Modifications of MS (Murashige and Skoog, 1962) media supplemented with different concentrations and combinations of IBA (indole-3-butyric acid), KN (Kinetin) and BA (6-benzyladenine) were prepared, pH adjusted top 5.7 using 0.1N HCl and 0.1N NaOH and solidified with agar-agar (Glaxo, Bacteriological Mediaid) 0.8% (w/v) as a gelling agent. The media were autocalved at 121°C and 15lb/in^2 pressure for 15-20 minutes in 150 ml Erlenmeyer flasks (Borosil, India) by dispersing 40 ml molten media in each flask plugged with non-absorbent cotton wrapped in one layer of muslin cloth. The cultures were incubated at 25±1°C in plant tissue culture chamber with 70% relative humidity under 16 hr photoperiod provided by cool fluorescent light (3000 lux).

The surface sterilized axillary shoot tips were cultured on MS media containing Benzyl adenine (0.5-2 mg/l), IBA (0.5-3 mg/l) and kinetin (1-4 mg/l) in different combinations. Out of 15 different media combinations tested for multiple shoot formation from shoot apices, MS medium containing KN (1.5mg/l) + IBA (0.5mg/l) + table sugar 3% (w/v) + agar-agar 0.8% (w/v) was found to be the best with 86.3% of shoot apices proliferating into multiple shoots within 7-8 days of inoculation (Table 3 ; Fig.1 A,B,C). Overall the supplementation of MS medium with KN and IBA resulted in

multiple shoot formation with non-significant difference in number of shoots per explants. The same medium was found suitable for obtaining maximum shoots (26.2) per explants (Fig.1D). For getting rapid and profuse shoot multiplication the above mentioned medium was manipulated in which agar component was completely omitted by keeping other components as such, therefore liquid medium was optimized for shoot multiplication However the cultures were kept at orbital shaker under same culture conditions for incubation and maximum no. of shoots obtained were 30 in number with in a time period of 5-6 days. The modifications in MS media by changing the concentrations and combinations of auxins and cytokinins did not help in obtaining shoots with more significant difference in their growth parameters.

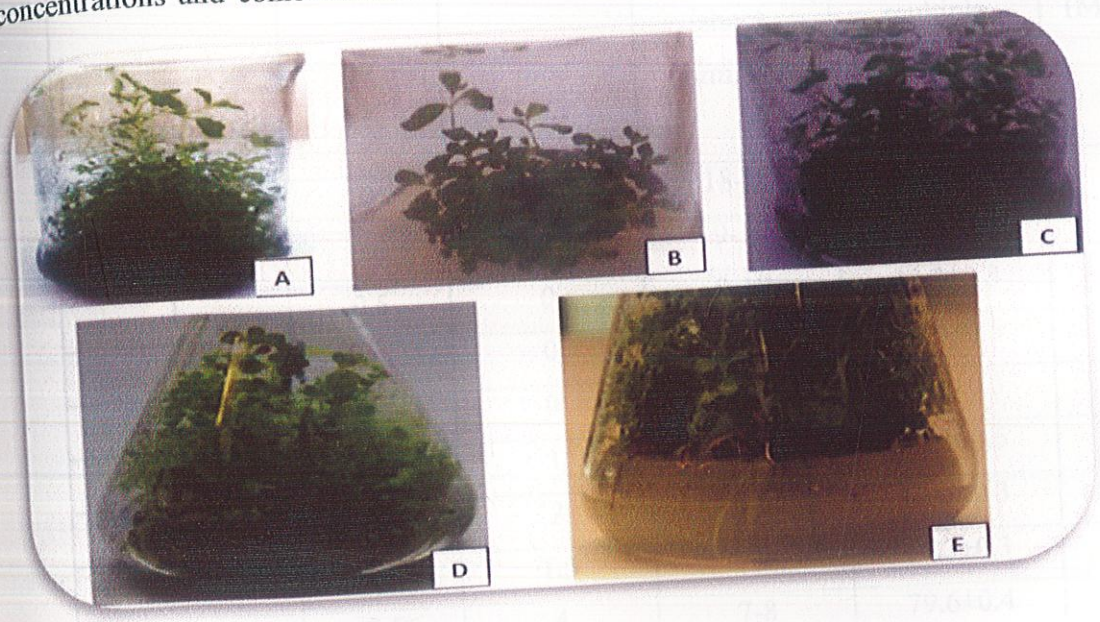


Figure 4.1: Micropropagation protocol for *H.perforatum* (B) Shoot apex (C) Multiple shoot formation (D) Shoot proliferation and growth (A) Plant grown in liquid media (E) Root induction

Table 4.1: Effect of different MS media combinations on *in vitro* shoot multiplication from shoot apices of *H.perforatum*

MS + Growth regulators (mg/l)			Parameters of shoot multiplication		
BA	IBA	KN	Days to multiple shoot formation	Percent shoot apices with multiple shoots (Mean ±S.E.)*	Shoots per explants (Mean ±S.E.)*
0	0	0	18-20	16.3±0.5	6.3±0.5
0	0.5	0	9-10	26.3±0.5	12.3±0.5
1	0.5	0	9-10	33.3±0.3	13.6±0.3
1	1	0	8-9	45.4±0.5	18.2±0.6
1	0.5	0	8-9	46.3±0.3	18.3±0.3
0	0.5	1	6-8	56.5±0.5	21.3±0.3
0	3	2	6-8	62.6±0.3	20.6±0.3
0	**0.5**	**1.5**	**7-8**	**86.3±0.3**	**26.2±0.3**
0	0.5	4	7-8	79.6±0.4	29.6±0.3
1	0	1.5	6-7	68.3±0.6	20.2±0.2
2	0	1.5	6-7	69.4±0.5	21.6±0.5
2	3	2	5-7	70.3±0.3	22.2±0.2
1	0.5	1.5	5-7	74.3±0.3	26.2±0.5
1	3	4	7-8	75.6±0.3	23.3±0.3
2	3	4	7-8	74.6±0.4	23.6±0.3

*Data represents mean of 20 replicates per treatment in three repeated experiments.

4.3 Induction of roots in shoots

Individual shoots were excised from the parent cultures and transferred onto half strength MS media supplemented with different concentrations and combinations of auxins such as IBA, IAA and NAA for root induction in culture tubes (25 × 150 mm) containing 10 ml of medium. The pH of medium was adjusted to 5.7 prior to autoclaving. The cultures were incubated under the same

photoperiod conditions as mentioned above. Data for days to root initiation, number of roots per shoot, average root length and percent rooting were recorded and analyzed for test of significance

4.4 Callus induction from different explants

Callus cultures were initiated from different explants such as leaf discs, nodal and root segments of *H.perforatum* on MS salts supplemented with different concentrations of 2,4-D (0.5-2mg/l) and/or IBA (0.5-2.0mg/l) in combinations with IAA (0.5-2.0 mg/l) and NAA (0.5-2.0 mg/l) (Fig.1.2 A,B,C). Callus formation initiated in all explants within 10-15 days at the cut surfaces in all test media combinations (Fig.3 D). All the explants were transformed into complete callus mass within 4 weeks of culture (Fig.3 E). Overall, MS medium supplemented either 2,4-D (2.mg/l):IBA(1mg/l) + table sugar 3% (w/v) + agar-agar (0.8%) was found to be the best for callus induction with frequencies of 70%, 56.3% and 38.3% from root segment, leaf discs and nodal segments, respectively (Table 4). Differences in callus morphology and appearance were observed among 3 explants with calli from leaf discs, root segments appearing as greenish, creamy white and light brown, respectively. Callus cultures derived from different explants were sub-cultured for 6-8 weeks so as to proliferate the calli on suitable callus induction media (Fig.3 F). During subculturing, some of the calli turned brown and eventually their proliferation stopped. Those calli were not carried further for regeneration. Calli with a creamy appearance were taken for regeneration.

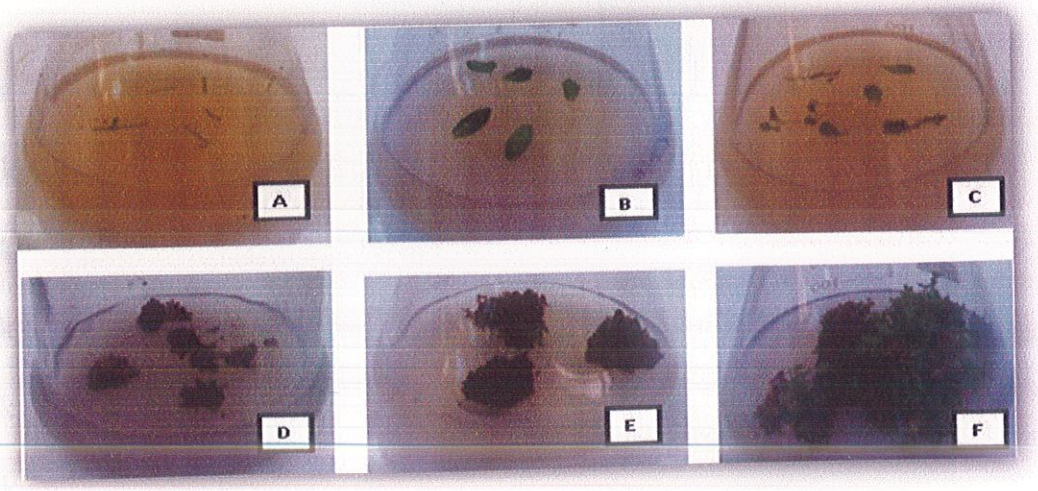


Figure 4.2: Callus initiation and proliferation from different explants of *H.perforatum*; (A) Leaf segments (B) Nodal segments (C) Root segments (D) Callus initiation (E) Callus growth (F) Callus regeneration

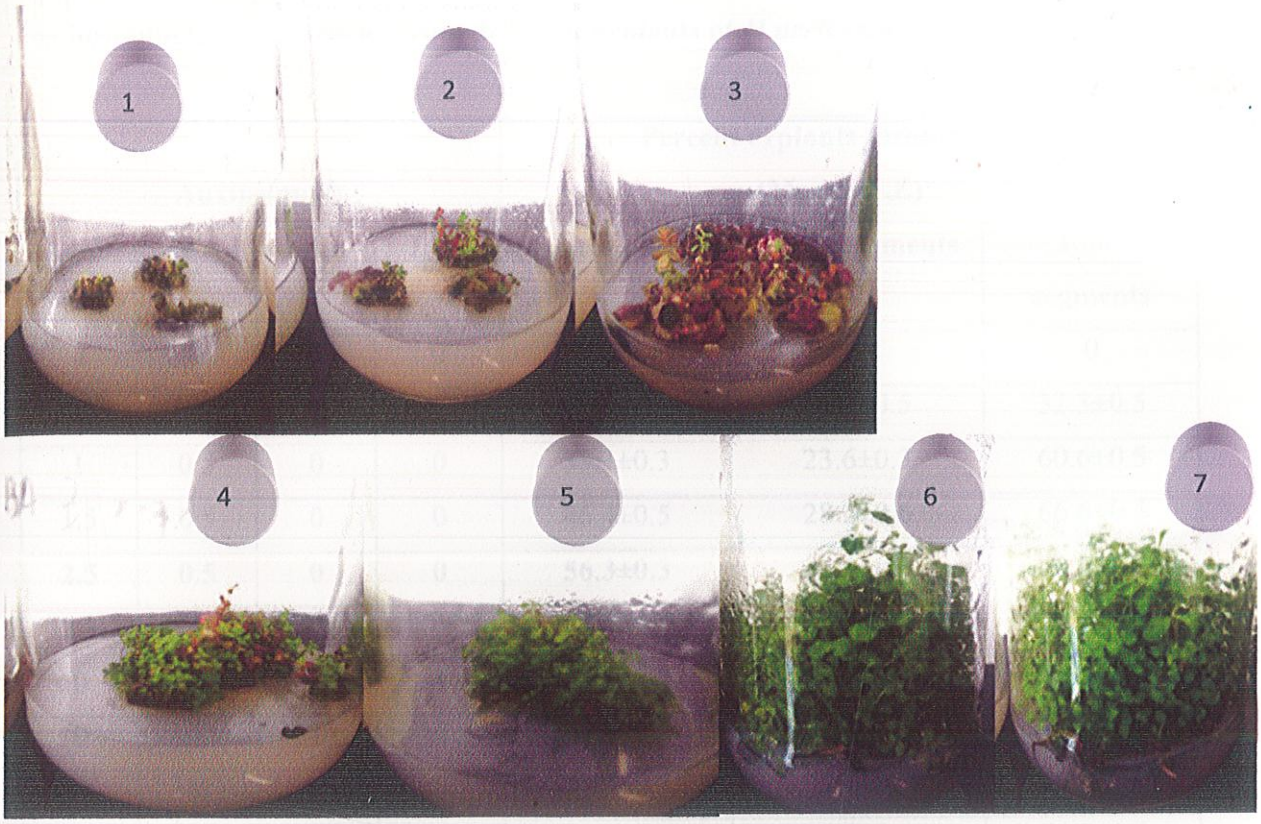


FIGURE 4.2.1: CALLUS INDUCTION AND REGENAERATION

1: 1st week

2: 2nd week

3: 3rd week

4: 4th week

5: 5th week

6: 6th week

7: 7th week

Table 4.2: Effect of MS medium supplemented with different concentrations of auxins on callus induction frequencies from different explants of *H.perforatum*

Auxins(mg/l)				Percent explants forming callus (Mean ±S.E)*		
2,4-D	IBA	NAA	IAA	Leaf disc	Nodal segments	Root segments
0	0	0	0	0	0	0
0.5	0	0	0	16.3±0.5	12.3±0.5	32.3±0.5
1	0.5	0	0	33.3±0.3	23.6±0.3	60.6±0.5
1.5	0.5	0	0	46.4±0.5	28.2±0.6	66.6±0.5
2.5	**0.5**	**0**	**0**	**56.3±0.3**	**38.3±0.3**	**70.0±0.5**
2.5	1	0	0.5	44.5±0.5	36.3±0.3	65.3±0.3
0	1	0.5	1	32.6±0.3	26.6±0.3	43.1±0.6
0	1	1	0	36.3±0.4	17.6±0.3	44.2±0.5
0	2	1.5	0	36.3±0.4	21.6±0.3	56.3±0.3
2.5	0	1	0.5	38.3±0.6	29.2±0.3	64.7±0.5
2.5	0	1.5	0.5	39.4±0.5	29.6±0.2	65.6±0.3
2.5	0	2	0	40.3±0.3	32.2±0.5	66.9±0.5
0	0	1.5	1	44.3±0.3	32.4±0.5	56.5±0.2
0	0	2	1.5	45.6±0.3	35.3±0.3	57.6±0.5
0	0	2	2	46.6±0.4	35.3±0.3	58.3±0.6

*Data represents mean of 20 replicates per treatment in three repeated experiments

4.5 Regeneration of callus cultures into shoots

Proliferating callus cultures or parts thereof were sub-cultured onto regeneration media containing MS salts supplemented with different concentration and combinations of BA, KN and IBA . Media containing BA and KN induced shoot primordial formation in the form of green nodular structures. No shoot primordia formation occurred in callus cultures derived from nodal segments on any of the test regeneration media. The MS+BA(1.5mg/l)+KN(2.5 mg/l) was found to be the best for regeneration with 76.7% and 72.2% calli differentiating into shoot primordia from calli derive from root segments and leaf discs, respectively . The same medium combination was found most suitable for obtaining primordial/callus and the highest number of shoot/callus from calli of both the explants(Fig3).To avoid browning of callus cultures and for increasing their efficiency of regeneration, the media was supplemented with activated charcoal 0.08%(w/v). The addition of activated charcoal in regeneration media not only reduced the browning of calli, but also enhanced their regeneration frequencies. The shoots regenerated from callus cultures were allowed to grow on regeneration medium for better growth and then all regenerated shoots were cultured in root induction medium as mentioned above for *in vitro* root induction.

Table 4.2: Effect of various media on shoot generation in calli- derived from leaf and root explants of *H.Perforatum*

Growth regulator(mg/l)			Days to shoot primordial initiation		Percent calli forming shoot primordial		Primordia/callus(no)		Shoot/callus(no.) (Mean±S.E)*	
BA	KN	IBA	Leaf disc	Root segments	Leaf disc	Root segments	Leaf disc	Root segments	Leaf disc	Root segments
0	0	0	0	0	0	0	0	0	0	0
0	1	0.5	15-20	15-18	13.0±0.6	14.0±0.6	3.0±0.5	3.0±0.5	3.0±0.5	3.6±0.3
0	1.5	1	15-21	14-18	22.2±0.6	24.9±0.7	3.3±0.5	3.6±0.6	3.3±0.3	4.6±0.3
0	2	1.5	15-20	14-18	34.4±0.6	35.6±0.6	4.0±0.3	4.3±0.7	3.6±0.6	5.0±0.5
0	2	2	16-20	15-20	36.6±0.7	40.5±0.5	4.3±0.3	4.3±0.8	4.0±0.5	5.3±0.3
0	2.5	2	16-20	15-18	40.5±0.5	42.4±0.3	5.0±0.5	5.2±0.2	4.6±0.3	4.6±0.6
0.5	0	1.5	16-21	15-18	22.2±0.6	29.7±0.6	5.3±0.5	5.3±0.3	4.0±0.0	4.7±0.3
1	0	2	15-22	14-18	46.6±0.7	50.0±0.5	3.3±0.2	4.0±0.5	4.3±0.3	4.7±0.3

1.5	0	2	15-22	14-18	51.2±0.2	53.7±0.6	4.3±0.4	4.3±0.4	4.6±0.5	5.3±0.4
1.5	0	2	15-20	14-17	59.4±0.4	64.2±0.4	4.6±0.5	4.6±0.5	6.3±0.3	5.0±0.5
1	2	0	11-17	10-15	64.2±0.4	64.8±0.5	5.3±0.6	5..6±0.6	6.3±0.3	6.0±0.6
1.5	2.5	0	11-18	10-14	66.4±0.6	70.5±0.5	5.6±0.7	5.6±0.6	6.6±0.5	6.3±0.3
1.5	**2.5**	**0**	**11-18**	**10-14**	**72.2±0.6**	**76.7±0.9**	**6.3±0.6**	**6.0±0.5**	**6.2±0.2**	**6.6±0.4**
1	2	1	11-17	10-15	62.4±0.4	64.2±0.2	5.3±0.3	5.3±0.3	6.3±0.3	6.3±0.3
1.5	2.5	1.5	11-17	10-15	63.0±0.3	64.8±0.6	5.3±0.3	5.4±0.6	6.3±0.3	6.3±0.3

Regeneration of plantlets and transfer to soil

The rooted shoots were gently removed from the culture vessels, washed under running tap water and transferred to pots containing sand: soil: vermiculite (1:1:1) in the greenhouse conditions for acclimatization and hardening. Different potting mixtures also were tested for increasing the survival percentage of these transferred plants. Initially the factors like temperature and relative humidity played a significant role for the survival of all *in vitro* grown plantlets the humidity kept very high and then we reduced it gradually.

4.6 Optimization of conditions for enriching hypericin in *Hypericum perforatum*

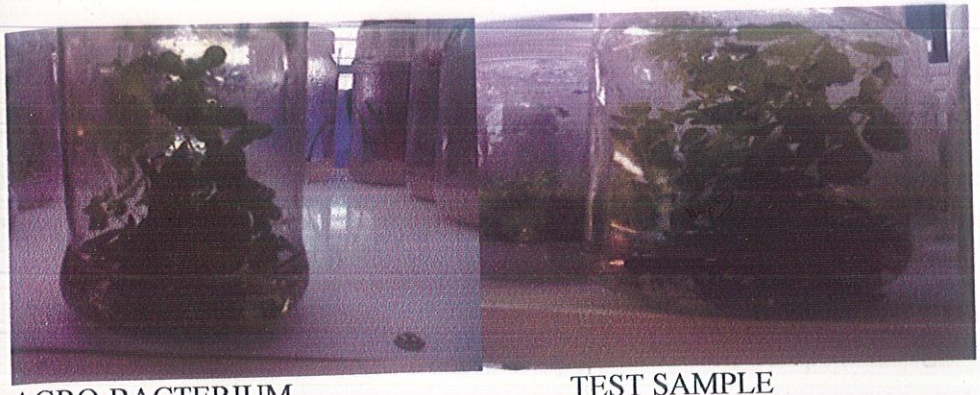
Effect of different incubation temperature on shoot biomass yield and Hypericin and Hyperforin biosynthesis and accumulation

During the early experiment on optimization of a suitable medium for micropropagation of *H.perforatum*, the cultures were incubated at 25±1^{0}C as reported previously in tissue cultures of H.perforatum as well as in other species. However, the shoot formed at 25±1^{0}C were weak were slow growing when compared to shoots that were grown at 15±1^{0}C. The shoot apices cultured on MS+KN(1.5mg/l)+IBA(0.5 mg/l) + sucrose 3%(w/v)+ agar-agar 0.8% (w/v) were incubated at two temperature regimes(15±1^{0}C, 25±1^{0}C) with 16hr/8hr light/dark cycle in order to see whether better shoot growth can be obtained . The shoot apices proliferated into multiple shoot within 5-6 days of culture at 2 test temperature with significant differences in various parameters of shoot growth and proliferation, shoot and root growth and total shoot biomass yield. The number of shoots per explants was not significantly different at 15±1^{0}C and 25±1^{0}C but significant differences for shoot morphology such as lead size and biomass yield were observed in shoot formed at 15±1^{0}C compared to 25±1^{0}C. However the shoots were sampled at regular interval of time period and stored at -80^{0}C for further analysis and estimation of bioactive ingredients.

For eliciting the amount of hypercin biotic elicitors were tried where *Agrobacterium rhizogenes* bacterial suspension was tried in different concentrations and methyl jasmonate . Increase in the amount was recorded in elicitation with methyl jasmonate whereas growth and development was not effected much but hypercin biosynthesis and accumulation elicited to good amount.

METHYL JASMONATE
(50 MICRO LITRE)

METHYL JASMONATE
(25 MICR0 LITRE)



AGRO-BACTERIUM TEST SAMPLE

Figure 4.4: *In vitro* grown plantlets in medium containing elicitors

Table 4.3: Status of *In vitro* grown plantlets in medium containing elicitors

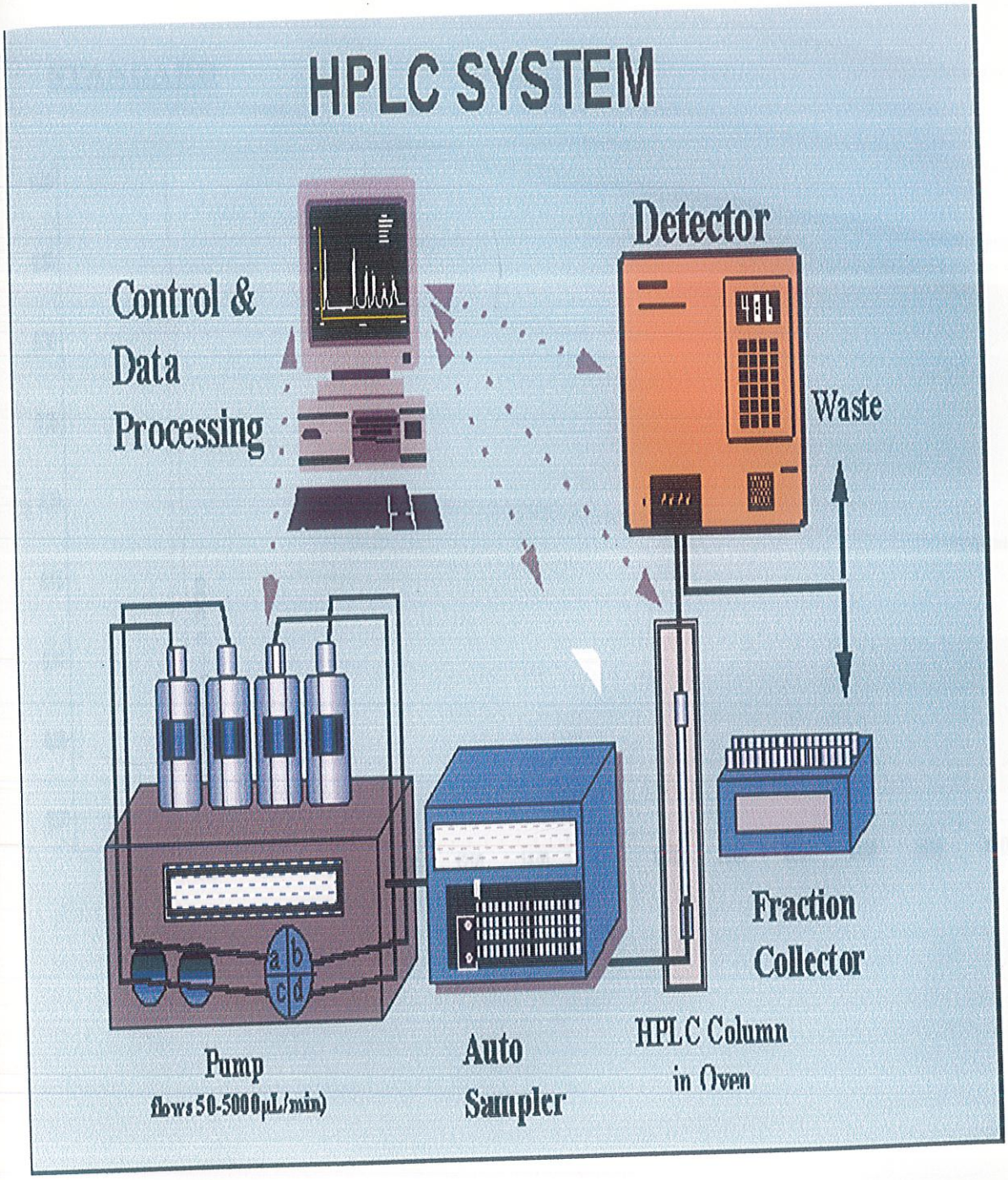
ELICITOR	CALLUS	SHOOT	ROOT	NO. OF ROOTS	NO. OF SHOOT
METHYL JASMONATE (25µL)	NO	YES	YES	30	20
METHYL JASMONATE (50 µ L)	NO	YES	YES	25	27
AGROBACTERIUM	NO	YES	YES	40	32
TEST	YES	YES	YES	75	50

4.7 ANALYSIS OF SECONDARY METABOLITE (HYPERCIN):

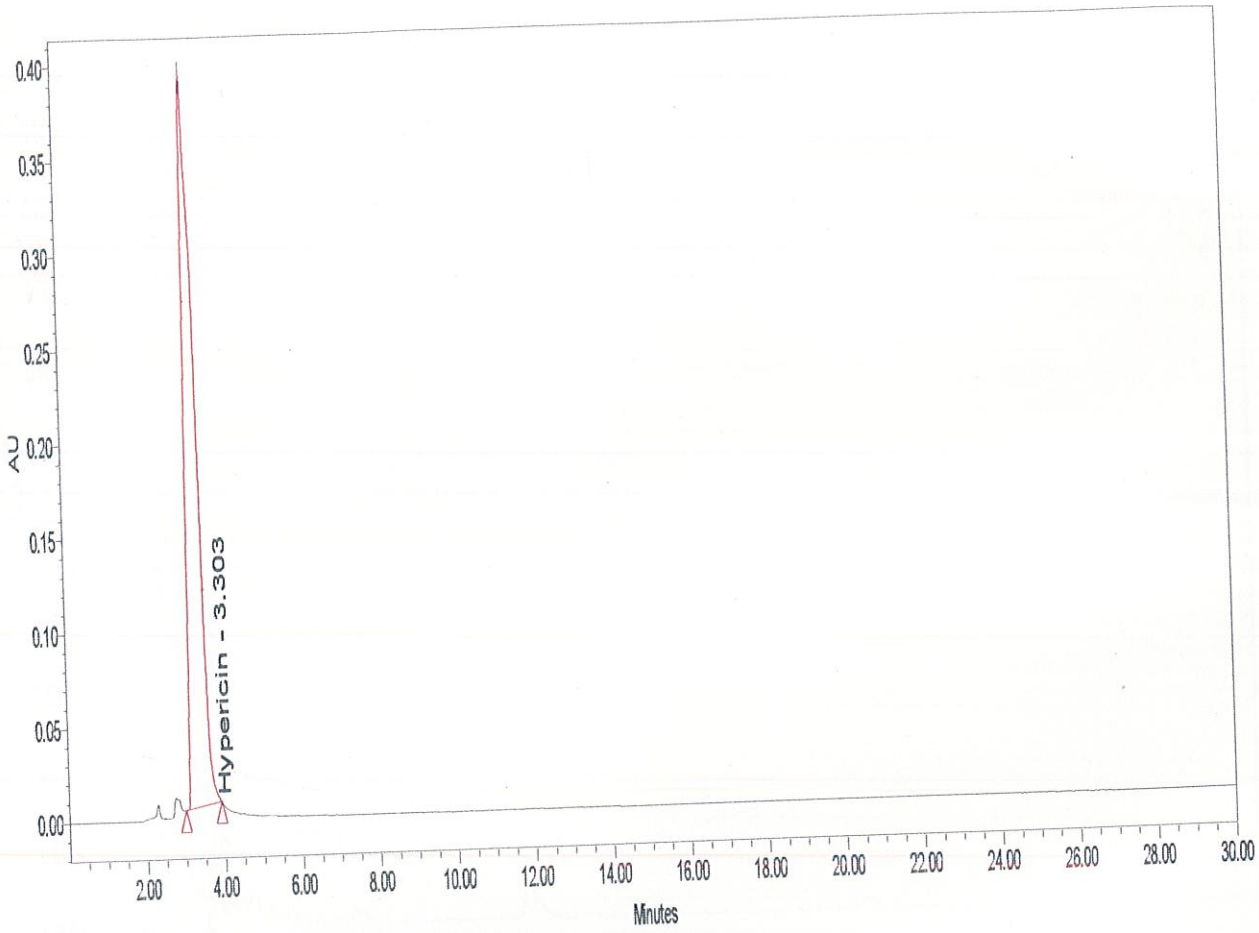
Different samples collected from test experiments were sampled at regular intervals and stored at -80^{0}C. Their analysis is carried out for hypercin biosynthesis and accumulation..Sensitive chromatographic methods were developed for the quantitative analysis of secondary metabolites in Hypericum perforatum (St. John's wort). Sample preparation methods were developed for plant, cell culture and biotransformation suspension matrixes. High performance liquid chromatography (HPLC) was used for the separation of analytes, and chromatographic data was acquired using photodiode array (PDA) detection or atmospheric pressure ionization mass spectrometry (API-MS).

High-performance liquid chromatography (sometimes referred to as high-pressure liquid chromatography), HPLC, is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying and purifying the individual components of the mixture. Some common examples are the separation and quantitation of performance enhancement drugs (e.g. steroids) in urine samples, or of vitamin D levels in serum.

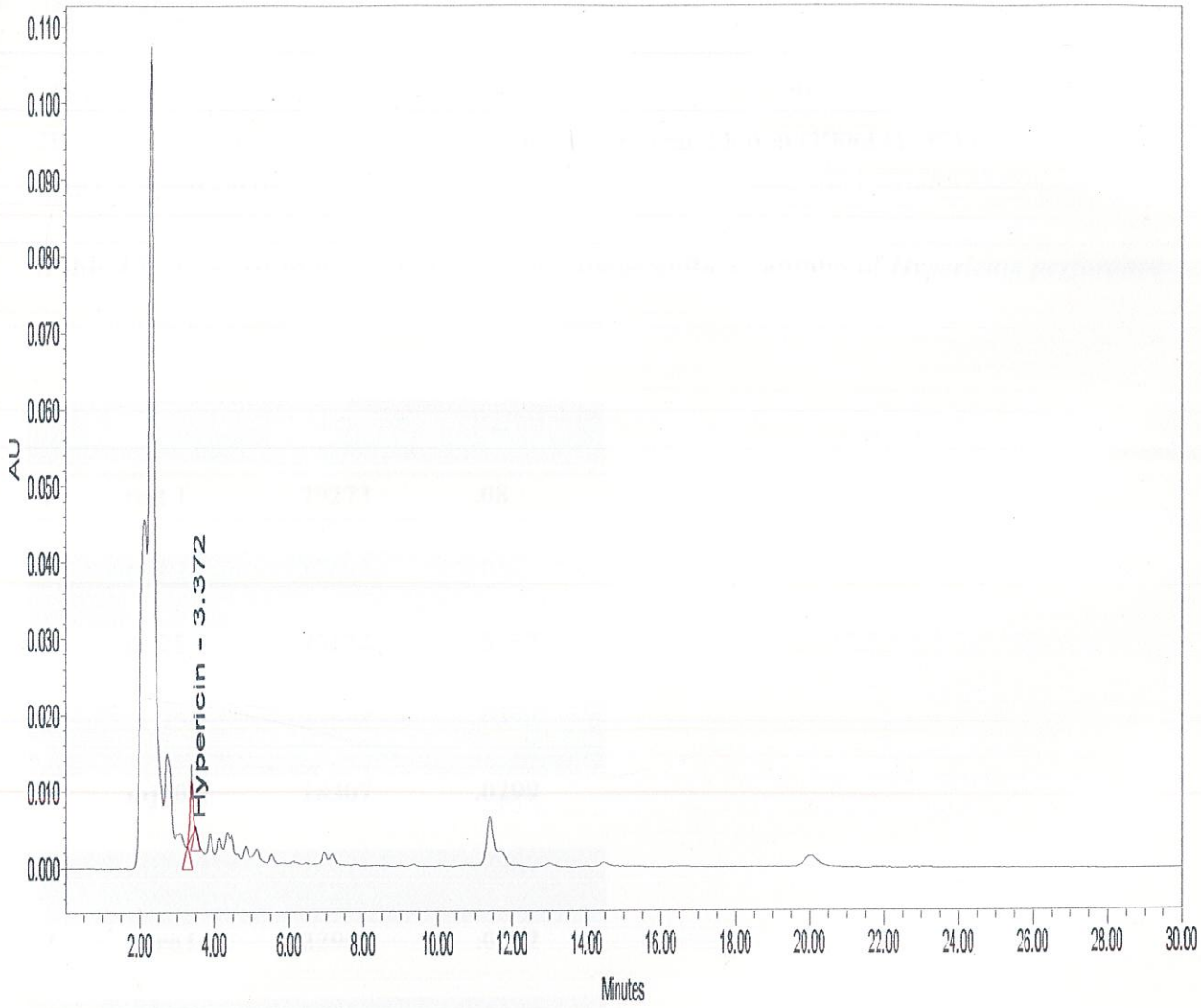
HPLC typically utilizes different types of stationary phases (i.e. sorbents) contained in columns, a pump that moves the mobile phase and sample components through the column, and a detector capable of providing characteristic retention times for the sample components and area counts reflecting the amount of each analyte passing through the detector.


HPLC SYSTEM
Control &
Data
Processing
Detector
Waste
Fraction
Collector
HPLC Column
in Oven
Auto
Sampler
Pump
flows 50-5000µL/min)
a
b
c
d

STANDARD


0.40
0.35
0.30
0.25
AU 0.20
0.15
0.10
0.05
0.00
Hypericin - 3.303
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
Minutes

SAMPLE


0.110
0.100
0.090
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
AU
Hypericin - 3.372
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00
Minutes

HPLC RESULT

As results obtained with given conditions for hypercin accumulation in tissue cultured samples of Hypericum perforatum. Standard (1/1) 1 mg in 1ml then dilution (100ul in 900ul of methanol) 3.303 peak -7553693

Table 4.5: Analysis of hypercin in different tissue culture samples of *Hypericum perforatum*

		Area	µg/ml
1	test 1	79273	.08
2	test 2	17694	.01
3	mj25 1	43174	.0457
4	mj25 2	22588	.0373
5	mj50 1	28307	.0299
6	mj50 2	144255	.2387
7	agro1	22904	.0242
8	agro 2	23826	.0252
9	nr 1	15330	.0162
10	nr 2	8821	.00934
11	shoot 1	35384	.0374
12	shoot 2	8816	.00933
13	callus1	20046	.0212
14	callus2	10269	.01087

CONCLUSION

In vitro propagation of *Hypericum perforatum* was carried out by taking shoot apices from the field grown plants and developed rapid micropropagation technique by optimizing cost effective medium (MS + Kn 1.5 mg/l +IBA 0.5 mg/l +sucrose 30 gm/l without agar) for maximum shoot proliferation and *in vitro* root induction was carried out by optimizing different concentrations and combinations of auxins.

Overall, MS medium supplemented either 2,4-D (2.mg/l):IBA(1mg/l) + table sugar 3% (w/v) + agar-agar (0.8%) was found to be the best for callus induction with frequencies of 70%, 56.3% and 38.3% from root segment, leaf discs and nodal segments, respectively (Table 4). Differences in callus morphology and appearance were observed among 3 explants with calli from leaf discs, root segments appearing as greenish, creamy white and light brown, respectively

The MS+BA(1.5mg/l)+KN(2.5 mg/l) was found to be the best for regeneration with 76.7% and 72.2% calli differentiating into shoot primordia from calli derive from root segments and leaf discs, respectively.

The number of shoots per explants was not significantly different at 15 ± 1^0C and 25 ± 1^0C but significant differences for shoot morphology such as lead size and biomass yield were observed in shoot formed at 15 ± 1^0C compared to 25 ± 1^0C.

The rooted shoots were gently removed from the culture vessels, washed under running tap water and transferred to pots containing sand: soil: vermiculite (1:1:1) in the greenhouse conditions for acclimatization and hardening

Elicitors Methyl jasmonate, agro bacterium didn't show good results as the growth of the plant Hypericum perforatum was restricted and number of leaves and growth was less than the test sample having just growth hormone.

But the secondary metabolite Hypericin was biosynthesized and accumulated in all tissue cultured plantlets even callus showed some amount of hypercin. Accumulation was seen in elicited shoots of Hypericum with methyl jasmonate and suspension of *Agrobacterium*. So this study is developed as an alternative technology for production of hypercin through cell culture and for their large scale production through elicitation.

REFERENCES

- Briskin DP & Gawienowski MC (2001) Differential effects of light and nitrogen on production of hypericins and leaf glands in Hypericum perforatum. Plant Physiol Biochem 39: 1075–1081.

- Brolis M, Gabetta B, Fuzzati N, Pace R, Panzeri F & Peterlongo F (1998) Identification by high-performance liquid chromatography–diode array detection–mass spectrometry and quantification by high-performance liquid chromatography–UV absorbance detection of active constituents of Hypericum perforatum. J Chromatogr A 825: 9–16.

- Butterweck V (2003) Mechanism of action of St John's wort in depression: what is known? CNS Drugs 17: 539–562.

- Bombardelli E & Morazzoni P (1995) Hypericum perforatum. Fitoterapia 66: 43–68.

- Couceiro MA, Afreen F, Zobayed SMA & Kozai T (2006) Variation in concentrations of major bioactive compounds of St. John's wort: Effects of harvesting time, temperature and germplasm. Plant Sci 170: 128–134.

- Čellárová E, Kimáková K, Daxnerová Z & Mártonfi P (1995) Hypericum perforatum (St. John's wort): in vitro culture and the production of hypericin and other secondary metabolites. In Bajaj YPS (ed) Biotechnology in Argiculture and Forestry 33: Medicinal and Aromatic Plants. Berlin, Springer-Verlag: 261–275.

- Draves AH, Walter SE (2003). Analysis of the hypericin and pseudohypericin content of commercially available St. John's Wort preparations. Can J ClinPharmacol. 2003; 10:114-118.

- Fabiane Raquel Pretto & Eliane Romanato Santarum,2010. Callus formation and plant regeneration from Hypericum perforatum leaves. 98: 025-810.

- Gadzovska S, Maury S, Delaunay A, Spasenoski M, Joseph C & Hagège D (2007) Jasmonic acid elicitation of Hypericum perforatum L. cell suspensions and effects on the production of phenylpropanoids and naphtodianthrones. Plant Cell Tiss Organ Cult 89: 1–13.

- Goel MK, Kukreja AK, Bisht NS (2009). In vitro manipulations in St. John's wort (Hypericum perforatum L.) for incessant and scale up micropropagation using adventitious roots in liquid medium and assessment of clonal fidelity using RAPD analysis. Plant Cell Tissue Organ Cult. 96: 1–9.

- Hostanska K, Reichling J, Bommer S, Weber M & Saller R (2003) Hyperforin a constituent of St John's wort (Hypericum perforatum L.) extract induces apoptosis by triggering activation of caspases and with hypericin synergistically exerts cytotoxicity towards human malignant cell lines. Eur J Pharm Biopharm 56: 121–132.

- Kamuhabwa AR, Agostinis P, Kasran A, De Witte PA (2000). Photodynamic Activity of Hypericin in Human Urinary Bladder Carcinoma Cell. Anticancer Res., 20: 2579–2584.

- Karppinen K, György Z, Tolonen A, Neubauer P, Jalonen J & Hohtola A (2006c) Comparison of various culture systems on growth and accumulation of hypericins in Hypericum perforatum shoot cultures. In Sorvari S & Toldi O (eds) Proceedings of the Second International Conference on Bioreactor Technology in Cell, Tissue Culture and Biomedical Applications. Turku, Karhukopio Oy: 96–101.

- Karppinen K, György Z, Kauppinen M, Tolonen A, Jalonen J, Neubauer P, Hohtola A & Häggman H (2006a) In vitro propagation of Hypericum perforatum L. and accumulation of hypericins, pseudohypericins and phloroglucinols. Propag Ornam Plants 6: 170–179.

- Kalaitzis JA & Moore BS (2004) Heterologous biosynthesis of truncated hexaketides derived from the actinorhodin polyketide synthase. J Nat Prod 67: 1419–1422. Karioti A & Bilia AR (2010) Hypericins as potential leads for new therapeutics. Int J Mol Sci 11: 562–594.

- Liu X-N, Zhang X-Q & Sun J-S (2007b) Effects of cytokinins and elicitors on the production of hypericins and hyperforin metabolites in Hypericum sampsonii and Hypericum perforatum. Plant Growth Regul 53: 207–214.

- Liu X-N, Zhang X-Q, Zhang S-X & Sun J-S (2007c) Regulation of metabolite production by precursors and elicitors in liquid cultures of Hypericum perforatum. Plant Cell Tiss Org Cult 91: 1–7.

- Patočka J (2003) The chemistry, pharmacology, and toxicology of the biologically active constituents of the herb Hypericum perforatum L. J Appl Biomed 1: 61–70.

- Pasqua G, Avato P, Monacelli B, Santamaria AR & Argentieri MP (2003) Metabolites in cell suspension cultures, calli, and in vitro regenerated organs of Hypericum perforatum cv. Topas. Plant Sci 165: 977–982.

- Pretto FR, Santarem ER (2000). Callus formation and plant regeneration from Hypericum perforatum L. leaves. Plant Cell Tissue and Organ Cult. 67: 107–113.

- Pavlíc M, Vacek J, Klejdus B & Kubáň V (2007) Hypericin and hyperforin production in St. John's wort in vitro culture: influence of saccharose, polyethylene glycol, methyl jasmonate, and Agrobacterium tumefaciens. J. Agric Food Chem 55: 6147–6153.

- Sirvent T & Gibson D (2002) Induction of hypericins and hyperforin in Hypericum perforatum L. in response to biotic and chemical elicitors. Physiol Mol Plant Pathol 60: 311–320.

- Santarem ER, Astarita LV (2003). Multiple shoot formation in Hypericum perforatum L. And hypericin production. Braz. J. Plant Physiol., 15 (1): 43–47.

- Wojcik A, Podstolski A (2007). Leaf explants response in in vitro culture of St. John's wort (Hypericum perforatum L.). Acta Physiol. Plant, 29: 151-156.

- Zobayed SMA, Afreen F & Kozai T (2007) Phytochemical and physiological changes in the leaves of St. John's wort plants under a water stress condition. Environ Exp Bot 59: 109–116.

- Zobayed SMA, Afreen F, Goto E & Kozai T (2006) Plant–environment interactions: accumulation of hypericin in dark glands of Hypericum perforatum. Ann Bot 98: 793–804.

- Zobayed SMA, Afreen F, Goto E & Kozai T (2006) Plant–environment interactions: accumulation of hypericin in dark glands of Hypericum perforatum. Ann Bot 98: 793–804.

- Zdunek K & Alfermann W (1992) Initiation of shoot organ cultures of Hypericum perforatum and formation of hypericin derivatives. Planta Med 58: 621–625.

- Zanoli P. Role of hyperforin in the pharmacological activities of St. John's wort. CNS Drug Rev. 2004; 10:203-218.

- Zobayed et al. (2005)