Biotic elicitors used to enhance plumbagin production in *Plumbago zeylanica* and assessment of antioxidant and antibacterial activity

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Submitted by

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CERTIFICATE

This is to certify that the dissertation entitled "**Biotic elicitors used to enhance plumbagin production in *Plumbago zeylanica* and assessment of antioxidant and antibacterial activity",** submitted by **Anshika Rastogi (2K18/IBT/02)** in the partial fulfilment of the requirements for the reward of the degree of Master of Technology, Delhi Technological University(Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under my guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

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DECLARATION

This is to certify that the thesis of Major Project II entitled "**Biotic elicitors used to enhance plumbagin production in *Plumbago zeylanica* and assessment of antioxidant and antibacterial activity,***"* Submitted by Anshika Rastogi (2KI8/IBT/02) in the partial fulfilment of the requirements for the reward of the degree of Mater of Technology, Delhi Technological University (Formerly Delhi college of Engineering, University of Delhi), is an authentic record of the my own work carried out under the guidance of my project supervisor **Dr. Navneeta Bharadvaja,** Assistant Professor, Department of Biotechnology, DTU. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

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ABSTRACT

Plumbago zeylanica (chitraka) is the most prominent herbal plant which is used as a medication to cure skin problems and infections such as ringworm, dermatitis, sores, acne and scabies. Traditionally all parts of *Plumbago zeylanica* have been utilized as a medication against many diseases. In Ayurveda chitraka is described as tumour- negating, appetizer, anti-anorexic and pain-reliever due to the presence of several important bioactive compounds such as alkaloid, tannins, phenols and naphthoquinones like plumbagin which is the most active constituent of this herb. Due to extensive use of this plant as a potent medicinal herb, micropropagation is necessary for higher biomass and plumbagin production. Since resistance to antibiotic against several microbes has become a critical issue in the whole world. So, to conquer this difficulty, identification and discovery new herbal drugs are necessary. In the current study, three elicitors namely yeast extract, malt extract and chitosan were used to enhance the plumbagin synthesis in *Plumbago zeylanica* as well as analysis of its antioxidant and antibacterial activity. Shoot culture of *Plumbago zeylanica* was performed in MS media which was supplemented with 200µl BAP and 150mg/l elicitors and then incubated for several weeks. Application of three biotic elicitors enhance the plumbagin production significantly. Phytochemical analysis of compound was carried out by UV-visible analysis which exhibited the presence of total phenol and tannin. DPPH free radical scavenging activity of all accession was performed to check their antioxidant potential. Cultures were also analysed for their antibacterial potential against *E,coli* and *S.aureus*. Results were expected that application of elicitors in *Plumbago zeylanica* shoot culture enhance the plumbagin production as well as other bioactive compounds.

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LIST OF ABBREVIATIONS

NBPGR - National Bureau of Plant Genetic Resources

BAP- 6-Benzylaminopurine

°C- degree Celsius

µg – microgram

mg- milligram

ml- millilitre

µL- microlitre

YE- yeast extract

ME- malt extract

HPLC- high performance liquid chromatography

DPPH - 2,2-diphenyl-1-picrylhydrazyl

FC- folin ciocalteu

PTC- plant tissue culture

INTRODUCTION

Medicinal plants played a very crucial role and are a source of many drug lead compounds. The extended use of medicinal plants in herbal medicines and healthcare is illustrated in Bible and Vedas. In present medicinal plant are used by pharma industries for the preparation of pharmaceutically important compounds. Active compounds which are produced as a result of secondary metabolism are used worldwide as a medication for treating several ailments. Medicinal plants are the "backbone" of herbal medicines which implies that more than 3.3 billion population in less developed countries depends on medicinal herbs (Singh R et al.,2015). Over the past 20 years around 28% of newly launched compounds into the market are considered as natural products (Ahmad et al.,1993). Around 80% population of developing countries depends on medicinal plants for herbal products or uses it as traditional medicines (Gupta et al.,2012).

India is known to be a great reservoir of medicinal herbs as the forests contains large number of aromatic and medicinal plants which are utilized as raw materials for the preparation of phytomedicines. In India around 8000 herbal medicines have been classified in AYUSH. Traditional medicines based on systems includes Ayurveda, Siddha, Unani, folk and among these systems, Unani and Ayurveda medicines are most developed and used in India.

Herbal medicines have many advantages over other chemotherapeutic drugs as herbal drugs are simple and effective in nature which also offer large range activity and have minimal side effects as compared to other synthetic drugs (Ahmad et al.,1993)

Medicinal plants are important due to the presence of various bioactive compounds known as secondary metabolites. These metabolites include alkaloids, flavonoids, terpenoids, tannins, phenols, saponin which are used in drug development. These compounds help in the growth of plant and maintain homeostasis. They are produced by plant directly or indirectly in response to various environmental stimuli.

For in vitro large scale production of secondary metabolites, plant tissue culture technology can be done under aseptic condition from explants such as stems, leaves, meristems and root (Thirumurugan.,et al 2018). Conventional method of whole plant cultivation for the production of secondary metabolite is very time taking and provides less yield. So as to overcome these problems adventitious root, shoot, cell suspension and hairy root culture are the alternative approach for the production of metabolite of interest.

There are more than 2,140,000 metabolites have been identified and are classified into five classes based on their function, structure and biosynthesis namely polyketides and fatty acid derived substances, non- ribosomal polypeptides, terpenoids, steroids, alkaloids and enzyme cofactors (Thirumurugan., et al 2018).

In vitro production of phytocompounds through cell culture can be increased by treatment of plant with elicitors and the process known as elicitation.

1.1 *Plumbago zeylanica*

Plumbago zeylanica is a indigenous pharmaceutically active which is a member of family plumbaginaceae and is commonly known as "Chitrak"or "White leadwort". It is an important perennial herb found in West Bengal, Uttar Pradesh, Maharastra and some part of South India. It is one of the important medicinal herbs because it contains many phytocompounds such as flavonoids, alkaloids, phenols, saponin, nepthoquinone, terpenes etc and is reported in Ayurveda for the treatment of various disorders.

The most important naphthoquinone present in the roots of *Plumbago zeylanica* is known as Plumbagin(5,7-dihydroxy-8-methoxy-2-methyl-1,4-napthoquinone) and is considered as the important phyotocompound of this plant which constitutes about 1% of whole plant (Pant et al.,2012). It is a yellow colour compound which is insoluble in water but is soluble many organic solvents. *Plumbago zeylanica* has many medicinal characterstics like anti-tumor, anti-fungal, anti-diabetic, anti-oxidant, anti-malarial, anti-fertility, anti-bacterial, anti-inflammatory, anti-cancer activity etc (Roy et al.,2017).

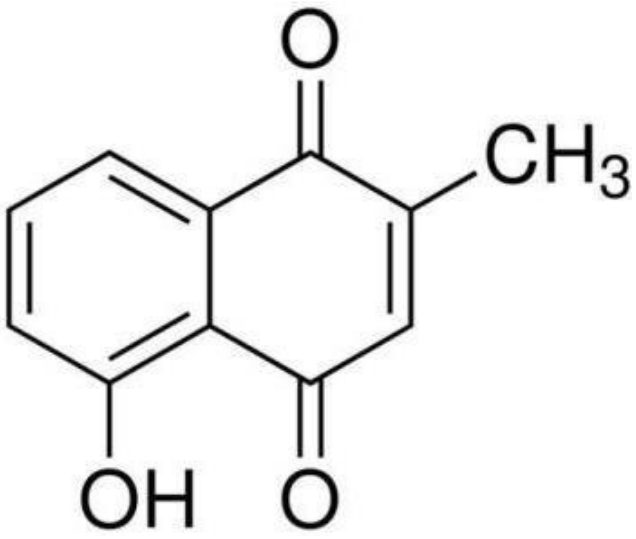

Figure 1: Structure of plumbagin (Roy et al.,2017)

Conventionally, all parts of *Plumbago zeylanica* have been used in the medicinal preparation for curing many diseases. Leaves, root and bark of this plant is used for the treatment of syphilis, tuberculosis, airthritis etc. Powdered roots of this plant mixed with water and vinegar is used to cure influenza (Pant et al.,2012).

Various micro elements (Mn, Zn, Cr, Fe and Co) and macro elements (Ca, Na, Mg and k) are present in the roots, stem and leaves of *plumbago zeylanica* in good amount and are determined

by ICP-AES (Inductively coupled plasma atomic emission spectrometry) (Sharma et al.,2015). Usually every part of this plant can be used for medicinal purpose but roots are considered as the most active part for the preparation of phytomedicine.

1.2 Micropropagation

Micropropagation is the method of producing multiple progeny of plant by using plant tissue culture techniques. It is an alternative technique for those plants which shows objection to traditional bulk propagation. This method produces large number of disease-free plants in less time period. Endangered species can also be conserved by this technique of plant tissue culture.

This study involves the use of different accessions of *plumbago zeylanica* and the motive of using these accessions is to select the best one for the production of secondary compounds. Different accessions denote the diversity between the plant population. Accession number is a unique number given to plant population which acts as identifier and used to maintain information in database. It involves change in the morphology of the plant such as flowering, leaf growth, frequency of stomata etc.

1.3 Phytochemical screening

Phytocompounds are the medicinally active constituents produced by the plants in stress condition by primary and secondary metabolism. These active constituents have biological activity and acts as defence system of the plant against various diseases. The process of extraction and identification of these phytocompounds is known as phytochemical screening. There are various phytocompounds present in *Plumbago zeylanica* such as steroids, phenols, alkaloids, flavonoids quinones and tannins (Rajakrishnan et al.,2017). Among all these the most important compound present in this plant is napthoquinone (plumbagin).

Currently there are 120 medicinally active compounds which are used in modern medicine and among these 80 % exhibits positive relationship between traditional use and modern therapeutic (Shrestha et al.,2015). The research on phytochemicals produced by the plant is very important for defining the bioactive profile of the plant of medicinal importance.

1.4 Elicitation

Elicitors are the compounds which triggers the stress response in plants tissues to increase the production of secondary metabolites and this process of enhancing the yield is known as elicitation. Not only elicitors can be used to enhance the production of bioactive compounds but also play a significant role in biosynthetic pathway to increase the yield of commercially useful elements (Patel et al.,2013). Various biotechnological approaches have been done to increase secondary metabolite yield but out of all, elicitation is considered as the best method for increasing phytocompounds production (Poornananda et al.,2016).

Elicitors are classified into two divisions based on their nature i.e, biotic and abiotic. In this study three biotic elicitors namely yeast extract, malt extract and chitosan are added to the

culture to elicit the yield of plumbagin in *Plumbago zeylanica.* It is very necessary to decide the correct amount of elicitor for the elicitation process as its high dose produces the hypersensitive response in plant which ultimately leads to death of cell (Poornananda et al.,2016).

- ## Biotic elicitors

The compounds which emerges either from host or pathogen and can produce defence response in plants are known as biotic elicitors. Many biological preparations which are complex in nature are more oftenly used as elicitors. For example, yeast extract.

Recently, number of elicitors are increasing very fast which includes oligosaccharides, glycoproteins, polysaccharides and their molecular structure has also been carefully explained.

Proteins- Biotic elicitors are divided into several other classes namely enzymes and proteins that activates plant defence system. A research indicates the enhanced production of phytoalexins in cell cultures of *N.tabacum.*

Oligosaccharides- According to the early research, it has been concluded that carbohydrate is a great source of overproducing the active compounds in plant culture.

Glycoproteins- These are the compounds which also enhances the phytoalexin production in plants. According to the research, glycoprotein which was prepared from baker's yeast was used to increase the synthesis of benzophenantridine alkaloids in *Eschscholzia californica* cell culture**.**

Fungal origin- The most important elicitors which activates the defence system of plants are pathogenic produced biotic elicitors. The most efficient way to activate the biosynthetic pathway of phenypropanoid is the use of fungal preparation which can be either pathogenic or non-pathogenic. In *Catharanthus roseus* suspension culture, it was observed that the cell wall of fungi enhanced the yield of ajmalicine, catharanthine and serpentine.

Yeast origin- Yeast extract is utilized by many scientists as a form of important biotic elicitor for past ten years. It triggers the synthesis of ethylene in tomato and also provide resistance to bacteria in *Phaseolus vulgaris.*

1.5 Objectives of the study

In Ayurveda, *Plumbago zeylanica* is considered as the most vital medicinal plant which is utilized for curing several disorders. Roots of *Plumbago zeylanica* is considered as the most active part because of the presence of plumbagin. Intensive use of this plant causes its exploitation and due to this it has become endangered (Tyagi et al.,2017). Moreover, natural environment is also affected by certain parameters like habitat destruction, suburbanization and development which ultimately causes exploitation to wild plant species. So, to conserve these endangered plants species, micropropagation play a major role in production of these plant species and is also useful in its germplasm conservation.

The objective of this study includes:

1. Shoot culture of *Plumbago zeylanica*.
2. Quantitative estimation of phytochemicals like tannins and phenols.
3. Quantitative estimation of plumbagin.
4. Evaluation of antioxidant potential of plant by DPPH method.
5. Assessment of antibacterial activity of plant.

REVIEW OF LITERATURE

2.1 Chemical composition

Plumbago zeylanica contains several phytocompounds. Different part of the plant contains different secondary compounds like alkaloids, saponin, steroids, flavonoids, tannins, glycosides, coumarins, tri-terpenoids, carbohydrates, phenolic compounds and napthoquinones (Plumbagin, 3-biplumbvagin, elliptone, chitranone, chloroplumbagin) (Min et al.,2011).

Like leaves of this plant contains chitranone and plumbagin (Ravikumar et al.,2011), Stem contains plumbagin, stigmasterol, zeylanone and sitosterol (Kumar et al.,2009), Fruit contains glucopyranoside. Plumbagin and sitosterol (Dhale et al.,2011), Flowers contains glucose, zeylanone and plumbagin (Subhash et al.,2013).

Napthoquinone

Napthoquinones are commonly known bioactive compounds which are synthesized through various pathways. The commonly known pathways for napthoquinone production are shikimic acid and acetate-malonate pathway but in higher plants plumbagin is synthesized via acetate-malonate pathway (Mallavadhani et al.,1998; Durand and Zenk, 1971).

Plumbagin

Plumbagin is a yellow coloured cryatalline medicinally active compound synthesize from the roots of *Plumbago zeylanica* through Soxhlet apparatus and further accompanied by silica gel column chromatography (Kishore et al.,2009).

Plumbagin shows various properties such as antibacterial (De paiva et al.,2003), antifungal (Mohana et al.,1980), antioxidant (Hsu et al.,2006), antimalarial (Likhitwitayawuid et al.,1997), cardioprotective (Itoigawa et al.,1990), anti-HIV activity (Min et al.,2002) and also enhances phagocytosis in human WBCs (Wagner et al.,1988).

A study reported the use of plumbagin in the synthesis of ROS (reactive oxygen species) like superoxide and hydrogen peroxide (Kawiak et al.,2007).

2.2 Pharmacological Activities

Plumbago zeylanica, a medicinally active plant shows various pharmacological activities. Anti-inflammatory, Wound healing , Anti-bacterial, Memory enhancing, Anti-diabetic, Larvicidal, Anti-cancer, Antioxidant activity etc.

- **Anti-inflammatory activity**

  300 and 500 mg/kg root extract of *Plumbago zeylanica* in methanol produces 31.03 and 60.3% inhibitory effects against acute inflammation (Jain et al.,2003).
  An in vivo experiment was done on rats to check the anti- inflammatory activity of different leaf extracts of *Plumbago zeylanica* and it was reported that acetone extract of leaves reduces the inflammation in rats which were induced with carrageenan (Sheeja et al.,2010).
  According to a comparative study on three medicinal plants, it was observed that *Plumbago zeylanica* reduces the oedema in comparison to *Phyllanthus emblica* and *Cyperus rotundus*(Dang et al.,2011).

- **Wound healing activity**

  A study was done on wistar rats to check the wound healing activity of *Plumbago zeylanica* root extracts and it was observed that wound healing activity of this plant is due to various bioactive compounds like flavonoids, terpenoids, saponins, alkaloids which are present in the plant (Jyothi et al.,2013).
  The antioxidant nature of *Plumbago zeylanica* accelerates the wound healing process by controlling wound oxidative stress (Honnegowda et al.,2015).
  Another study investigated wound healing activity in rats and reported that methanolic extracts of *Plumbago zeylanica* roots showed significant activity (Reddy et al.,2001)

- **Anti-bacterial activity**

  Anti- bacterial activity of 82 plants were investigated and among all only ethanolic extract of certain plants like *Plumbago zeylanica, Terminalia chebula and Emblica officinalis* showed potent anti-bacterial activity (Ahmad et al.,1998).
  Chloroform extracts of roots of *P.zeylanica* was evaluated for the anti-bacterial activity against some bacterias and it was observed that maximum zone of inhibition was shown against *Salmonella typhi, Escherichia coli and Staphylococcus aureus* (Jeyachandran et al.,2008).
  A study reported that maximum anti-bacterial activity of *Plumbago zeylanica* was shown in ethanolic extract against *Bacillus subtillis, Salmonella typhi, Staphylococcus aureus and Pseudomonas aeruginosa* whereas moderate activity was shown in chloroform and acetone extract (Banik et al.,2014).

- **Memory enhancing activity**

  An experiment was performed on mice to check the effect of *Plumbago zeylanica* and the mice were subjected to scopolamine which induces amnesia. It was observed that 200 mg/kg chloroformic extract of this herb showed promising memory enhancing effect by reducing the amnesia (Mittal et al.,2010).

- **Anti-diabetic activity**

  A research was done on diabetic rats to check the effect of *Plumbago zeylanica*. It was observed that when 100mg of ethanolic extract along with 200mg/kg tolbutamide was given orally to diabetic rats which were treated with streptozotocin caused decline in glucose-6-phosphate activity and simultaneously increases the hexokinase activity (Zarmouh et al.,2010).
  Another study reported that in diabetic rats GLUT4 mRNA expression and protein is enhanced by plumbagin which indicates the translocation of GLUT4 and also contribute to glucose homeostasis (Sunil et al.,2012).
  Plumbago zeylanica and haridra powder when given to obese patient in the form of capsule, it was observed that *Plumbago zeylanica* helps in significant weight loss (Kotecha et al.,2007).

- **Anti-cancer activity**

  It was reported that the medicinally active compound plumbagin found in *Plumbago zeylanica* showed anti-cancer activity by inhibiting the proliferation of cell and blocks the cell cycle which ultimately leads to apoptosis (Zhao et al.,2006).
  Extracts of Plumbago zeylanica was evaluated for their anticancer activity against Ehrlich Ascites Carcinoma and it was observed that ethanolic extracts acquires powerful anticancer activity, thus causing reduction in the lipid peroxidation level (Hiradeve et al.,2010).
  Plumbagin inhibits the apoptosis in human gastric cancer cells that may be due to its ability to supress the STAT3 and Akt phosphorylation (Li et al.,2007).

- **Central nervous system activity**

  A study was done to evaluate the CNS activity of *P.zeylanica* leaf extracts and the result states that the leaf extracts possess powerful CNS depressant activity along with the properties of muscle relaxation (Vishnukanta et al.,2009).
  Leaf extracts of *P.zeylanica* were also evaluated for anti-convulsant activity and it was observed that Plumbago zeylanica did not acquire this activity (Vishnukanta et al.,2010).
  A study was performed on rats to check the effect of *P.zeylanica* root extracts on central dopaminergic activity and locomotor behaviour. It was reported that the root extract precisely enhanced the spontaneous ambulatory activity (Bopaiah et al.,2001).

- **Anti-oxidant activity**

  A study was conducted to study the anti- oxidant activity of different root extracts and plumbagin. Three methods were used to check the anti-oxidant activity these are- 1,1diphenyl-2-picryl hydrazyl (DPPH), Ferric reducing/antioxidant power (FRAP) and 2,2'-azobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS).
  Many studies reported that extracts of *Plumbago zeylanica* and its secondary metabolite plumbagin show anti-oxidant potential and also have therapeutic effect (Tilak et al.,2004).
  In another study, antioxidant activity and phenolic content of methanolic extracts of various plants were carried out such as *A.calamus, H.antidysenterica, P.zeylanica* and *H.indicus*. It was observed that antioxidant activity was highest in root extract if *P.zeylanica* (Zahin et al 2009).

- **Blood Coagulation activity**

  A study was conducted in albino rats to check the effects of *Plumbago zeylanica* extracts and napthoquinone.
  Individual groups were given *Plumbago zeylanica* extracts and napthoquinone and were checked for their effect on clotting time, platelet count, bleeding time and platelet adhesion.
  The result shows that platelet adhesion was decreased in those rats which were given *Plumbago zeylanica* extracts and napthoquinone (Vijayakumar et al., 2006).

- **Lipid metabolism activity**

  Plumbagin, an active constituent of *Plumbago zeylanica* was found to reduce the serum cholesterol when given to hyperlipidaemic rabbits. Reduction in LDL level was also observed (Alpana et al.,1996)

- **Larvicidal activity**

  A study showed that hexane and chloroform extracts of *Plumbago zeylanica* exhibit highest larvicidal activity as compared to various other species of Plumbago (Maniafu et al., 2009).
  Another study revealed the larvicidal activity of methanolic extracts of *Plumbago zeylanica* against Aedes aegypti and Anopheles stephensi (Patil et al., 2010).

- **Antifertility activity**

  Some workers prepared the complex of plumbagin with hydroxyl propyl betacydodextron tom enhance its solubility. This complex was screened for its antifertilty activity when entrapped in the lipid layer of niosomes (D'souza et al.,1997). According to a research highest anti-implantation activity was seen in hydroalcoholic extract of Plumbago zeylanica due to its anti-estrogenic activity which causes changes in uterus (Vishnukanta et al.,2010**).**
  It was also observed that *Plumbago zeylanica* interrupts the estrous cycle which results in temporary delay in ovulation (Edwin et al., 2009).

2.3 Importance of plant tissue culture

Recently, a great attention has been given to in-vitro techniques which provides rapid propagation of plant species and also helpful in the conservation of plants which are extinct in nature (Sahoo and Chand, 1998 and Prakash et al., 1999). Conventional method of plant propagation is very challenging and also unsatisfactory to fulfil the increasing demand of bioactive compounds because of poor seed germination and its death (Anonymous 1989).

Micropropagation is enforced on various species of medicinal plants but it is still costly as it requires manual handling throughout the process. Therefore, it is crucial to establish the proper technique to propagate *Plumbago zeylanica* for the identification of superior clones and their dispersion.

All part of the plants can be used as an explant but it was observed that in most cases nodal part is used as an explant for micropropagation. Nodal explants of *Hypericum perforatum* was utilized for the production of Hypericin (Santarem and Astarita,2003), Sreeranjini et al.,2014 reported the propagation of *Morinda citrifolia L*.nodes in MS media. Nodal explant of *Plumbago zeylanica* is used in MS media for plumbagin production (Verma et al.,2002). Fattahi et al.,2013 reported the use of stem and leaves explant of Dracocephalum *kotschyi*.

A productive method has been evolved for the micropropagation of *Plumbago zeylanica* by using nodal culture (Rout et al.,1999). Result shows the induction of various shoots from nodal explants which were cultured on MS media.

Rout (2002) cultured leaves of *Plumbago zeylanica* and *Plumbago rosea* on MS medium for in-vitro regeneration of plants.

Chaplot et al., (2006) used axillary bud proliferation and organogenesis method to propagate *Plumbago zeyalnica*. After 2 weeks various shoots were induced from nodal explants.

2.4 Plumbagin content

0.91% of plumbagin present in the roots of plumbago species found in India (CSIR,1969). However, the plumbagin content changes with age, season, locality and condition of the soil. It was found that old and dry soil helps in higher production of active components in the roots. A study showed that fresh roots produces higher amount of plumbagin as compared to old roots.

It was reported that greater plumbagin content was found in *Plumbago rosea* which is approximately 0.69-1.4% when compared to *Plumbago zeylanica* i.e.,0.19-0.33% (Menon et al.,1999). The level of plumbagin changes consequently at different growth stages in *Plumbago rosea.*

Plumbago zeylanica contains remarkably low plumbagin content when compared to various ecospecies of *Plumbago rosea* (Arya et al.,1999).

2.5 In-vitro production of secondary metabolite

Gopinath et al. (2009) performed a very easy and quick experiment using RP-HPLC to quantify the amount of plumbagin and embelin. It was observed that when acetonitrile was used as mobile phase, it gives the maximum resolution at pH 3.5. Both quantitative and qualitative result are then described which helps in the formation of herbal medicine.

Pawar et al. (2010) established a very easy and effective procedure for the assessment of plumbagin in various root extracts of *P.zeylanica* by using HPTLC.

Panichayupakaranant and Tiwarkul (2002) demonstrated the root culture of *P. rosea* on solid Gamborg's B_5 medium having 0.1-0.5, g/l kinetin and 0.5-2.0 mg/l NAA. The plumbagin content was estimated by TLC-densitometry. The result showed the highest plumbagin production was found in the root culture having 0.1mg/l kinetin and 1mg/l NAA.

Karuppusamy (2002) perfomed a study to check the effect of immobilization on the production of plumbagin. Immobilization of *P.rosea* cell cultures was done on calcium alginate and then cultured in MS medium having $CaCl_2$. It was observed that plumbagin production was increased by one, two and three folds when immobilized in calcium alginate as compared to control.

2.6 Factors affecting in-vitro cultures

For accomplishing the objectives of in vitro cultures, it is very important to have a systematic culture system. There are various factors that have impact on plant tissue culture are -plant genotype, medium composition, growing conditions, culture environment.

- **Genotypic differences**

There is a huge proof that proves the existence of variation among different genotypes of plants in culture systems. If genotypic alterations arise at the beginning of callus culture, type of callus & its growth rate shows genetic manipulation. Variation in regenerative capacity of plant is also observed in plant kingdom. For example, dicotyledons have better tendency of regeneration than monocotyledons.

- **Medium composition**

The most important factor that helps to regenerate a plant is the composition of nutrient media. Murashige and Skoog developed a formulation in 1962 which is used as a most common composition in tissue culture technology. In vitro morphogenesis route is determined by altering the amount and type of plant growth regulators. Normally, higher amount of auxin supplemented media induces callus and if cytokinin is added with auxin, it may lead to the promotion of callus. Direct shoot production from cultured explant depends on the auxin to cytokinin ratio.

There are several other constituents of the media that have a impact on developmental pathway of the culture. The culture media has two main functions. Firstly, to provide nutrients to explant that are essential for its growth and development.

Second is the hormonal control that directs the explant growth and its development. It is applied by plant growth regulator and depends on the type and amount of hormone which have been used. According to the study, it is observed that media composition do not have a significant role in in vitro as compared to the type and level of hormones. Apart from plant hormones, there are various other parameter that have impact on in vitro culture systems such as carbon and nitrogen source and pH of culture media.

- **Culture conditions**

Culture environment play a very important role in plant growth and its development. Each plant grows differently in the environment in which they are subjected to. Various environmental factors such as light duration, oxygen, carbon dioxide, temperature and humidity play a major role in plant tissue culture.

MATERIALS AND METHOD

3.1 Plant material

Various accessions of *Plumbago zeylanica* were collected from National Bureau of Plant Genetic Resources (NBPGR), New Delhi and maintained in MS media at PTC laboratory of Department of Biotechnology, DTU.

3.2 Culture Medium preparation

MS media was prepared by mixing all the constituents i.e., macronutrients (NH_4NO_3,KNO_3 ,$CaCl_2.2H_2O$,$MgSO_4.7H_2O$,KH_2PO_4),micronutrients(H_3BO_3,$MnSO_4.4H_2O$,$ZnSO_4.4H_2O$,KI, $Na_2MoO_4.2H_2O$,$CoCl_2.6H_2O$,$CuSO_4.5H_2O$,$FeSO_4.7H_2O$,Na_2EDTA),vitamins(niacin,thiamine ,glycine,pyridoxine),M-inositol,carbon source(sucrose) in 500ml distilled water and then pH was balanced to 5.8 by using 1N HCl. After that final volume was made up to 1000ml with distilled water. Then 0.8% solidifying agent agar was added and the media was sterilized by autoclaving it at 15psi or 121° C for 15 minutes. After autoclaving the media was then allowed to cool at room temperature before adding some hormone (200µl BAP) and lastly pouring was done in flasks and culture tubes.

3.3 Preparation of elicitors

Three different biotic elicitors-yeast extract, malt extract and chitosan were used for elicitation. Stock solution of these elicitors were prepared. Yeast and malt extract(150mg/l) were prepared in distilled water & chitosan(150mg/l) was prepared in glacial acetic acid and then autoclaved. These stocks were then cooled down and filter sterilized using syringe filter before adding into culture medium.

3.4 Inoculation of explants

In this study, nodal stem *Plumbago zeylanica* were used as explants. Stems of suitable size of *plumbago zeylanica* were trimmed using scalpel and blade. A cut was made on both bottom and top part of the stem so as to remove the dead portion. These nodal explants were then aseptically inoculated into the culture medium containing 200µl BAP and 150mg/l elicitors with the help of forceps. Culture tube containing media without elicitor were kept as control. The lid of the culture tube was then closed thoroughly and fixed with parafilm and then incubated at 25±2°C temperature maintained in a culture room with a photoperiod of 16 hr light, 8 hr dark and 65% humidity.

3.5 Quantification of plumbagin

Plumbagin was quantified by using High performance liquid chromatography (HPLC). Methanol and water in the ratio of 80:20 was taken as mobile phase in C18 column with a rate of flow of 1.0ml/min. Wavelength of 272nm was determined using UV detector. Plumbagin

calibration curve was plotted using standard plumbagin over concentration range 100-500µg/ml.

3.6 Phytochemical analysis

Chemical tests were performed on different extracts of elicited and non-elicited in vitro cultured *Plumbago Zeylanica*. for the estimation of total phenolic content, total tannin content and antioxidant potential.

Preparation of plant extracts

1g of plant material was measured and was finely grounded using mortar and pestle and then soaked in 10 ml methanol which was then placed in a shaker device for 1-2 minutes at room temperature. After that the mixture was then stored in fridge for 2 days. Then organic fraction of the mixture was filtered by using syringe filter and was stored at 4°C in the fridge for further use.

Quantification of phytochemical constituents.

Total phenol

Quantification of phenol was done by using spectrophotometer. 200µl of plant extract was taken into test tube, and 1.5ml of Folin-Ciocalteu reagent is added. After the addition of FC reagent, the solution was kept in dark for 5 minutes. After that, 1.5ml of 5% Na_2CO_3 was added to it. The solution was then made up to the mark and kept aside for 2 hours for colour development and whose absorbance was then measured at 750 nm.

Total tannin

Quantification of tannin was done by using spectrophotometer. 100µl of plant extract was taken into test tube, and 7.5ml of distilled water was added. After that 0.5ml of FC reagent and 1ml of 35% Na_2CO_3 was added to the mixture. Shake the constituents of the test tube and kept aside for half an hour at room temperature for colour formation whose absorbance was then measured at 700nm.

3.7 Analysis of antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity

(a) Preparation of DPPH Stock Solution: Initially 3.9mg of DPPH was weighed for preparing the stock solution of 0.06M by using solvent absolute methanol.

(b) Determination of Antioxidant potential: A solution was made by mixing 3.9ml of DPPH stock solution with 100µl of plant extract. The resulting mixture was then vortexed for 30 seconds and kept in dark room for about half an hour; after that the absorbance was read at 517nm wavelength in UV-vis spectrum.

3.8 Analysis of antibacterial activity

Sub culturing of bacterial strains

Bacterial strains were sub cultured in nutrient broth which were incubated overnight at 37 °C. The antibacterial potential against *E. coli* and *S.aureus* was determined by agar disc diffusion method.

Disc diffusion assay

Nutrient agar media was prepared and poured into Mueller-Hinton agar plates. After pouring, plates were then allowed to solidify for about half an hour. Bacterial culture was inoculated on each plate which were then left for drying. The sterile filter paper disks (Whatman No. 1) were soaked in 20µl of each plant extract. The soaked discs were then put down on to the agar plates which were previously inoculated by the bacterial strain. Streptomycin soaked disc was used as the control. Plates were then kept for 18 hours of incubation at $35 \pm 2°C$ and after that the zone of inhibition were recorded.

Figure 2: In vitro shoot culture of *P.zeylanica* (IC-398891) in MS medium with elicitor

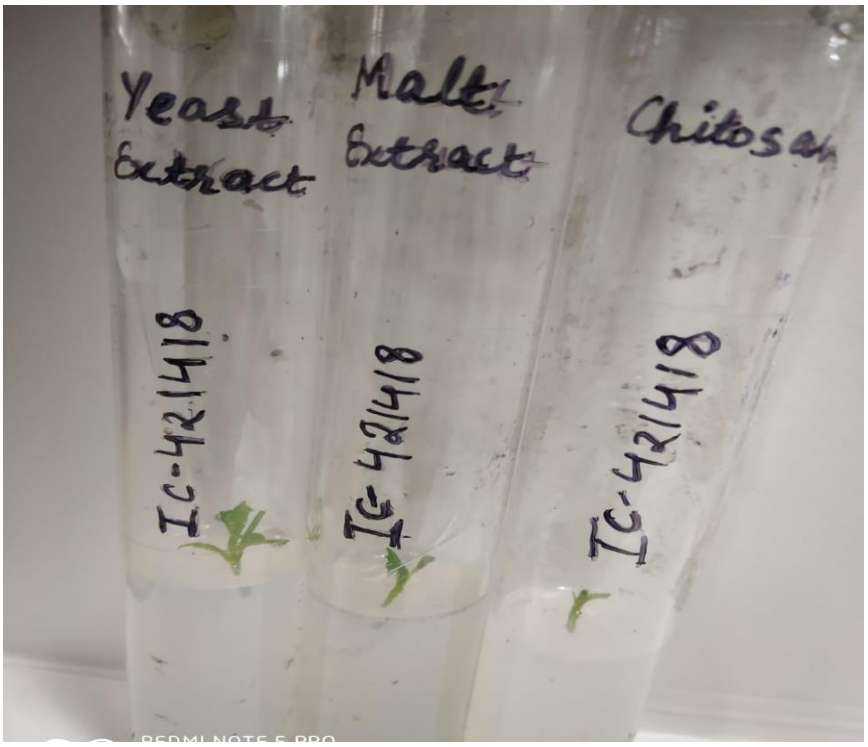

Figure 3: In vitro culture shoot of *P.zeylanica* (IC-421418) in MS medium with elicitor

Figure 6: In vitro shoot culture of *P.zeylanica* (IC-524441) in MS medium with elicitor

Figure 7: In vitro shoot culture of *P.zeylanica* (IC-439214) in MS medium with elicitor

RESULTS AND DISCUSSION

All the cultures were pre-treated with three types of elicitors that is yeast extract, malt extract and chitosan and then analysed for their plumbagin production. It was observed that those cultures which were treated with elicitors showed higher plumbagin production as compared to the control.

4.1 Effect of yeast extract on plumbagin production

Yeast extract (YE) is a basic elicitor that contains vitamin B-complex and many important compounds such as N-acetyl-glucosamine, ergosterol, chitin and glycopeptides which activates the plant defence system by enhancing the metabolite production. 150mg/l of yeast extract was successfully added in the culture to enhance the plumbagin production. Shoot treated with 150mg/l yeast extract is expected to produce higher plumbagin content as compared to that of control.

4.2 Effect of malt extract on plumbagin production

Malt extract is basically a carbohydrate source and also has a very significant role in the production of bioactive compounds. It was observed that some PGR_S like auxins & gibberellins are also found in malt extract. A study showed the elicitation of secondary metabolites in *Centella asiatica* by using malt extract. In current experiment, 150mg/l of malt extract was added in the media to enhance the plumbagin production and it is expected that media supplemented with malt extract synthesize higher plumbagin than control.

4.3 Effect of chitosan on plumbagin production

Chitosan is a well-known constituent of fungi cell wall and is also known as natural polycationic polymer. Various research proved that chitosan triggers the natural defence mechanism of many plants. Several studies showed that chitosan enhances the bioaccumulation of napthoquinones, terpenoids, alkaloids and phenylpropanoids in callus cultures. A research indicate that chitosan enhances the plumbagin synthesis in *Plumbago rosea* and resveratrol and viniferins *in Vitis vinifera.* 150mg/l chitosan was successfully added to the media to influence the plumbagin production in *Plumbago zeylanica* and it was expected that chitosan supplemented media produces higher plumbagin as compared to control.

4.4 Analysis of antioxidant activity

Natural antioxidant has the tendency to scavenge free radical which is estimated by decrement in absorbance by UV-vis spectroscopy. DPPH free radical scavenging test was done for estimating the antioxidant power of various accession extracts of *Plumbago zeylanica.* It was

expected that elicited extracts show higher antioxidant activity as compared to non -elicited extracts.

4.5 Analysis of antibacterial activity

In this study, antibacterial activity of different extracts of *Plumbago zeylanica* with three biotic elicitors namely-yeast extract, malt extract, chitosan was evaluated against *E. coli* and *S. aureus* by disc diffusion assay. The result was expected that elicited culture extract shows maximum antibacterial activity than non- elicited culture extracts.

4.6 Phytochemical studies

Prior screening of herbal drugs is very essential to examine their phytochemical and pharmacological value. Several studies reported various types of qualitative tests for the determination of phytocompounds associated in *P.zeylanica* roots. In current experiment quantitative test was conducted on *P.zeylanica* to quantify tannin, phenol and plumbagin content. Three biotic elicitors (yeast extract, malt extract, chitosan) were used for the preparation of plant extracts and each extract was analysed for their phytocompound production, antioxidant potential and antibacterial activity. DPPH free radical scavenging activity of different extracts was carried out and elicited extracts are expected to show maximum free radical activity than non-elicited extracts. This difference in the activity is due to variation in several compounds like tannins, phenols and flavonoids. Standard gallic acid was used to determine total tannin content of different plant extracts. Tannins are universal, water-soluble phenolic compound in nature that are almost present in all plants. The difference in total phenolic content of different extracts of *P.zeylanica* is due to several parameters that are linked with the accessions like location, environmental factors and location.

CONCLUSION

P.zeylanica is pharmaceutically important plant which is used for treating several ailments due of the presence of phytocompound called plumbagin. It is very important to conserve this plant due to its overexploitation for its medicinal properties. So, to conserve these endangered plants species, micropropagation play a major role in production of these plant species and is also useful in its germplasm conservation. Changes in environmental condition and genotype interaction alters the phytocompound production. So, to overcome this, selection and screening of individual accession is necessary to enhance the plumbagin production.

Demand of products from herbal sources is growing day by day due to its utilization in various disease treatment. Naturally derived products provide great prospect to evaluate its role in various applications. For continuous production of natural products an efficient method is required. Traditional methods have various problems, to overcome the limited availability of biologically active from medicinal plants, an alternative approach is required. Biotechnological methods have various advantages such as continuous production, reliable source of natural products, controlled environment independent from climate and soil condition, synthesis of bioactive compounds at higher rate takes place. Increased production of secondary metabolites which has medicinal use can be generated in large scale culture of plant cells. For controlled and successful biotechnological production of specific compound, continued and intensified efforts are needed.

In organ and cell culture, elicitors have been involved to elicit the yield of bioactive compounds. The biotic and abiotic elicitors react with secondary metabolite, and their effect depends of the type of metabolite being produced.

Present study justified that traditionally used medicinal plant *P.zeylanica* was evaluated for secondary metabolite production (tannins, phenols, plumbagin), antioxidant activity and antibacterial activity. The presence of such phytocompounds like tannins and phenols indicates the medicinal characteristics of *P.zeylanica*. Hence, further study on these compounds is necessary to understand their biological nature which is very useful for treating different types of diseases. Three biotic elicitors were used namely, yeast extract, malt extract, chitosan to enhance the phytocompound production. From the expected results, conclusion has been made that elicited culture showed higher secondary metabolite production as compared to the non-elicited one. Their quantitative analysis gives almost approximate idea for their quantity present whereas pharmacological study showed their application part. The different extracts were also evaluated for antioxidant and antibacterial activity also. Extracts with elicitors were expected to show higher antioxidant and antibacterial activity as compared to control.

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