SELECTION OF HIGH CONTENT CELL LINES OF

Picrorhiza Kurroa

BY

ARUN SHARMA (051508)

SHIVAM SHARMA (051504)

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JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY-WAKNAGHAT

CERTIFICATE

This is to certify that the work entitled, "**Selection of high picroside content cell lines of *Picrorhiza kurroa***" submitted by **Arun Sharma(051508)** and **Shivam Sharma(051504)** in partial fulfillment for the award of degree of Bachelors of Technology in **Bioinformatics** of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Dr. R S Chauhan

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Arun Sharma
(051508)

Shivam Sharma
(051504)

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ABSTRAC T

P. *kurroa* Royle ex Benth (Family: Scrophulariaceae) is a medicinal herb mainly found in the North-Western Himalayas at altitudes of 3000-4300 meters. *P. kurroa* is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers, to treat dyspepsia, chronic diarrhea, scorpion sting, etc. A commercial formulation named as Picroliv has been prepared from *P. kurroa* extracts containing Picroside-1 and launched as a highly effective hepatoprotective drug. Picroliv has also been shown to have other medicinal properties such as immunostimulating effect in hamsters and prevention of infections. Kutkin is the active principal of *Picrorhiza kurroa* and is comprised of kutkoside and the iridoid glycoside picrosides I, II, and III. Other identified active constituents are apocynin, drosin, and nine cucurbitacin glycosides. Successful induction of callus has been achieved in M.S. Media. Different explants (root, leaf, node) were taken for callus initiation. Repeated subculturing was done to create the selection pressure. Some of the elicitors like salicylic acid and sodium acetate were also used in different concentrations in selective media. Precursor Phenylalanine was also tried as a component of selective media in different concentrations. After using the above mentioned selective media browning of callus occurred after 2-4 weeks of inoculation.

OBJECTIVES

- **Selection of high yielding cell lines of *Picrorhiza kurroa* for picroside content**

- **Standardization of conditions for increasing picroside content**

CHAPTER 1

INTRODUCTION

1.1 *Picrorhiza kurroa*

Picrorhiza kurroa is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers, and to treat dyspepsia, chronic diarrhea, and scorpion sting. It is a small perennial herb from the Scrophulariaceae family, found in the Himalayan region growing at elevations of 3,000-5,000 meters. *Picrorhiza kurroa* has a long, creeping rootstock that is bitter in taste, and grows in rock crevices and moist, sandy soil.

The leaves of the plant are flat, oval, and sharply serrated. The flowers, which appear June through August, are white or pale purple and borne on a tall spike; manual harvesting of the plant takes place October through December. The active constituents are obtained from the root and rhizomes. The plant is self-regenerating but unregulated over-harvesting has caused it to be threatened to near extinction.

Current research on *Picrorhiza kurroa* has focused on its hepatoprotective, anticholestatic, antioxidant, and immune-modulating activity.

1.2 Active Constituents

Kutkin is the active principal of *Picrorhiza kurroa* and is comprised of kutkoside and the iridoid glycoside picrosides I, II, and III. Other identified active constituents are apocynin, drosin, and nine cucurbitacin glycosides. Apocynin is a catechol that has been shown to inhibit neutrophil oxidative burst in addition to being a powerful anti-inflammatory agent, while the curcubitacins have been shown to be highly cytotoxic and possess antitumor effects.

1.3 Mechanisms of Action

The hepatoprotective action of *Picrorhiza kurroa* is not fully understood but may be attributed to Picrorhiza's ability to inhibit the generation of oxygen anions and to scavenge free radicals.

Picrorhiza's antioxidant effect has been shown to be similar to that of superoxide dismutase, metal-ion chelators, and xanthine oxidase inhibitors. In rats infected with malaria, Picrorhiza restored depleted glutathione levels, thereby enhancing detoxification and antioxidation, and helping maintain a normal oxidation-reduction balance. In this same animal model, Picrorhiza also demonstrated an anti-lipid peroxidative effect. Like silymarin, Picrorhiza has been shown to stimulate liver regeneration in rats, possibly via stimulation of nucleic acid and protein synthesis. Picrorhiza's anti-inflammatory action is attributed to the apocynin constituent, which has been shown to have potent anti-inflammatory properties in addition to inhibiting oxidative burst in neutrophils.

1.4 Study results

Leaves and roots show differential accumulation of picrosides. Picroside-I accumulated in leaves whereas roots contained higher amounts of picroside-II. Leaves and roots show differential accumulation of antioxidants. Ascorbate was more abundant in leaves than roots whereas glutathione is most abundant in roots. This analysis of picroside contents in leaves and roots provides the first indication of differential metabolism of Picrosides in these plant organs. This provides the foundation for comparative analysis of biosynthesis and transport between tissues.

1.5 Application

- *Picrorhiza kurroa* are effective at preventing liver toxicity and the subsequent biochemical changes caused by numerous toxic agents.
- Picrorhiza extracts may be of therapeutic value in treating viral hepatitis.
- Effective in treatment of Liver cirrrosis.
- The curcubitacin has shown cytotoxic effects and is effective against tumor.

1.6 Selection of high secondary metabolite producing strains

Plant cell culture presents a heterogeneous population in which physiological characteristics of individual plant cells are different.

Cell cloning method provides a promising way of selecting cell lines yielding increased level of product. A strain of ***Euphoria milli*** accumulated about 7-fold the level of anthocyanin produced by parent cell culture after 24 selections.

Cell cloning using cell aggregates of coptis japonica and obtain strain, which grow faster and produced a higher amount of *berberin* .Production of high berberin took place after 27 generations. Increased capsaicin and romarinic acid in PEP cell lines of *Capsicum annuum* were reported. Selective agents such as 5-methyl tryptophan, glyphosate and biotin have also been studied to select high-yielding cell lines.

1.7 Precursor feeding

Exogenous supply of a biosynthetic precursor to culture medium may also increase the yield of the desired product. The concept is based on the idea that any compound which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic rout, stands a good chance of increasing the yield of the final product. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases. For example amino acids have been added to cell suspension culture media for production of tropane alkaloids, indole alkaloids etc. addition of phenylalanine to *Salvia officinalis* cell suspention cultures stimulated the production of rosmarinic acid. Addition of same precursor resulted stimulation of taxol production in texus cultures. Feeding ferulic acid to cultures of vanilla planifolia resulted in increase in vanillin accumulation. Furthermore addition of leucine , led to enhancement of volatile monoterpenes in cultures of perilla frutiscens, where an addition of geraniol to rose cell culture led to accumulation of nerol and citro-nellol.

1.8 Elicitation

Plants produce secondary metabolites in nature as a defense mechanism against attack by pathogens. Elicitors are the signals triggering the format of secondary metabolites. Use of elicitors of plant defense mechanisms, i.e. elicitation has been one of the most effective strategies for improving the productivity of bioactive secondary metabolites. Biotic and abiotic elicitors which are classified on their origin are used to stimulate secondary metabolite formation in plant cell cultures, thereby reducing the process time to attain high product concentrations. Production of many valuable secondary metabolites using various elicitors were reported .

CHAPTER 2

MATERIALS AND METHODS

2.1 REQUIREMENT AND APPARATUS

- ***Flasks (150ml)*** -Initially we have started our experiment with 10 flasks.
- ***Explant*** – We took previously grown cultured Explant from tissue culture room.
- ***Media components*** – Stock solution (7) were prepared, agar-agar, sucrose, growth hormones (auxins and IBA).

2.2 MEDIA PREPARATION

- MS Media was prepared by using above components chemicals. pH set to 5.6
- After pouring the media into flasks and covering it with cotton plugs it was autoclaved (15-20 min).
- After solidification media was ready for inoculation.

2.3 INOCULATION

- Place the flask containing media, Petri plate, test tube, burner, ethanol, match box and scalpel and forceps in Laminar Air Flow for 20 min for sterilization by UV light.
- Take different Explant (leaf, root and node) and inoculate these 2-3 injured explants portions in flasks.
- For subculturing callus is used for inoculation.

2.4 STARTING OF PROJECT

- We have started by inoculating fresh explants into MS Media in sterilized condition in laminar air flow. Inoculate flasks were kept in tissue culture room under standard condition (Temperature, photoperiod etc).
- Observations were taken after regular intervals.

2.5 PROGRESS IN PROJECT

2.5.1 Subculturing

- To increase the amount of callus subculturing of induced callus was done in sterilized condition.
- Observations were taken after regular interval and further subculturing was done.

2.5.2 Use of selective media

- Selective agents like salicylic acid and sodium acetate were used for preparation of selective media .
  - Salicylic acid -1)50mg/l, 2)30 mg/l
  - Sodium acetate – 1)0.5mg/l, 2)1 mg/l
- Inoculation and sub culturing (induced callus) was done in both types of selective media(Flasks kept under same standard conditions).
- Observations were taken after regular intervals.

2.5.3 Precursor

After using various elicitors we tried following concentrations of precursor **phenylalanine.** Phenylalanine was used in *Salvia officinalis* cell suspension cultures to increase the production of rosmarinic acid.

1)0.05 mM

2) 0.10 mM

3)0.15 mM

2.5.4 Callus induction

In spite of continuing with subculturing and culturing in selective media we continued to grow more amount of callus by inoculating fresh explants so we can get ample amount of callus.

CHAPTER 3

METHODOLOGY

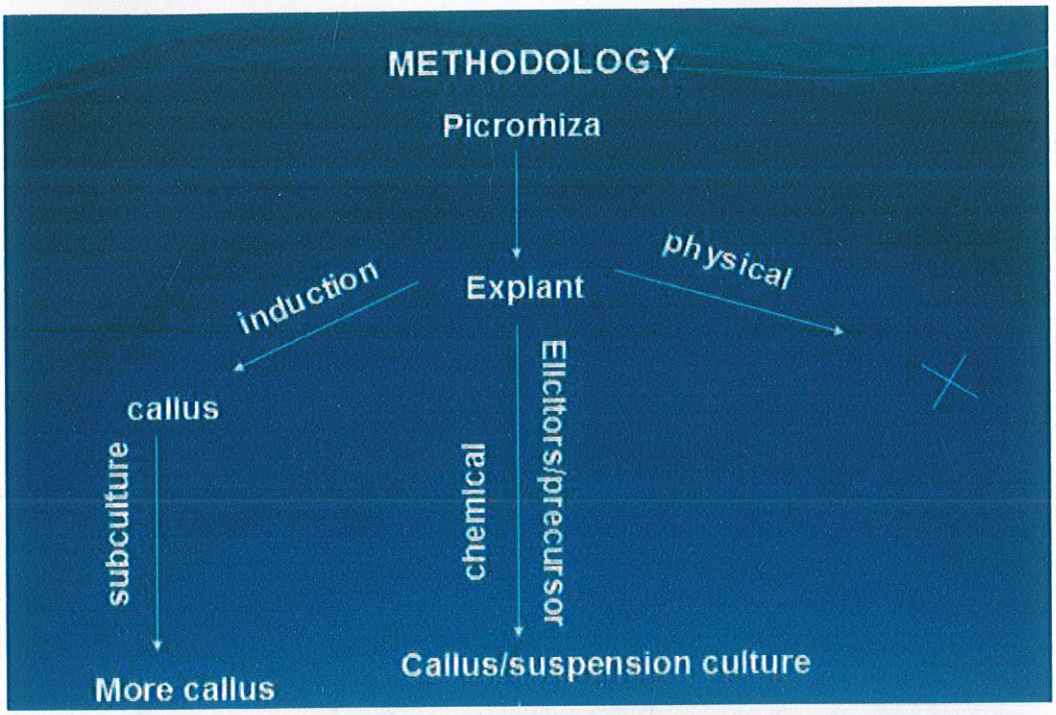


Figure- Methodology

3.1 Methodology

For selection of high picroside content cell lines following methodologies can be adopted:

1) By varying physical agents like light, temperature, pressure etc. but for our experiment we didn't adopt this methodology.
2) By using various elicitors and precursors:
   Chemical agents like salicylic acid and sodium acetate are used to enhance the production of secondary metabolites. we tried both the chemicals in different concentrations to observe the effect on callus.
3) By creating selection pressure:
   Continuous sub culturing was done so that soma clonal variation can induce which can yield cell lines with higher content of picroside.

CHAPTER 4

RESULT

Effect of different elicitors on picroside accumulation in callus

Sno	*Elicitor*	*Concentration used*	*Time of incubation*	*Status/observation*
1	Salicylic acid	50mg/l	2 weeks	Browning of callus
		30 mg/l	2 weeks	Browning of callus
2	Sodium Acetate	0.5mg/l	4 weeks	Browning of callus
		1 mg/l	4 weeks	Browning of callus

Effect of precursor on picroside accumulation in callus

Sno	*Precursor*	*Concentration used*	*Time of incubation*	*Status/observation*
1	Phenylalanine	0.05Mm	2 weeks	Browning of callus
		0.10Mm	2 weeks	Browning of callus
		0.15Mm	2 weeks	Browning of callus

Culture status

Sno	*Type of Explant*	*Time period*	*% of callus*
1	Leaf	3 weeks	70
2	Node	4 weeks	10
3	Root	3 weeks	20

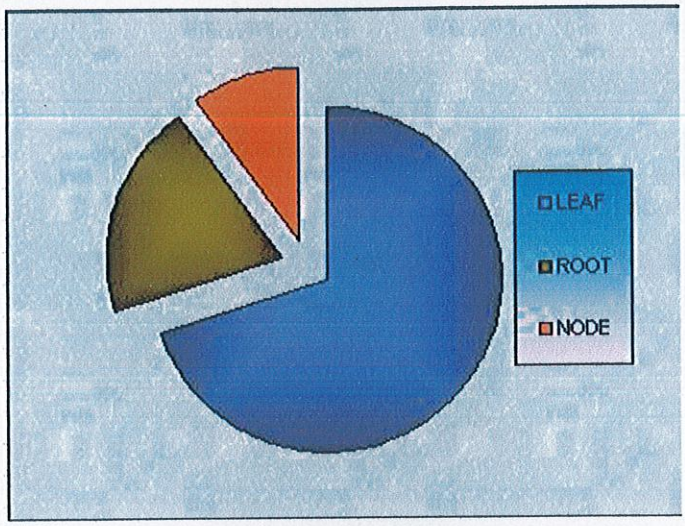
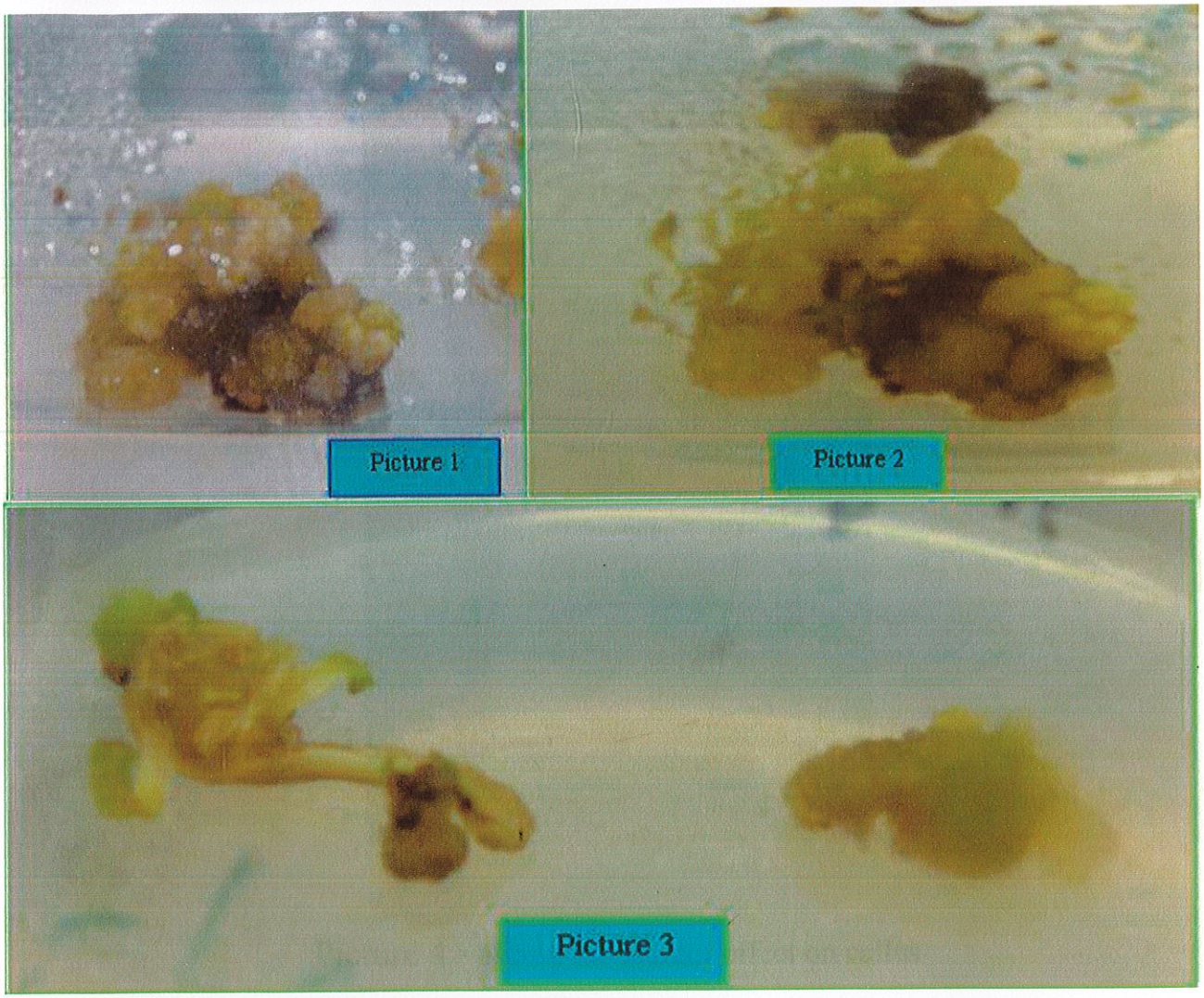


Figure- Culture status

Below there are picture of some callus culture.

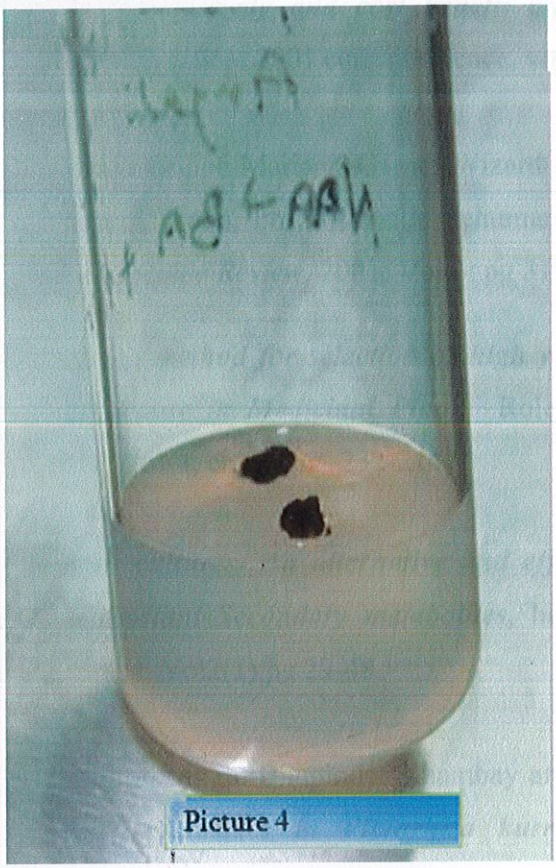


Details

Picture 1 - Callus culture of leaf

Picture 2 - Subculture of root segment

Picture 3 - Callus culture of nodal segment



Picture 4 - Shows the elicitor effect on callus

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