INVESTIGATION OF MORPHOGENETIC DIFFERENCES AND SEAWEED EXTRACT STIMULATED INCREASE IN BIOMASS AND PICROSIDE-I CONTENT IN *PICRORHIZA* SPECIES

Thesis submitted in fulfilment of the requirements for the Degree of

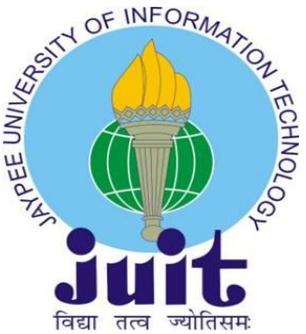
DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

By

NEHA SHARMA



Department of Biotechnology and Bioinformatics

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT, DISTRICT SOLAN, H.P., INDIA

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DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the Ph.D. thesis entitled **"Investigation of Morphogenetic Differences and Seaweed Extract Stimulated Increase in Biomass and Picroside-I Content in *Picrorhiza* Species"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Hemant Sood.** I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. thesis.

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Investigation of Morphogenetic Differences and Seaweed Extract Stimulated Increase in Biomass and Picroside-I Content in *Picrorhiza* Species",** submitted by **Neha Sharma** at **Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

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Neha Sharma

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26S	26S rRNA
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HMGS	3-Hydroxy-3-methylglutaryl-CoA synthase
HMGR	3-Hydroxy-3-methylglutaryl-CoA reductase
PMK	Phosphomevalonate kinase
DXPS	1-Deoxy-D-xylulose-5-phosphate synthase
DXPR	1-Deoxy-D-xylulose-5-phosphate reductoisomerase
ISPD	2-C-methylerythritol 4-phosphate cytidyl transferase
ISPE	4-(Cytidine-5-diphospho)-2-C-methylerythritol kinase
MECPS	2-C-methyl-D-erythritol 2,4-cyclopyrophosphate synthase
GS	Geraniol synthase
G10H	Geraniol-10-hydroxylase
10-HGO	10-Hydroxygeraniol dehydrogenase
IS	Iridoid synthase
DAHPS	3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase
PAL	Phenylalanine ammonia lyase
HK	Hexokinase
PK	Pyruvate kinase
ICDH	Isocitrate dehydrogenase
MDH	Malate dehydrogenase
G6PDH	Glucose-6-phosphate dehydrogenase
RBA	RuBisCO activase
ARP	Auxin response protein
ARF7	Auxin response factor 7
HisK	Histidine kinase
CytO	Cytokinin oxidase
MVA	Mevalonate
MEP	2-C-methyl-D-erythritol phosphate
EX	Explant stage

CI	Callus initiation stage
CM	Callus mass stage
SP	Shoot primordia stage
MS	Multiple shoots stage
FD	Fully developed stage
MS medium	Murashige and Skoog medium
P-I	Picroside-I
P-II	Picroside-II
G6P	Glucose-6-phosphate
FBP	Fructose-1,6-bisphosphate
DHAP	Dihydroxyacetone phosphate
G3P	Glyceraldehyde-3-phosphate
BPG	1,3-Bisphosphoglycerate
PEP	Phosphoenolpyruvate
6PGL	6-Phosphogluconolactone
Ru5P	Ribulose-5-phosphate
E4P	Erythrose-4-phosphate
F6P	Fructose-6-phosphate
DAHP	3-Deoxy-D-arabino-heptulosonate 7-phosphate
AKG	α-Keto glutarate
OAA	Oxaloacetate
HMG CoA	3-Hydroxy-3-methylglutaryl coenzyme A
IPP	Isopentenyl pyrophosphate
DOXP	1-Deoxy-D-xylulose-5-phosphate
CDP-ME	4-(CDP)-2-methyl-D-erythritol
CDP-ME 2-P	4-(CDP)-2-methyl-D-erythritol 2-phosphate
DMAPP	Dimethylallyl pyrophosphate
GPP	Geranyl pyrophosphate
10-HG	10-Hydroxy geraniol
SWE	Seaweed extract

SWM	SWE media
MSS	MS media supplemented with SWE
C1	Control medium
SM	Standard medium
SNP	Sodium nitroprusside
ABA	Abscisic acid
MeJa	Methyl jasmonate
IBA	Indole-3-butyric acid
KN	Kinetin
HPLC	High Performance Liquid Chromatography

ABSTRACT

Picrorhiza kurroa and *P. scrophulariiflora* are the two endangered species of genus *Picrorhiza* which possess a broad range of pharmacological activities. Picroside-I (P-I) is an important iridoid glycoside used in several herbal formulations for treatment of various disorders. P-I is synthesized in shoots of *P. kurroa* and *P. scrophulariiflora*. Over exploitation of these species necessitates the development of conservation strategies and enhanced production of secondary metabolites.

No information exists on molecular basis of improving shoot biomass and P-I biosynthesis in different morphogenetic stages of *P. kurroa* and *P. scrophulariiflora*; thus, limiting genetic interventions towards genetic improvement of these plant species. Therefore, shoot development along with P-I biosynthesis was studied in different morphogenetic stages of *P. kurroa* and *P. scrophulariiflora.* Expression analysis of genes involved in primary metabolism *viz.* RBA, HisK, CytO, HK, PK, ICDH, MDH and G6PDH showed high transcript abundance in MS and FD stages vis-à-vis shoot development in *P. kurroa* and *P. scrophulariiflora.* Genes such as HMGR, PMK, DXPS, ISPE, GS, G10H, DAHPS and PAL of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways showed significant modulation of expression in SP, MS and FD stages in congruence with P-I content as compared to CM stages of *P. kurroa* and *P. scrophulariiflora.* Quantitative expression analysis of secondary metabolism genes at two temperatures revealed that 7 genes *viz.* HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL showed 47-87 and 38-73 folds high expression in fully developed shoots of *P. kurroa* and *P. scrophulariiflora*, respectively originated from leaf explants at 15 ± 2 °C compared to 25 ± 2 °C. Further screening of these genes at species level showed their high expression pattern in *P. kurroa* (6-19 folds) vis-à-vis *P. scrophulariiflora* that was in corroboration with P-I content.

Moreover, limited progress has been made so far with respect to development of rapid and cost effective approach for enhancement of shoot biomass vis-à-vis P-I production in these plant species under in vitro conditions. So, the effect of seaweed extract (SWE) obtained from *Kappaphycus alvarezii* with and without growth hormones was studied for in vitro propagation

and production of P-I in *P. kurroa* and *P. scrophulariiflora*. Murashige and Skoog (MS) media supplemented with SWE (MSS) showed enhancement of 2.79, 2.61 fold in shoot biomass and 2.67, 2.61 fold in number of shoots after one month in *P. kurroa* and *P. scrophulariiflora*, respectively as compared to control C1 at 15 ± 2 °C. MSS with growth hormones also improved the plant growth with increment of 2.67, 2.65 fold in shoot biomass and 1.47, 1.37 fold in number of shoots after one month in *P. kurroa* and *P. scrophulariiflora*, respectively as compared to control SM at 15 ± 2 °C. SWE as a medium (SWM) showed comparable results and proved to be an economic alternative to MS medium. Both MSS and SWM increased P-I accumulation at 25 ± 2 ºC and 15 ± 2 ºC by ~2-4 and ~2-3 folds in *P. kurroa* and *P. scrophulariiflora*, respectively.

Also, there are no reports on use of seaweed extract as a biostimulant and/or media replacement for in vitro micropropagation and P-I production in *P. kurroa* and *P. scrophulariiflora.* So, a comparative analysis of SWE with other elicitors *viz.* MeJa, SNP and ABA was carried out to identify an efficient elicitor for enhanced plant growth and P-I content in *P. kurroa* and *P. scrophulariiflora*. Results indicated that treatment with SWE showed highest shoot biomass and P-I content in *P. kurroa* and *P. scrophulariiflora* plants as compared to SNP, ABA and MeJa. Interestingly, SWE modulated all the four integrating secondary metabolic pathways, covering almost all critical steps in MEP, MVA, iridoid and shikimate/phenylpropanoid pathways to stimulate P-I biosynthesis. SNP targeted MVA/MEP pathways in conjunction with iridoid pathway while ABA modulated shikimate/phenylpropanoid pathway to increase the P-I content in *P. kurroa* and *P. scrophulariiflora*.

Hence, the current study has revealed developmental regulation of P-I biosynthesis in *P. kurroa* and *P. scrophulariiflora.* Key genes contributing to P-I biosynthesis have been identified which could be targeted for enhancing P-I production. This study has also provided a first time insight into the potential of SWE as an elicitor as well as an alternative to MS media for enhancing shoot biomass and P-I production in *P. kurroa* and *P. scrophulariiflora.*

CHAPTER 1

INTRODUCTION

Himalayan region has rich diversity of medicinal plants having more than 800 valuable medicinal plant species in India. These medicinal herbs are extensively used by the locals as well as tribal communities since ancient times for curing various human ailments. Amongst many useful herbs, *Picrorhiza* species have been used in treating various liver disorders. The name has been derived from its bitter roots, where "Picros" means bitter, while "rhiza" means root. *Picrorhiza kurroa* Royle ex Benth and *P. scrophulariiflora* Pennell (family Scrophulariaceae) are the two important species of genus *Picrorhiza* which have been widely used in traditional as well as in modern medicinal system in India, China, Tibet and Nepal [1]. *P. kurroa* is distributed in Western Himalayas at 3,000–4,300 m altitude, while *P. scrophulariiflora* is restricted to Eastern Himalayas at 4,300–5,200 m altitude (Fig. 1.1). Both species contain various pharmaceutical compounds like picroside I (P-I), picroside II (P-II), kutkoside, vanillic acid, phenylethanoids, apocynin, androsin, cucurbitacins, picrotin, picrotoxinin, etc. P-I and P-II are the main bioactive constituents of these species, which are used in various herbal formulations such as Picroliv, Katuki, Arogya, Kutaki, Livocare, Livomap, Livomyn, Livplus, Pravekliv and Vimliv for the treatment of liver diseases, fever, allergy, hepatitis-B, leukoderma, gastrointestinal and urinary disorders etc [1-2]. They also possess anti-oxidant, immunomodulatory, anti-malarial, anti-inflammatory, anti-cancerous, neuroprotective, anti-asthmatic and anti-diabetic properties [3]. The rhizomes of *Picrorhiza* have been used as adulterant for *Gentiana kurrooa* [4]. *P. kurroa* and *P. scrophulariiflora* are freely traded as 'kutki' leading to estimated consumption of 416 MT by herbal industries in India [5]. However, the consumption is much higher today as the annual growth rate of herbal medicines has increased in recent years with 20% leading to increase in demand for medicinal plants by 11.1% [6]. *P. kurroa* and *P. scrophulariiflora* are self-regenerating in nature, but overexploitation and indiscriminate as well as unscientific collection practices has led to considerable depletion of their natural populations.

As a result, *P. kurroa* and *P. scrophulariiflora* plants have been listed as endangered species by International Union for Conservation of Nature and Natural Resources [7] and red data book of endangered species, respectively [8]. Therefore, immediate thrust has to be given for their conservation, micropropagation and enhancement of in vitro production of secondary metabolites.

Fig. 1.1 Mature field grown plants of *P. kurroa* (a) and *P. scrophulariiflora* [1] (b)

Different tissue culture techniques including micropropagation, synthetic seed production, conservation of germplasm through encapsulated microshoots, plant regeneration via direct and indirect organogenesis have been employed for conservation of *P. kurroa* and *P. scrophulariiflora* [8-16] but limited progress has been made so far with respect to development of an efficient approach for enhancing secondary metabolite production in these plant species under in vitro conditions.

P. kurroa and *P. scrophulariiflora* share almost similar chemical composition in terms of biological activities and phytoconstituents [1]. The biosynthesis and accumulation of P-I and P-II take place differentially in different tissues of these species *viz.* P-I in shoots and P-II in roots or stolons, whereas both accumulate in rhizomes [3, 11-12, 17-18]. P-I is a monoterpenoid glycoside which possess anti-microbial, anti-inflammatory, anti-cancerous properties and is also used for the treatment of hepatitis B [19-21]. The biosynthesis of P-I involves non-mevalonate (MEP), mevalonate (MVA), shikimate/phenylpropanoid and iridoid pathways [22-23] (Fig. 1.2). Iridoid backbone of P-I is derived from geranyl pyrophosphate (GPP) by condensation of

isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP) via MVA and MEP pathways [24]. Biosynthesis of P-I involves the synthesis of iridoid moiety from GPP through series of oxidation and cyclization steps followed by condensation of glucose moiety and cinnamate from phenylpropanoid pathway [25-26]. Recently, studies related to NGS transcriptome analysis for picrosides production, differential biosynthesis of picrosides and involvement of primary metabolic enzymes vis-à-vis picrosides content have provided valuable cues towards P-I production [18, 22-23, 27] but these studies have limited their interest towards *P. kurroa*, thereby neglecting the species diversity and leaving *P. scrophulariiflora* unexplored.

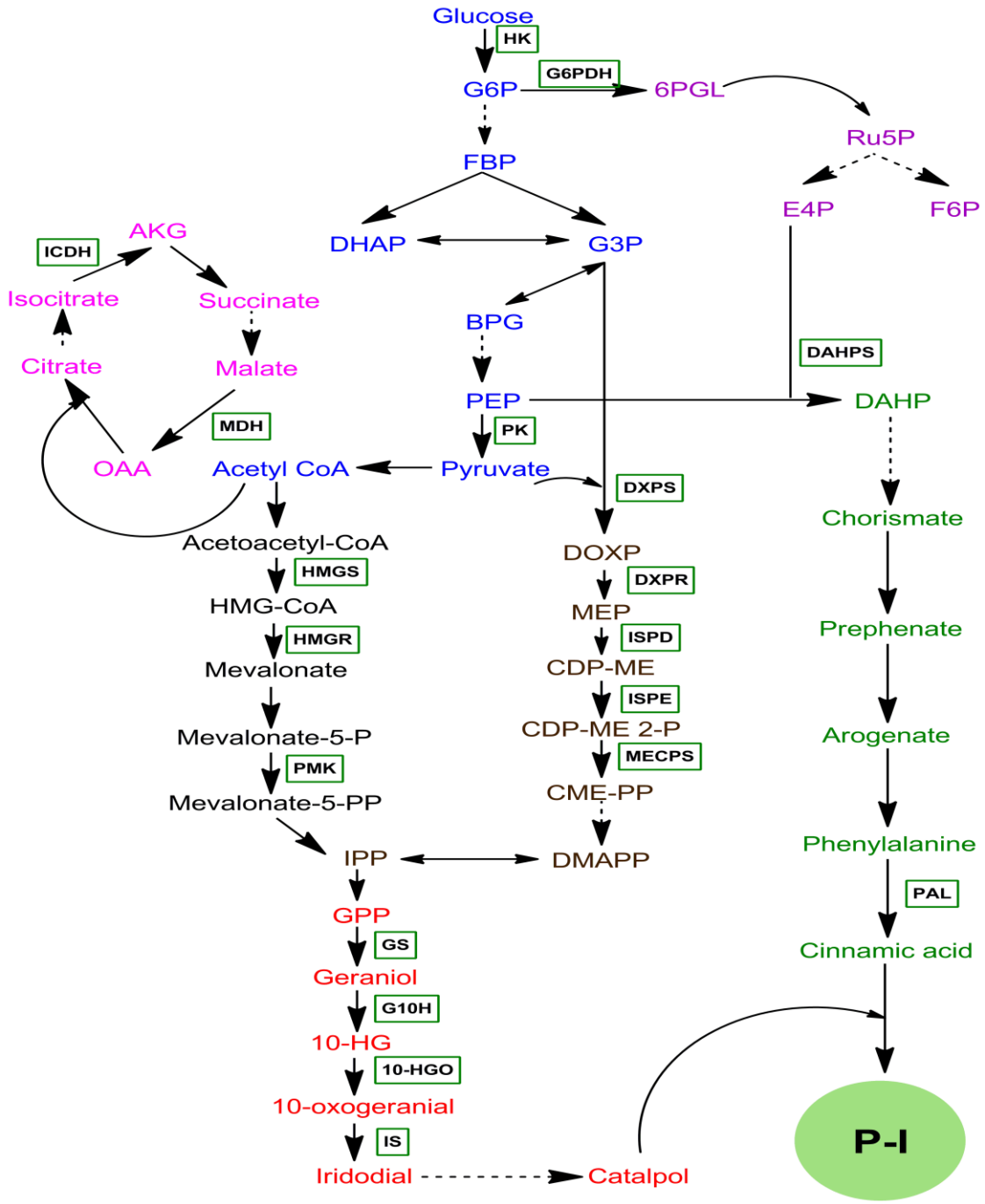


Fig. 1.2 Linking of primary and secondary metabolic pathways integrating to P-I biosynthesis. The metabolic network has been reconstructed by including glycolysis (blue color), TCA cycle (pink color), pentose phosphate pathway (purple color), MVA (black color), MEP (brown color), shikimate/phenylpropanoid (green color) and iridoid pathways (red color). Single and multiple steps were indicated by solid and dotted lines, respectively [27]

The biosynthesis and accumulation of medicinally important metabolites have been reported to occur in different tissues and organs of plants and is largely influenced by the developmental stage of a particular organ/tissue [28]. P-I biosynthesis occur in differentiated tissue (shoot) rather than the undifferentiated mass of cells suggesting growth and developmental stages playing imperative role in regulation of P-I production [11]. The environmental factors such as high altitude and seasonal variations complicate the process of understanding biology of P-I biosynthesis in *P. kurroa* and *P. scrophulariiflora*; hence, cell cultures offer a suitable biological system under homeostasis wherein the morphogenetic events and developmental fate of regenerating tissues can provide the clear picture of P-I biosynthesis. However, no molecular data is available till date to fully substantiate the developmental stages vis-à-vis P-I biosynthesis in *P. kurroa* and *P. scrophulariiflora* under in vitro conditions. Therefore, the role of key genes as regulatory or control points for P-I production can be ascertained by unraveling the dynamics of P-I biosynthesis at different developmental stages of *P. kurroa* and *P. scrophulariiflora*.

Moreover, there is dearth of knowledge on development of rapid and cost effective micropropagation technique for enhancing shoot biomass along with secondary metabolite production in *P. kurroa* and *P. scrophulariiflora*. Since in vitro grown shoots of these species produce P-I, they can be used as promising alternative to the plants grown in natural habitat. Seaweed extracts have been reported to influence plant growth and development, early shoot formation and enhanced secondary metabolite production in various plant species such as *Lycopersicon esculentum, Brassica oleraceae, Arabidopsis thaliana* etc [29-31]. Seaweeds are macroscopic, multicellular marine alga whose extracts contains macronutrients, micronutrients, amino acids, vitamins, cytokinins, auxins, gibberellins, carbohydrates, betaines, and abscisic acid-like growth substances [32]. These components enhance plant growth and development by improving N and S uptake along with their assimilation, basal metabolism and transportation of various nutrients. Commercially available seaweed extracts are mainly derived from brown and red alga such as *Ascophyllum nodosum, Macrocystis pyrifera, Ecklonia maxima, Lithothamnium calcareum, Porphyra perforate, Kappaphycus alvarezii, Gracilaria salicornia, Gelidiella acerosa* [32-33]. As of now, no information exists on use of seaweed extracts for plant biomass and P-I enhancement in in vitro grown *P. kurroa* and *P. scrophulariiflora* plants. Therefore, seaweed extracts can be evaluated as a biostimulant or a cost effective alternative to Murashige

and Skoog medium (MS medium) [34] for enhancing shoot biomass along with P-I production in *P. kurroa* and *P. scrophulariiflora*.

A number of different elicitors such as abscisic acid (ABA), salicylic acid, methyl jasmonate (MeJA), sodium nitroprusside (SNP), hydrogen peroxide, yeast extract, chitin and chitosan have been investigated for plant based secondary metabolite production. ABA, MeJa and SNP are known to increase oxidative stress by initiating signal transduction processes which mediates various pathophysiological and developmental processes, thereby triggering secondary metabolism for production of bioactive compounds [35]. Elicitors enhance secondary metabolites content by regulating the expression of genes involved in biosynthesis of plant secondary metabolites [35]; therefore, understanding the molecular basis of their biosynthesis in context to elicitor treatment is required for the metabolic engineering of iridoid glycosides in *P. kurroa* and *P. scrophulariiflora.* Singh et al. [36] have studied the regulation of MVA and MEP pathway genes vis-à-vis picrosides content in response to different modulators in *P. kurroa* but their study did not provided the information of their effects in vivo which could benefit the micropropagation of *Picrorhiza* species with increased P-I content. Therefore, comparative analysis of seaweed extract with different elicitors including ABA, MeJa and SNP can aid in identification of an efficient elicitor which could elicit shoot biomass and P-I content in *P. kurroa* and *P. scrophulariiflora.* Hence, the effect of seaweed extract and other elicitors on P-I production can be ascertained by expression analysis of key genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways for P-I biosynthesis in these species under in vitro conditions.

Thus, the current research work focused on in vitro studies for growth and development status along with molecular aspects for P-I production in *P. kurroa* and *P. scrophulariiflora*. Genes of primary and secondary metabolic pathways involved in shoot development and P-I biosynthesis in different morphogenetic stages of *P. kurroa* and *P. scrophulariiflora,* starting from explants through de-differentiation of original explants into callus mass and then re-differentiation of callus into shoot primordia and fully developed shoots under in vitro conditions were identified. Effects of different temperatures and explants were also studied at different developmental stages of *P. kurroa* and *P. scrophulariiflora* to ascertain the role of key genes involved in P-I

biosynthesis. Moreover, potential of seaweed extract as a biostimulant with MS medium was evaluated for enhancement of shoot biomass and P-I content in in vitro grown *P. kurroa* and *P. scrophulariiflora* plants. For in vitro mass propagation of these species, seaweed extract has also been investigated as a nutrient medium alternative to MS medium. Further, a comparative analysis of seaweed extract with other elicitors *viz.* MeJa, SNP and ABA was carried out to identify an efficient elicitor for enhanced plant growth and P-I content in *P. kurroa* and *P. scrophulariiflora*. Expression analysis of key genes was also done to evaluate the effect of different elicitors on MEP, MVA, iridoid and shikimate/phenylpropanoid pathways involved in P-I biosynthesis.

Therefore, the current work was undertaken to fill the research gaps in knowledge on P-I biosynthesis with the following objectives:

Objective 1: Understanding P-I biosynthesis in different in vitro morphogenetic stages of *P. kurroa* and *P. scrophulariiflora*

Objective 2: Investigating the effect of seaweed extract for increase in biomass and P-I production in *P. kurroa* and *P. scrophulariiflora*

Objective 3: Discerning the expression status of key pathway genes vis-à-vis P-I biosynthesis in *P. kurroa* and *P. scrophulariiflora*

CHAPTER 2

REVIEW OF LITERATURE

Medicinal plants have been used by mankind for the treatment of various ailments since ancient times. The traditional systems of medicines such as Unani, Ayurveda and ancient texts like Rig Veda, Atharva Veda, Charaka Samhita and Sushruta Samhita mention the properties and use of various plants as medicines. According to World Health Organization (WHO), 80% population of developing countries cannot access the expensive modern pharmaceutical products and rely on traditional medicine to meet their primary healthcare needs [37]. These herbal medicines are relatively cheaper and safer than synthetic or modern medicines [38-39]. Higher plants also contribute directly or indirectly in about 25% of modern medicine [40]. Around the world, 21,000 plants have been listed for medicinal purposes by the WHO and among these, 2500 species are in India; 150 species being used commercially on a fairly large scale [41].

The therapeutic value of medicinal plants is attributed to the presence of secondary metabolites. All biochemical processes in plant cell can be conditionally classified as primary and secondary metabolism. Compounds and processes which are necessary for growth, development and breeding belong to primary metabolism. It includes the metabolism of proteins, nucleic acids, carbohydrates, lipids and essential for basic photosynthetic or respiratory processes. On the other hand, secondary metabolism is not directly essential for plant cell life and function in defence (against herbivores, microbes, viruses or competing plants), signalling (to attract pollinating or seed dispersing insects/birds) and chemical adaptations to environmental stresses [42-43]. Plants generally produce many secondary metabolites which are biosynthetically derived from primary metabolites. The pattern of secondary metabolites in a given plant is complex and can vary in a tissue- and organ specific way during different developmental stages [43]. Classification of secondary metabolites may be based on the chemical structure or biological characteristics of substances. In general, three big groups of secondary compounds can be assigned: terpenoids (derived from acetyl coenzyme A or glycolysis cycle intermediates), phenolics (aromatic rings

bearing a hydroxyl functional group) and alkaloids (nitrogen containing compounds). Their number in plants is estimated to be more than 500,000 [44].

These pharmacologically active compounds are derived either from different organs like leaves, stem, bark, root, flower, seed or whole medicinal plant, which are required for herbal drug formulations. As per the estimates, world market for plant derived drugs by year 2050 is likely to reach ~US $5 trillion. Therefore, the demand of high-yield/high-quality medicinal plants will continue to increase in future. Today, only 10% of all medicinal plant species used are cultivated, with by far the larger majority being obtained from wild collections [45]. The rising demand of plant-based drugs is creating heavy pressure on some selected high-value medicinal plant populations in the wild. Harvesting from the wild has led to loss of genetic diversity and habitat destruction of many plant species. Slow growth rate, complex accumulation patterns, low population densities and narrow geographic ranges of medicinal plants are making them more prone to extinction [46]. Also, uneconomical chemical synthesis (mainly for large complex molecules), non-availability of uniform and unadulterated quality plant material in sufficient quantities impedes in meeting the ever increasing industrial demands. Therefore, biotechnological tools offer an excellent platform for production of desirable natural products by enhancing their biosynthesis and accumulation. Plant tissue culture techniques in combination with molecular techniques can help in understanding the process of biosynthesis and accumulation of secondary metabolites along with the multiplication and conservation of medicinal plants.

Out of many important medicinal plants in present era, *Picrorhiza* genus finds a key position for conservation and enhancement of secondary metabolite production. The current status of literature on various aspects of *Picrorhiza* research has been reviewed as under:

2.1 *Picrorhiza*

The genus *Picrorhiza* belongs to the tribe Veroniceae of Scrophulariaceae family. This family is arranged in the order Scrophulariales, subclass Asteridae and class Dicotyledonae of the Angiospermae according to the taxonomical system of Cronquist. The genus *Picrorhiza* was

considered monotypic, with only *P. kurroa* species, until Pennell [47] noted *P. scrophulariiflora* (originally written as "*P. scrophulariaeflora*") the second species of this genus. Recently, *Picrorhiza tungnathii* has also been identified in Western Himalayas from Uttarakhand, India [48]. In Indian languages, the common names of *P. kurroa* and *P. scrophulariiflora* are 'Kutki' and 'Nepalese Kutki', respectively. Both species are quite similar in morphology and known by same vernacular names. Their vernacular names include 'Kutki' in Nepali and Hindi, 'Katuki' in Bengali, 'Karu' in Punjabi, 'Putising' in Dzongkha; 'Katurohini' in Sanskrit and 'Hellebore' in English [1]. It is called 'Hun-hunglien' in China, 'Kaur' in Kashmir Himalayas, 'Kadu' in Himachal Himalayas and 'Kadvi' in Uttarakhand Himalayas. Both species grow in Himalayan region in rocky slopes as well as in organic soils but are mostly found on the rocky crevices.

2.2 P. kurroa

P. kurroa is a perennial herb and propagates through seeds and stolons. Stolons eventually mature into a rhizome with independent shoots and roots. These separate looking plants at above ground level are actually joined together by stolons and when get detached from the mother stock, becomes independent plants (Fig. 2.1) [49]. It yields off-shoots of 5-9 cm in length from joints of rhizomes with leaf blades 7–11 cm in length. Leaves are basal, spathulate to narrow elliptic, coarsely saw-toothed and narrowed to a winged stalk. Its flowers are sessile, zygomorphic, bilipped, bisexual, pale or purplish blue in color and appear in June through August. Corolla is 4-5 mm long, 5-lobed, nearly actinomorphic and stamens are didynamous with 2 long (15.50 mm) and 2 short (13.25 mm) stamens. The flower architecture prefers mostly cross-pollination but self-pollination also occurs to some extent. Manual harvesting of the plant takes place in October through December [1, 49-50].

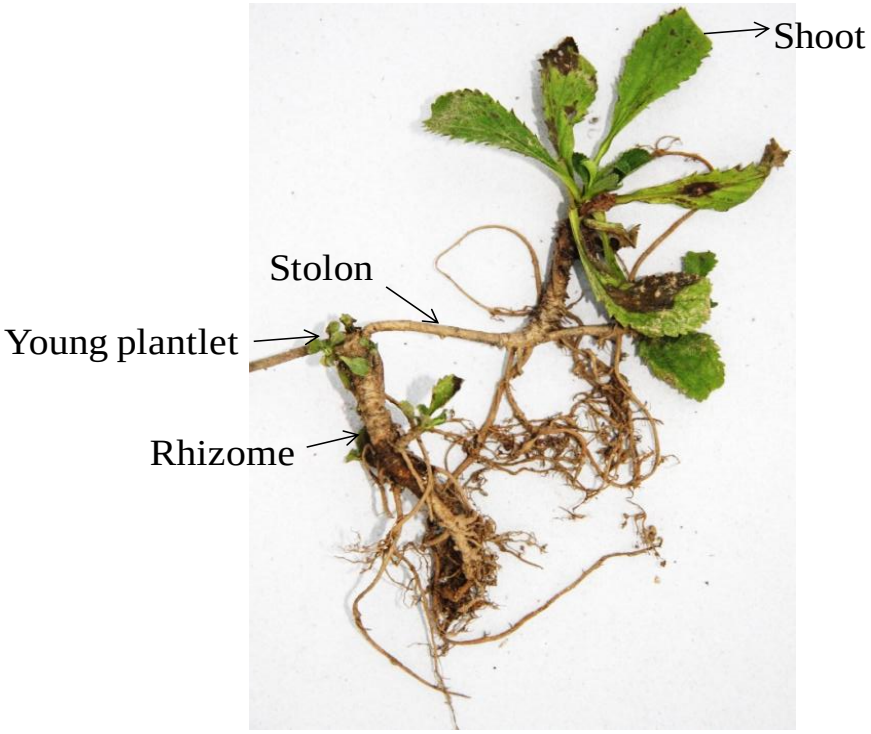


Fig: 2.1 Mature *P. kurroa* plant showing different organs

2.3 *P. scrophulariiflora*

P. scrophulariiflora is about 10-20 cm in height and have stout creeping rootstock with jointed and zigzag growing underground rhizomes. Propagation occurs through seeds and rhizomes. Its leaves are basal, oblanceolate or narrowly spathulate, serrated in upper half and 10–14 cm in length. Flowers are dark blue-purple in color. Corolla is 9-10 mm long, 4-lobed, bilabiate and stamens are slightly didynamous and equalling corolla in size. Flowering time is from July through August. Fruits are capsule of 6-10 mm and fruiting occurs is October through November. Seeds are pale brown in color and 1 x 0.8 mm in size. Harvesting time is September through December [1, 51-52]

2.4 Geographical distribution

P. kurroa grows naturally in dry western Himalayan region and can be found from Kashmir to Kumaon [47] and Pakistan to Uttarakhand [51] at 3000-4300 m altitudes, while *P.*

scrophulariiflora is distributed from moist Eastern Himalaya to mountains of Yunnan at 4300-5200 m altitudes [47] (Fig. 2.2; Table 2.1).

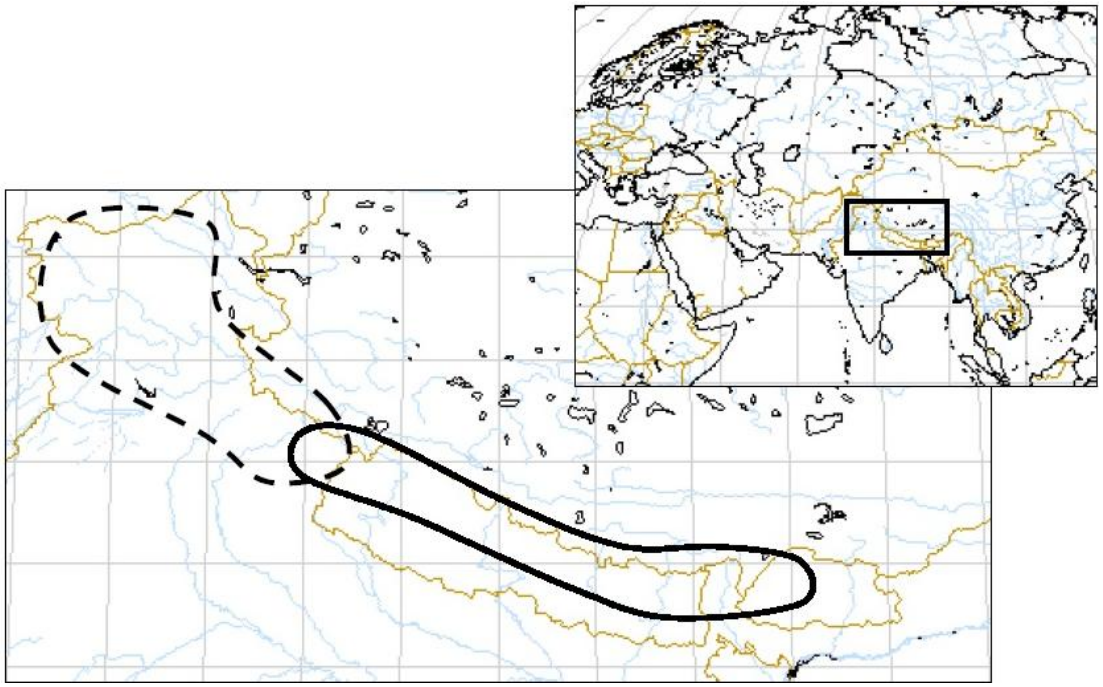


Fig. 2.2 Distribution of *Picrorhiza* species in the Himalayan region: — — *P. kurroa*; —— *P. scrophulariiflora* [50]

Table 2.1 Distribution of *P. kurroa* and *P. scrophulariiflora* in the Himalayan region

	State/Country	Location	Reference
P. kurroa	Jammu & Kashmir	Pir Panjal range	[53-54]
		Gumri , Kolohoi, Zojpal, Sonsa Nag	[55-56]
	Himachal Pradesh	Pangi, Bharmour, Lahaul, Dauladhar valleys	[57]
		Lahaul, Kinnaur, Kulu, Rohru, Kangra, Pangi, Bharmour	[58]
		GHNP, Dhauladhar WLS, Kugti WLS	[59-60]
	Uttarakhand	Harsil, Raithal, Sukhi, Sayara, Tehri-Garhwal in Bhagirathi Valley	[61]

		Kedarnath, Har-ki-dun, Ponwati, Tali, Harshil, Gangotri in Garhwal Hills	[54]
		Kumaon hills	[53]
		VOF NP, Kedarnath WLS, NDBR, alpine ranges of Ralam, Dhauli, Kali valleys	[62-64]
P. scrophulariiflora	Nepal	Bagmati zone, Rasuwa, Langtang, Gorkha, Gosaikunda, Jaljale Himal, Jaljale–Tin Pokhari, Jumla, Karnali, Laurivinayak, Murkhagari, Thaple Himal	[50, 65-67]
	Sikkim	Naku Chhu, North Sikkim, East Sikkim, Zemu valley, Llonakh, Choktsering Chhu, North of Jongri, Gamothang (Gopethang), Jongri (Dzongri), Jongri-Olothang, Preig Chu–Jongri (Prek Chhu)	[47, 53, 68-69]
	Bhutan	Bhutan	[70]

2.5 Phytochemistry of *P. kurroa* and *P. scrophulariiflora*

The genus *Picrorhiza* has been widely studied for its various phytoconstituents. A total of 132 constituents have been identified from different parts of plants such as rhizomes, roots, leaf, stem and seeds [3]. *P. kurroa* and *P. scrophulariiflora* have similar chemical composition but *P. scrophulariiflora* contains additional compounds such as phenylethanoids, glycosides and plantamajoside [1, 50, 71]. *Picrorhiza* species have been widely used in pharmaceuticals due to presence of various active constituents. The major classes of chemical compounds isolated from these species are iridoid glycosides, cucurbitacins, phenolic and phenylethanoids (Table 2.2). P-I and P-II are the two important iridoid glycosides of *Picrorhiza* species (Fig. 2.3) which are used in various herbal formulations. The basic structure of P-I and P-II is same except P-I having cinnamate moiety and P-II having vanillate moiety. Kutkin and Picroliv are the main herbal

preparations of *P. kurroa*. Kutkin is a mixture of P-I and kutkoside in a ratio of 1:2 and other minor glycosides [72-73], whereas Picroliv is a similar but less purified fraction, having about 60% of an equal mixture of Picroside-I and kutkoside [74].

Table 2.2 Major chemical constituents isolated from *Picrorhiza* species

Class	***P. kurroa***		***P. scrophulariiflora***	
	Compound	**Reference(s)**	**Compound**	**Reference(s)**
Iridoid	Picroside I	[75]	Picroside I	[76]
	Picroside II	[77]	Picroside II	[76]
	Picroside III	[78]	Picroside III	[76]
	Picroside V	[79]	Picroside IV	[71]
	Minecoside	[80]	Minecoside	[71]
	Catalpol	[81]	Catalpol	[17]
	Kutkoside	[72]	Specioside	[71]
	6-Feruloylcatalpol	[80]	6-Feruloylcatalpol	[71]
	Pikuroside	[82]	Pikuroside	[83]
	Veronicoside	[80]	Acubin	[84]
			Verminoside	[71]
Cucurbitacins	Arvenin	[80]	Arvenin	[84]
	2-β-glucosyloxy-3,16,20,25-tetrahydroxy-9-methyl-19-norlanosta-5, 23-diene-22-one	[80]	2β-glucopyranosyloxy-3, 16,20,22-tetrahydroxy-9-methyl-19-norlanosta-5,24-diene	[17]
	2-β -glucosyloxy-3,16, 20,25-tetrahydroxy-9-	[80]	2-(β-D-glucopyranosyloxy)-3,16,20,25-	[71]

	methyl-19-norlanost-5-ene-22-one		tetrahydroxy-9-methyl-19-norlansota-5-ene -22-one	
	25-Acetoxy-2-β-glucosyloxy-16,20-dihydroxy-9-methyl-19-norlanosta-5,23-diene-3,11,22-trione (2-O-glycolside of cucurbitacin B)	[80]	25- Acetoxy-2β glucopayanosyloxy-3,16, 20-trihydroxy-9-methyl-19-norlansota-5,23diene-22-one	[17]
Phenolics	Picein	[80]	Picein	[85]
	Androsin	[80]	Androsin	[85]
	Ellagic acid	[86]	Bergenin	[87]
	Vanillic acid	[88]	Catechin	[89]
	Apocyanin	[90]	Umbelliferon	[84]
			Luteolin	[89]
Phenylethanoids			Scroside A	[71]
			Scroside B	[71]
			Scroside C	[71]
			Scroside D	[91]
			Scroside E	[91]
			Scroside F	[84]
			Scroside G	[89]
			Plantamajoside	[71]

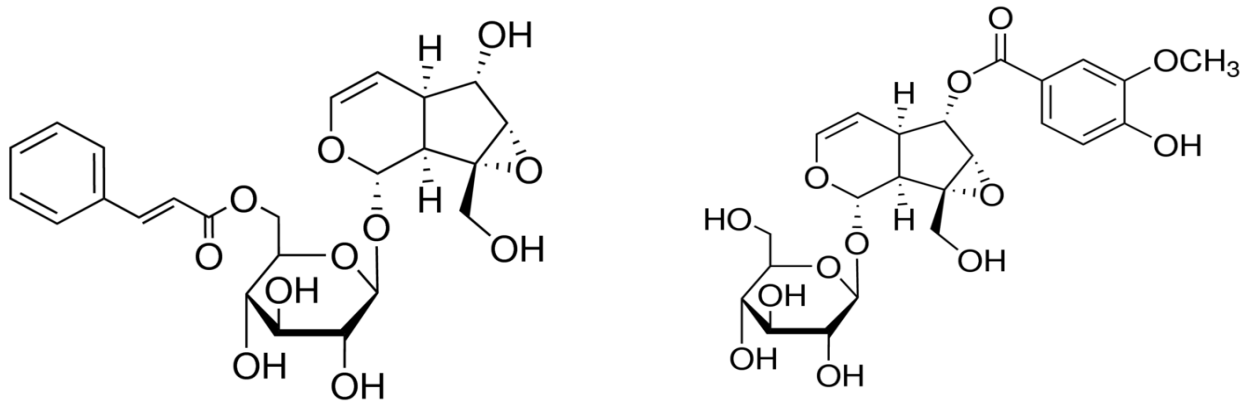


Fig. 2.3 Chemical structures of Picroside-I and Picroside-II

2.6 Medicinal properties of *P. kurroa* and *P. scrophulariiflora*

Picrorhiza species have been used in the traditional as well as in modern systems of medicine. *P. kurroa* is used for the treatment of various disorders including liver ailments, fever, asthma and jaundice, gastrointestinal and urinary problems, leukoderma, snake bite, scorpion sting etc. [3]. Its extracts have anti-inflammatory [92], hepatoprotective [93], immunomodulatory [94], free radical scavenging [95], anti-allergic and anti-anaphylactics [96] and anti-hepatitis B surface antigen activities [97]. *P. scrophulariiflora* is used in Tibetan and Chinese traditional medicines to treat various ailments and have pharmaceutical value for hepatoprotective [83], immunomodulator [98], antidiabetic [99], antioxidant [100], antimalarial [101] and neuroprotective [102] activities. Some of the medicinal properties of *P. kurroa* and *P. scrophulariiflora* extract are listed in Table 2.3.

Table 2.3 Major medicinal properties of *P. kurroa* and *P. scrophulariiflora*

Property	***P. kurroa***	***P. scrophulariiflora***
Hepatoprotective	[93]	[83]
Antioxidant	[100]	[100]
Immunomodulatory	[94]	[98]
Antimalarial	[103]	[101]
Anti-inflammatory	[92]	[104]
Anticancerous	[105]	-
Neuroprotective	[106]	[102]
Antiasthmatic	[107]	-
Antidiabetic	[108]	[99]

2.7 Medicinal properties of P-I and P-II

2.7.1 P-I

P-I is biosynthesized in shoots of *P. kurroa* and *P. scrophulariiflora* [3, 11]. P-I has been found to be effective against jaundice and chronic liver injury induced by carbon tetrachloride. It enhances humoral immune response and is reported to cure hepatitis B [19]. Singh et al. [109] have demonstrated anti-inflammatory activity of P-I in different test models. Rathee et al. [21] have reported anti-invasion activity of P-I against MCF-7 cell lines (human breast cancer). It inhibits MCF-7 cell invasion, migration and down-regulation of the expression of matrix metalloproteinases (MMPs) at mRNA and protein level.

2.7.2 P-II

Biosynthesis of P-II occurs in roots or stolons of *P. kurroa* and *P. scrophulariiflora* [3, 18]. P-II reduces the content of free radicals and enhances the activity of antioxidase and GSHPx, thus preventing cerebral ischemic injury in rats [110-111]. Guo et al. [112] have shown that P-II

downregulates the expression of TLR4, NFκB and TNFα by hampering apoptosis and inflammation induced by cerebral ischemic reperfusion injury, thereby improving the neurobehavioral function of rats. P-II has also been reported to protect the cardiomyocytes from hypoxia induced apoptosis [113].

2.8 Molecular basis of picrosides biosynthesis

The biosynthesis and accumulation of terpenoids is controlled by structural and regulatory genes in different plant species [114]. Various studies have been taken up to understand the molecular basis of picrosides biosynthesis. Kawoosa et al. [25] cloned two regulatory genes of terpenoid metabolism *viz.* 3-hydroxy-3-methylglutaryl coenzyme A reductase (pkhmgr) and 1-deoxy-D-xylulose-5-phosphate synthase (pkdxs) from *P. kurroa*. Further eight full-length cDNA sequences from MEP and MVA pathway including, 1-deoxy-D-xylulose-5-phosphate synthase (DXPS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXPR), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK), 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDS), acetyl-CoA acetyltransferase (ACTH), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), isopentenyl pyrophosphate isomerase (IPPI), geranyl diphosphate synthase (GDPS) and two partial sequences viz. PAL, COMT from phenylpropanoid pathway, were cloned and their expression analysis vis-à-vis picrosides content was carried out in different tissues [Singh et al. 2013]. Pandit et al. [22] cloned five genes of MEP and MVA pathway namely ISPD, MECPS, HDS, HMGS and PMK using comparative genomics and did expression analysis of all 15 genes of these pathways vis-à-vis picrosides content in different tissues of *P. kurroa.* Kumar et al. [26] for the first time proposed a plausible complete biosynthetic pathway for picrosides biosynthesis in *P. kurroa* using a bio-retrosynthetic approach by assembling the biosynthetic pathway i.e. from end-product to their precursor. Bhat et al. [115] cloned phenylalanine ammonia lyase (PAL), an important rate-limiting gene from phenylpropanoid pathway and observed its expression pattern in relation to picrosides content in different tissues of *P. kurroa.* Recently, Shitiz et al. [23] made an important endeavour towards picrosides biosynthesis in *P. kurroa* by completely elucidating the P-I biosynthetic pathway using NGS transcriptomes and enzyme inhibitor studies. Kumar et al. [27] have also determined the gene expression patterns of primary

and secondary metabolic pathway genes vis-à-vis P-I content in in vitro grown shoots of *P. kurroa* at different time intervals *viz.* 0, 10, 20, 30 and 40 days.

2.9 Tissue culture status of *P. kurroa* and *P. scrophulariiflora*

Tissue culture techniques offer a useful tool for conservation of germplasm and mass propagation of threatened plant species [116-117]. These techniques also help in large scale production of high value medicinal plants for their commercialization and sustainable utilization in herbal industries. Mondal et al. [1] have reviewed the studies related to shoot proliferation for mass propagation of *P. kurroa* and *P. scrophulariiflora* under in vitro conditions (Table 2.4). First attempt for in vitro proliferation of *P. kurroa* was done by using shoot tips on MS medium supplemented with varying concentrations of Kinetin (KN) (3–5 mg/L) [118]. Later, stem cuttings on MS medium containing 6-benzyladenine (BA) (0.11–2.25 mg/L) alone or in combination with Indole-3-acetic acid (IAA) (0.02–0.2 mg/L) or gibberellic acid (GA3) (0.03–0.35 mg/L) [119]; runners, axillary shoots on MS medium containing BA at a lower concentration (0.23 mg/L) [120] have been used for the multiplication of *P. kurroa*. Sood and Chauhan [9] developed a low-cost micropropagation protocol for *P. kurroa*. Direct regeneration via shoot organogenesis from leaf explants derived from in vitro grown shoot cultures have been reported on Gamborg's B5 medium [121] supplemented with 3 mg/L KN and 1 mg/L indole-3-butyric acid (IBA) [14]. Indirect shoot organogenesis via callus was established from different explants including leaf, nodal and root segments of *P. kurroa*. MS media supplemented with 2, 4-dichlorophenoxyacetic acid (2, 4-D) (2 mg/L) + IBA (0.5 mg/L) led to high frequency callus formation and regeneration of shoots was observed in MS medium containing BA (2 mg/L) + KN (3 mg/L) [10, 122]. A regeneration protocol was standardized by using leaves from aseptic shoot cultures, raised from ex vitro leaves at 2.32 μM of KN [15]. Sharma et al. [123] reported that in vitro-derived nodal explants of *P. kurroa* cultured on MS medium supplemented with 0.11 mg/L thidiazuron (TDZ) along with 0.5 mg/L IBA developed somatic embryos. In vitro grown microshoots of *P. kurroa* were also encapsulated in alginate beads for their storage [13]. Recently, Chand et al. [124] have reviewed that High Altitude Plant Physiology Research Centre, HNB Garhwal University, Srinagar, Uttarakhand (India) has taken an initiative in cultivation of *P. kurroa* which is the first step towards reducing the pressure on wild medicinal plants

population, ensuring regular supply of raw material to industries and uplifting the economy of local farmers.

On the other hand, very limited work has been done on tissue culture of *P. scrophulariiflora*. A study by Bantawa et al. [4] have reported first time shoot proliferation of *P. scrophulariiflora* using shoot-tips and nodal explants at lower concentrations of KN (0.5 mg/L). Then, a reproducible in vitro regeneration system from leaf derived callus was developed for *P. scrophulariiflora* on a Woody Plant Medium (WPM) [125] supplemented with 0.1 mg/L 1-Naphthaleneacetic acid (NAA) and 0.05 mg/L KN [12]. Bantawa et al. [8] also achieved synchronous maturation of somatic embryos from leaf-derived callus by transferring these somatic embryos onto a solidified MS medium containing 0.5 mg/L ABA for 2 weeks, followed by transfer to a fresh MS medium containing 0.5 mg/L KN for another 4 weeks.

Cell cultures offer a suitable biological system for rapid production of plant metabolites of pharmaceutical importance [126]. However, limited information is available on biosynthesis and accumulation of P-I and P-II in *P. kurroa* and no report exist on picrosides production in *P. scrophulariiflora* under in vitro conditions. Sood and Chauhan [11] have reported the accumulation of P-I in shoot cultures of *P. kurroa* with no detection of P-II. The P-I content was 1.9, 1.5, and 0.04 mg/g dry weight in leaf sections, stem, and root segments, respectively, while P-I was absent in callus derived from these explants. Kawoosa et al. [25] have shown that picrosides biosynthesis is regulated by light and temperature in in vitro raised *P. kurroa* plants and found high picrosides content in shoots grown at low temperature (15°C) and under illumination as compared to 25°C and dark conditions. Sood and Chauhan [127] have also reported highest P-I accumulation in shoots grown at 15°C (2.03 mg/g) as compared to 0.12 mg/g in shoots formed at 25°C.

In the recent past, several studies related to optimization of genetic transformation protocols have been attempted in *P. kurroa*. Verma et al. [128] for the first time established *Agrobacterium rhizogenes* mediated hairy root cultures of *P. kurroa*. In terms of biomass and glycoside content, superior clone was obtained which superseded all other hairy root clones along with the non-transformed in vitro-grown control roots of *P. kurroa*. Mishra et al. [129] and Rawat et al. [130]

have also developed hairy root lines of *P. kurroa* through *A. rhizogenes*, for picrotin and picrotoxinin production. Recently, Verma et al. [131] have reported yield enhancement strategies for production of picroliv from hairy root cultures of *P. kurroa*. A protocol for genetic transformation using *A. tumefaciens* strain GV3101 harboring binary vector pCAMBIA1302 has also been developed in *P. kurroa* [14].

Table 2.4 In vitro propagation of *Picrorhiza* species

***Picrorhiza* Species**	**Objective**	**Explant type**	**Nutrient medium**			**Hardening and field transfer**	**References**
			Shoot initiation	**Shoot proliferation**	**Rooting**		
P. kurroa	Micropropagation	Shoot tips	MS + KN (5 mg/L) + IAA (1 mg/L)		MS + NAA(1 mg/L)	Survival of 86.7% was observed after transferring the rooted plantlets to clay pot containing sterile sand, soil	[118]
	Micropropagation	Terminal and nodal cuttings	MS + BA (0.69 mg/L)	MS + BA (0.23 mg/L)	MS + NAA (0.2 mg/L)		[119]
	Plant regeneration via callus mediated organogenesis	Leaf and nodal cutting from mature plant	MS + 2, 4 D (0.5–2 mg/L) +NAA (4 mg/L) + KN (1 mg/L)	MS + BA (0.25 mg/L)	MS + NAA (0.2 mg/L)	Four-week-old plantlets were successfully established in soil	[132]
	Micropropagation	Nodal	MS + BA (0.23 mg/L)		MS + IBA		[120]

		segments			(3 mg/L)		
	Development of a low cost micropropagation technology	Shoot apices	MS + IAA (2) + KN (3 mg/L) + table sugar (3%)		MS + IBA (3 mg/L) + table sugar (3%)		[9]
	High frequency callus induction and plantlet regeneration	Leaf disc, nodal segment and root segments	MS + 2,4 D (2 mg/L) + IBA (0.5 mg/L) for callus induction	MS + BA (2 mg/L) + KN (3 mg/L)	MS + IBA (3 mg/L)	Hardening mixture used was sand: soil: Vermiculite (1:1:1)	[10]
	Micropropagation	Nodal segments		MS + NAA (0.6 mg/L)	MS + NAA (0.4 mg/L) + IAA (0.1 mg/L) + IBA (0.5 mg/L)	The survival percentage was 81.5% in hardening chamber	[133]
	Micropropagation and somatic embryogenesis	Nodal segment, leaf tissue	MS + KN (2 mg/L) + IBA (0.5 mg/L)	MS + KN (2 mg/L) + IBA (0.5 mg/L)	MS + IBA (1 mg/L)		[123]

	Micropropagation via synthetic Seeds	In vitro derived leaf, shoot tips, nodal segments	Nodal segments on MS + TDZ (0.11 mg/L) and IBA (0.5 mg/L) for somatic embryos		MS + NAA (1 mg/L)		[13]
	Plant regeneration via adventitious (de novo) shoot organogenesis	Leaf explants from in vitro shoot cultures	B5 + KN (3 mg/L) + IBA (1 mg/L)	B5 + KN (3 mg/L) + IBA (1mg/L)	B5 + KN (3mg/L) + IBA (1 mg/L) + activated charcoal (1%)		[14]
	A regeneration protocol was standardized	Leaf	MS + TDZ (0.5 μM)	MS + KN (2.32 μM)	Rooting was observed on PGR free medium.	The survival of plants grown at 25°C was higher (80%) under green house conditions as compared to plants grown at 25°C (50%)	[15]
P. scrophulariiflora	Micropropagation	In vitro shoot tips,	WPM + BA (0.05 mg/L)		MS + NAA (1mg/L)	The regenerated plantlets were	[4]

		nodal segments				hardened in plastic cups containing 9:1 virgin soil and sand with 97% survival	
	Micropropagation	Shoot tips, nodal segments	WPM + BA (0.05 mg/L)	MS + KN (0.5 mg/L)	MS + NAA (1mg/L)	Rooted plantlets were transferred to plastic cups containing sterile virgin soil and sand (9:1)	[8]
	Plant regeneration via callus mediated organogenesis	In vitro leaf	WPM + NAA (0.1mg/L) + KN (0.05 mg/L)	WPM + BA (0.1 mg/L)	WPM + NAA (1 mg/L)	Survival rate was 90% and well hardened plants were distributed to the local farmers for planting	[12]
	Plant regeneration via somatic embryogenesis	In vitro leaf derived callus	MS + BA (0.1–2.0 mg/L)	MS + ABA (0.1–1.0 mg/L)		Survival rate was 82%	[8]

2.10 Morphogenesis and developmental status vis-à-vis picrosides biosynthesis

Plants synthesize large numbers of different secondary metabolites out of which some are restricted to specific tissues or organs, e.g. benzoyloxylated glucosinolates and proanthocyanids are only found in seeds [134-135]. Biosynthesis and accumulation of metabolites is largely influenced by the developmental stage of a particular organ/tissue as well as in response to external stimuli [28]. Site-specific accumulation of metabolites suggests specialized functions in different stages of plant development [136]. There are very few reports showing the influence of growth and developmental stages of *P. kurroa* on P-I content, while no such study has been reported for *P. scrophulariiflora*. The biosynthesis of P-I and P-II is known to occur differentially in shoots and roots of *P. kurroa* wherein P-I accumulates preferentially in shoots and P-II in roots of field grown plants of *P. kurroa* [11, 18]. Sood and Chauhan [11] showed that P-I biosynthesis occur in shoots rather than undifferentiated mass of cells. Pandit et al. [18] have analyzed P-I and P-II contents in field grown plants of *P. kurroa* and found 0.05% to 0.76% P-I content in shoots; 0.15–0.50 % P-I and 0.1–0.45 % P-II content in rhizomes of different developmental stages. However, molecular studies can provide a clear picture of P-I biosynthesis. Various environmental factors such as altitude, light, temperature, seasonal variations are known to influence the biosynthesis and accumulation of secondary metabolites in plants grown in wild, thus complicating the process of understanding biology of P-I biosynthesis. Hence, cell culture techniques along with molecular approaches could be efficiently utilized for better understanding of P-I biosynthesis at different developmental stages of *P. kurroa* and *P. scrophulariiflora*.

2.11 Elicitors and biostimulants

2.11.1 Elicitors

Plants grown under tissue culture conditions can be used as a promising alternative for metabolites which are not easily available through chemical synthesis or extraction methods. However, one of the major obstacles is the low yield of plant secondary metabolites in plant cell

cultures. Elicitors have been widely used for production of metabolites under in vitro conditions [35]. These are chemicals or biofactors from various sources that can induce physiological and morphological responses and secondary metabolites accumulation in plants and/or plant cells in vitro. Elicitation is the process of inducing or enhancing secondary metabolites biosynthesis by plants to ensure their survival, persistence and competitiveness. Various elicitors such as ABA, MeJa, SNP, salicylic acid, hydrogen peroxide, yeast extract, chitin, chitosan etc. have been investigated for plant based secondary metabolite production [35, 137]. Elicitors enhance secondary metabolites content by triggering signal transduction process that regulates gene expression for biosynthesis of plant secondary metabolites [35]. There are very few reports on use of elicitors in *Picrorhiza* species for secondary metabolite enhancement. Recently, Rawat et al. [138] have studied the effect of MeJa and yeast extract on production of picrotin and picrotoxinin and found yeast extract more efficient than MeJa for their production in roots of *P. kurroa*. Singh et al. [36] have also studied the effect of different modulators *viz.* hydrogen peroxide, MeJa, ABA and salicylic acid on MVA/MEP pathway genes for enhanced production of P-I in *P. kurroa,* but their effect remained unclear in vivo. These findings therefore, will not benefit micropropagation of these plant species with high P-I content.

2.11.2 Biostimulants

Various biostimulants are also known to enhance plant growth along with secondary metabolites content in plant species. Plant biostimulants contain substance(s) whose function is to stimulate natural processes to enhance nutrient uptake, nutrient efficiency, tolerance to abiotic stress, facilitating nutrient assimilation, translocation and use, enhance secondary metabolite production, improve plant quality and yield [139]. Biostimulants are available in a variety of formulations with varying ingredients and include humic substances, amino acid containing products and hormone containing products like seaweed extracts [140]. There is no report till date on use of any biostimulant for enhancing plant growth and secondary metabolite production on *Picrorhiza* species.

2.12 Seaweed extracts

Seaweed extracts have been used for long for the improvement of plant growth and development as well as secondary metabolite production [32, 141]. Seaweeds are macroscopic, multicellular marine alga and their extracts are widely used as biostimulants for the growth and development of plants [142]. Commercially available seaweed extracts are mainly derived from brown and red alga such as *Ascophyllum nodosum*, *Macrocystis pyrifera*, *Ecklonia maxima*, *Lithothamnium calcareum*, *Porphyra perforate*, *Kappaphycus alvarezii*, *Gracilaria salicornia*, *Gelidiella acerosa* [32-33] and are reported to contain macronutrients, micronutrients, amino acids, vitamins, cytokinins, auxins, gibberellins, carbohydrates, betaines, and abscisic acid (ABA)-like growth substances [32, 143-146]. A number of commercial seaweed extract products available for use in agriculture and horticulture are reviewed by Khan et al. [32] and have been listed in Table 2.5.

Table 2.5 List of commercially available seaweed extract products

Product name	**Seaweed name**	**Company**
Acadian®	*Ascophyllum nodosum*	Acadian Agritech
Agri-Gro Ultra	*A. nodosum*	Agri Gro Marketing Inc.
AgroKelp	*Macrocystis pyrifera*	Algas y Bioderivados Marinos, S.A. de C.V.
Alg-A-Mic	*A. nodosum*	BioBizz Worldwide N.V.
Bio-Genesis™ High Tide™	*A. nodosum*	Green Air Products, Inc.
Biovita	*A. nodosum*	PI Industries Ltd
Emerald RMA	Red marine algae	Dolphin Sea Vegetable Company
Espoma	*A. nodosum*	The Espoma Company
Guarantee®	*A. nodosum*	Maine Stream Organics
Kelp Meal	*A. nodosum*	Acadian Seaplants Ltd
Kelpak	*Ecklonia maxima*	BASF
Kelpro	*A. nodosum*	Tecniprocesos Biologicos, S.A. de

		C.V.
Kelprosoil	*A. nodosum*	Productos del Pacifico, S.A. deC.V.
Maxicrop	*A. nodosum*	Maxicrop USA, Inc.
Nitrozime	*A. nodosum*	Hydrodynamics International Inc.
Profert®	*Durvillea Antarctica*	BASF
Sea Winner	Unspecified	China Ocean University Product Development Co., Ltd
Seanure	Unspecified	Farmura Ltd.
Seasol®	*D. potatorum*	Seasol International Pty Ltd
Soluble Seaweed Extract	*A. nodosum*	Technaflora Plant Products, LTD
Stimplex®	*A. nodosum*	Acadian Agritech
Synergy	*A. nodosum*	Green Air Products, Inc.
Powdered seaweed extract	*Kappaphycus alvarezii*	Sea6 Energy Pvt Ltd

Previous reports have highlighted the importance of seaweed extracts on plant growth and development wherein seed germination in *Triticum aestivum* [147]; in vitro mass propagation of *Lycopersicon esculentum* L. [30] and brinjal [33]; improved yield and fruit quality in winter rapeseed [148], *Malus domestica* [149], strawberry [150], tomato [151-152], spinach [153], okra [154], *Olea europaea* [155], and broccoli [156]; lateral root formation in maize [157], tomato [158], Arabidopsis [29], grape [159], strawberry [150], winter rapeseed [148], Norway spruce [160], *Pinus contorta* [161] and leaf chlorophyll content was enhanced upon their treatment [148, 153, 162]. Jannin et al. [148] assessed the effects of algal extract through microarray analysis and found majority of differentially expressing genes involved in photosynthesis and cellular metabolism. Studies have shown that seaweed extract treatment upregulated the expression of genes coding for proteins involved in N and S uptake, assimilation and transportation, which play major role in growth and development of plant [148, 163-164].

Seaweed extracts are also reported to enhance secondary metabolite production in variety of species including, phenolic and flavonoid compounds in *Brassica oleraceae* [31], free radical scavenging and iron chelating activities in *Kappaphycus Doty* [165], protection against viral, fungal and bacterial infections in *Nicotiana tabacum* and *Cicer arietinum* [166-168]. Fan et al.

[153] have correlated the increase in antioxidant capacity along with phenolics and flavonoid content in seaweed extract treated spinach with increase in transcript abundance of key enzymes involved in antioxidative capacity (glutathione reductase) and glycine betaine synthesis (betaine aldehyde dehydrogenase and choline monooxygenase). Activity of a key enzyme *viz.* Chalcone isomerase involved in biosynthesis of flavanone precursors and phenyl propanoid plant defense compounds also increased upon treatment with seaweed extract. Seaweed extracts have been shown to alleviate a variety of abiotic stresses including drought, salinity and extreme temperature by inducing genes that code for proteins that are regulatory in nature such as transcription factors, protein kinases, phosphatases and other proteins which directly protect against stress, including osmoprotectants, detoxifying enzymes and transporters [139]. The modes of action of seaweed extracts are not yet well understood, but presence of bioactive molecules such as plant growth hormones, unique polysaccharides, polyphenols, betaines, oligo-alginates (brown algae), oligo-carrageenans (red algae) may play an important role in growth and secondary metabolite production [31, 139, 148, 153].

Overall, various studies have been reported on clonal propagation of *P. kurroa* and *P. scrophulariiflora* but limited efforts have been made for cost effective micropropagation of quality plant material with enhanced shoot biomass and secondary metabolite content which can be used for the cultivation of these species, thereby can aid in their conservation. Also, molecular studies are required for better understanding of P-I biosynthesis and ascertaining the role of genes involved in P-I production in *Picrorhiza* species under in vitro conditions which can aid in enhancement of secondary metabolite content.

Thus, the review of literature has shown the following research gaps:

- No information on molecular basis of improving shoot biomass and P-I biosynthesis in different morphogenetic stages of *P. kurroa* and *P. scrophulariiflora*
- Limited efforts on development of rapid and cost effective approach for enhancement of shoot biomass vis-à-vis P-I production under in vitro conditions in *P. kurroa* and *P. scrophulariiflora*
- No information on use of seaweed extract as a biostimulant and/or media replacement for in vitro micropropagation and P-I production in *P. kurroa* and *P. scrophulariiflora*

CHAPTER 3

MATERIAL AND METHODS

3.1 Plant material

P. kurroa and *P. scrophulariiflora* were procured from Himalayan Forest Research Institute, Jagatsukh, Manali, H.P., India and National Bureau of Plant Genetic Resources, New Delhi, India, respectively. Both species were cultured and maintained in plant tissue culture chambers with subculturing after every 4 weeks at 25 ± 2 °C and 15 ± 2 °C with 70% relative humidity, 16 h day/8 h night photoperiod at photosynthetic photon flux density of 40 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Philips, India) on MS medium supplemented with IBA (3 mg/l), KN (1 mg/L), sucrose (30 g/L) and agar (9 g/L). The pH was adjusted to 5.7 using 0.1 N HCl or 0.1 N NaOH and 50 ml of medium was dispensed in each jar prior to autoclaving at 121°C, 15 lb $inch^{-2}$ pressure for 20 minutes [10].

3.2 Callus induction

Leaf and root segments (0.5-1 cm each) from 4 to 5 weeks old in vitro grown *P. kurroa* and *P. scrophulariiflora* cultures were used for callus induction. MS media having different growth hormone combinations, sucrose (30 g/L) and agar (9 g/L) were prepared. Different growth hormones used were: 2, 4-D (0.50, 1.00, 1.50, 2.00 mg/L) and/or IBA (0.50, 1.00, 1.50, 2.00 mg/L) and TDZ (0.25, 0.50 and 1.00 mg/L). In all the experiments, 5-6 leaf and root explants were taken from both plant species maintained in two plant growth chambers at 25 ± 2 °C and 15 ± 2 °C incubation temperatures. These explants were inoculated on different media combinations at aforementioned incubation temperatures, respectively for 4 weeks. Medium without growth hormones served as control. Callus initiation started in TDZ containing medium within 10-15 days and callus mass was achieved in next 15 days for both species at 25 ± 2 °C and 15 ± 2 °C.

Cultures were then transferred to shoot induction media. The experiment was performed in triplicates and repeated thrice. Samples of both species from different explants at 25 ± 2 °C and 15 ± 2 °C for different stages *viz.* explants (EX), callus initiation (CI) and callus mass (CM) stages were frozen and kept in -80°C for further experimentation.

3.3 Plant regeneration

The calli initiated from different explants at 25 ± 2 °C and 15 ± 2 °C of both plant species were then transferred to MS media supplemented with IBA (1.0, 2.0 mg/L) and/or BAP (0.5, 2.0 mg/L) and/or KN (0.2, 0.5, 1.0, 2.0, 3.0 mg/L) for regeneration and multiple shoot formation. Sucrose (30 g/L) and agar (9 g/L) were invariably added to the media. Cultures were incubated at same conditions as described earlier for 4-6 weeks. Medium without growth hormones served as control. Regenerated multiple shoots were then transferred to MS medium supplemented with IBA (3 mg/L), KN (1 mg/L), sucrose (30 g/L) and agar (9 g/L) for shoot elongation, root induction and full growth of both plant species. The experiment was performed in triplicates and repeated thrice. Samples of both species from different explants at 25 ± 2 °C and 15 ± 2 °C for different stages *viz.* shoot primordia (SP), multiple shoots (MS) and fully developed (FD) stages were frozen and kept in -80°C for further experimentation.

3.4 Preparation of MS media supplemented with seaweed extract as a biostimulant (MSS)

Seaweed extract (SWE) (Appendix Table A1) obtained from red seaweed *Kappaphycus alvarezii* was provided by Sea6 Energy Pvt Ltd. (Bangalore, India). Stock solution of SWE was prepared by dissolving 10 g of soluble SWE powder in 10 ml of distilled water by constant stirring with magnetic stirrer for 15 minutes followed by filter sterilization with 0.22 µm syringe filters. Further, MSS having sucrose (30 g/L), agar (9 g/L), different concentrations of SWE (0.1, 1.0, 2.0, 3.0 g/L) alone (MSS1 to MSS4) (Table 3.1) and in combination with growth hormones (IBA: 3 mg/l and KN: 1 mg/l) (MSS5 to MSS8) (Table 3.2) were prepared. MS media without growth hormones (C1) and with growth hormones (SM) were used as control media for analysis. In vitro grown *P. kurroa* and *P. scrophulariiflora* shoots (0.5-1.0 cm) were cultured on the above media combinations in tissue culture chambers maintained at 25 ± 2 °C and 15 ± 2 °C and data

was recorded for total plant length, shoot biomass, number of shoots and roots after 10^{th}, 20^{th} and 30^{th} day. Further, to compare the effect of different seaweed extracts on growth of *P. kurroa* and *P. scrophulariiflora*, shoots of both species were cultured on MS media supplemented with optimized concentration of SWE (*Kappaphycus alvarezii*) and different concentrations of Biovita – an *Ascophyllum nodosum* extract (PI Industries Ltd, Udaipur, India) (MSB1: 0.1% and MSB2: 0.2%) obtained from (Table 3.3). Data was recorded in triplicates and repeated thrice for total shoot biomass after 10^{th}, 20^{th} and 30^{th} day at 25 ± 2 °C and 15 ± 2 °C.

Table 3.1 MS medium supplemented with different concentrations of SWE (g/L), sucrose (30 g/L) and agar (9 g/L)

S. No	Medium name	Medium composition
1.	MSS1	MS + SWE (0.1) + sucrose + agar
2.	MSS2	MS + SWE (1.0) + sucrose + agar
3.	MSS3	MS + SWE (2.0) + sucrose + agar
4.	MSS4	MS + SWE (3.0) + sucrose + agar
5.	C1	MS + SWE (0.0) + sucrose + agar

Table 3.2 MS medium supplemented with different concentrations of SWE (g/L), sucrose (30 g/L), growth hormones (3 mg/L IBA and 1 mg/L KN) and agar (9 g/L)

S. No	Medium name	Medium composition
1.	MSS5	MS + SWE (0.1) + sucrose + IBA + KN + agar
2.	MSS6	MS + SWE (1.0) + sucrose + IBA + KN + agar
3.	MSS7	MS + SWE (2.0) + sucrose + IBA + KN + agar
4.	MSS8	MS + SWE (3.0) + sucrose + IBA + KN + agar
5.	SM	MS + SWE (0.0) + sucrose + IBA + KN + agar

Table 3.3 MS medium supplemented with different concentrations of Biovita, sucrose (30 g/L), growth hormones (3 mg/L IBA and 1 mg/L KN) and agar (9 g/L)

S. No	Medium name	Medium composition
1.	MSB1	MS + Biovita (0.1%) + sucrose + IBA + KN + agar
2.	MSB2	MS + Biovita (0.2%) + sucrose + IBA + KN + agar

3.5 Preparation of SWE media (SWM) for *P. kurroa* and *P. scrophulariiflora* micropropagation

Shoot apices of *P. kurroa* and *P. scrophulariiflora* (0.5-1.0 cm) were taken and cultured on 6 different concentrations of SWE (0.01, 0.1, 1.0, 2.0, 3.0, 5.0 g/L), pH 5.7, agar (9 g/L) at 25 ± 2 °C and 15 ± 2 °C. Optimized concentration (2.0 g/L) of SWE was tested with different combinations of sucrose (30 g/L) and growth hormones (IBA: 3 mg/l, KN: 1 mg/l) on solid (with agar) and liquid (without agar) media (SWM1 to SWM8). Data was recorded in triplicates and repeated thrice for total plant length, shoot biomass, number of shoots and roots on 10^{th}, 20^{th} and 30^{th} day. Results were compared with similar combinations of MS media (MSM1 to MSM8) (Table 3.4).

Table 3.4 Different combinations of SWM (SWE 2 g/L) and control media with presence (+) and absence (-) of sucrose (30 g/L), growth hormones (3 mg/L IBA and 1 mg/L KN) and agar (9 g/L)

S. No.	SWM	SWM composition	Control media	Control media composition
1.	SWM1	SWE - sucrose - growth hormones - agar	MSM1	MS - sucrose - growth hormones - agar
2.	SWM2	SWE - sucrose - growth hormones + agar	MSM2	MS - sucrose - growth hormones + agar
3.	SWM3	SWE + sucrose - growth hormones - agar	MSM3	MS + sucrose - growth hormones - agar
4.	SWM4	SWE + sucrose - growth hormones + agar	MSM4	MS + sucrose - growth hormones + agar
5.	SWM5	SWE - sucrose + growth hormones - agar	MSM5	MS - sucrose + growth hormones - agar
6.	SWM6	SWE - sucrose + growth hormones + agar	MSM6	MS - sucrose + growth hormones + agar
7.	SWM7	SWE + sucrose + growth hormones - agar	MSM7	MS + sucrose + growth hormones - agar
8.	SWM8	SWE + sucrose + growth hormones + agar	MSM8	MS + sucrose + growth hormones + agar

3.6 Hardening of in vitro grown plantlets

In vitro grown rooted shoots of *P. kurroa* and *P. scrophulariiflora* were gently removed from culture vessels, washed under running tap water and transferred to pots containing sand:soil:vermiculite (1:1:1) for hardening in greenhouse. The plantlets were covered with glass jars for 10-15 days to avoid desiccation. Glass jars were taken off every day for 1-2 h to acclimatize them to external environment. Data was recorded in triplicates and repeated thrice for percent survival of plants.

3.7 Elicitor treatment

To compare the effect of various elicitors with SWE, different concentrations *viz.* 50, 100 and 200 µM of ABA (Sigma-Aldrich, USA), SNP (Sigma-Aldrich, USA) and MeJa (Sigma-Aldrich, USA) were filter sterilized and employed in optimized MS media. The optimum concentrations of elicitors were compared with 2 g/L SWE for plant growth and P-I production. *P. kurroa* and *P. scrophulariiflora* shoots grown at 25 ± 2 ºC were taken and cultured in the above mentioned media supplemented with elicitors at 15 ± 2 ºC. The shoot samples were collected after 30 days and data was recorded for total shoot biomass in both plant species. *P. kurroa* and *P. scrophulariiflora* shoots grown at 15 ± 2 ºC without any elicitor treatment were used as controls. The experiment was performed in triplicates and repeated thrice.

3.8 Quantification of P-I by HPLC

P. kurroa and *P. scrophulariiflora* fresh samples corresponding to different morphogenetic stages, shoots grown on different media combinations (Tables 3.1, 3.2 and 3.4) and different elicitors (SWE, ABA, SNP, MeJa) were subjected to P-I estimation by HPLC analysis. All the samples were ground in liquid nitrogen and 100 mg of powdered sample was percolated in 10 ml 80% methanol. The samples were vortexed, sonicated for 30 minutes at room temperature and filtered through 0.22 µm filter (Millipore). The filtrate was diluted 1:10 for estimation of P-I content by following the method described by Sood and Chauhan [11]. P-I quantification was done on Waters HPLC System equipped with Waters 515 HPLC pumps, Waters 717 autosampler, Waters 2996 photodiode array detector and Empower software. For analysis, 20 µl of sample was injected into Waters Spherisorb reverse phase C18 column (4.6 mm x 250 mm, 5 µm). The mobile phase used for the analysis was solvent A (0.05% trifluoro- acetic acid in water) and Solvent B (1:1 methanol/acetonitrile mixture). Solvent A and B were used in the ratio of 70:30 (v/v). The column was eluted in isocratic mode with a flow rate of 1ml/min at detection wavelength of 270 nm. The cycle time of analysis was 30 min at 30°C. The compound was identified on the basis of its retention time and comparison of UV spectra with P-I standard (Fig. 3.1) procured from Chroma Dex, Inc. and calculated in µg/mg fresh weight (FW). The experiment was performed in triplicates.

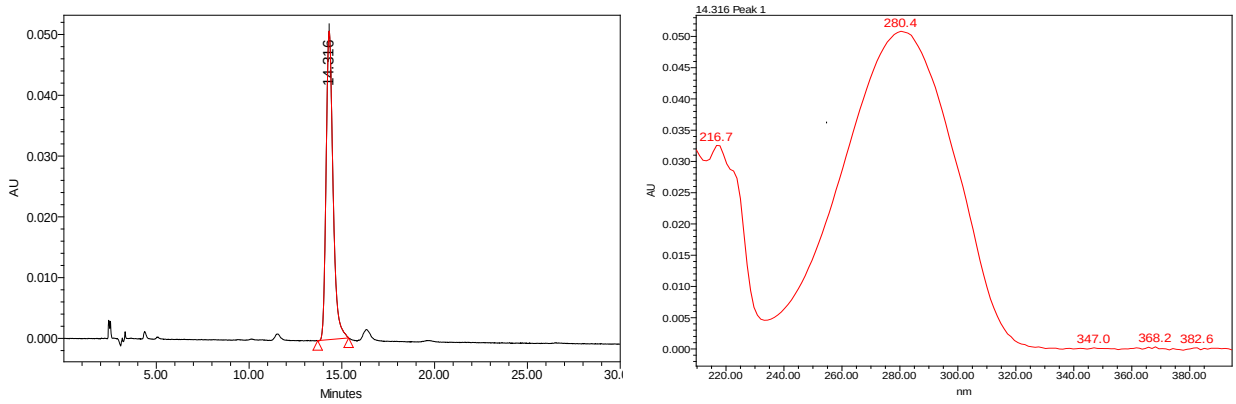


Fig. 3.1 HPLC chromatogram and absorption spectra of P-I standard

3.9 Selection of genes

Genes pertaining to primary and secondary metabolism in plants such as HK, PK of glycolysis; ICDH, MDH of TCA cycle; G6PDH of pentose phosphate pathway; RBA of photosynthetic apparatus; ARP, ARF7, CytO of hormone metabolism; HisK involved in signal transduction and HMGR, PMK of MVA pathway; DXPS, ISPD, ISPE of MEP pathway; GS, G10H, 10-HGO, IS of iridoid pathway; DAHPS and PAL of shikimate/phenylpropanoid pathway were selected to study their effect on different morphogenetic stages vis-à-vis shoot regeneration and P-I biosynthesis in *P. kurroa* and *P. scrophulariiflora* under in vitro conditions. Furthermore, to study the effect of different elicitors at molecular level, genes encoding enzymes HMGS, HMGR, PMK, DXPS, DXPR, ISPD, ISPE, MECPS, G10H and PAL were selected on the basis of their role as rate limiting enzymes for P-I production in both plant species (Table 3.5).

Table 3.5 Genes implicated in primary and secondary metabolism in different plant species

S. No.	Gene	Plant species	Reference
1.	3-Hydroxy-3-methylglutaryl-CoA synthase (HMGS)	*P. kurroa*	[22]
2.	3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR)	*P. kurroa*	[25]
3.	Phosphomevalonate kinase (PMK)	*P. kurroa*	[22]
4.	1-Deoxy-D-xylulose-5-phosphate synthase (DXPS)	*P. kurroa*	[25]
5.	1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXPR)	*P. kurroa*	[22]
6.	2-C-methylerythritol 4-phosphate cytidyl transferase (ISPD)	*P. kurroa*	[22]
7.	4-(Cytidine-5-diphospho)-2-C-methylerythritol kinase (ISPE)	*P. kurroa*	[22]
8.	2-C-methylerythritol-2, 4-cyclophosphate synthase (MECPS)	*P. kurroa*	[22]
9.	Geraniol synthase (GS)	*P. kurroa*	[23]
10.	Geraniol-10-hydroxylase (G10H)	*Ophiorrhiza pumila*	[169]
11.	10-Hydroxygeraniol dehydrogenase (10-HGO)	*P. kurroa*	[23]
12.	Iridoid synthase (IS)	*P. kurroa*	[23]
13.	3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS)	*A. thaliana*	[170]
14.	Phenylalanine ammonia lyase (PAL)	*P. kurroa*	[115]
15.	Hexokinase (HK)	*P. kurroa*	[27]
16.	Pyruvate kinase (PK)	*P. kurroa*	[27]
17.	Isocitrate dehydrogenase (ICDH)	*P. kurroa*	[27]
18.	Malate dehydrogenase (MDH)	*P. kurroa*	[27]
19.	Glucose-6-phosphate dehydrogenase (G6PDH)	*P. kurroa*	[27]
20.	RUBISCO activase (RBA)	*A. thaliana*	[171]
21.	Auxin response protein (ARP)	*A. thaliana*	[171]

22.	Auxin response factor 7 (ARF7)	*A. thaliana*	[171]
23.	Histidine kinase (HisK)	*A. thaliana*	[171]
24.	Cytokinin oxidase (CytO)	*A. thaliana*	[171]

3.10 RNA isolation and cDNA synthesis

Total RNA from *P. kurroa* and *P. scrophulariiflora* samples was isolated by using TRIzol® Reagent (Life Technologies, USA) according to the manufacturer's instructions and quality was assessed in 1% (w/v) ethidium bromide-stained agarose gel. RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). cDNA synthesis was done by using Verso cDNA synthesis kit (Thermo Scientific) from total RNA (1 µg) as per manufacturer's instructions. Concentration of each cDNA sample was adjusted to100 ng/µl for expression analysis.

3.11 Quantitative real time-PCR (qRT-PCR) analysis

Primer pairs for HMGR, PMK, DXPS, ISPD, ISPE, HK, PK, GS, G10H, 10-HGO, IS, DAHPS, PAL, MDH, ICDH and G6PDH were procured from Pandit et al. [22], Kumar et al. [27] and Shitiz et al. [23] while RBA, ARP, ARF7, HisK and CytO genes primers were designed from transcriptomic sequences of *P. kurroa* (data not published) by using Primer3 software [172] (Table 3.6). These gene specific primers were tested on cDNA of *P. kurroa* and *P. scrophulariiflora* samples. The reaction was performed in triplicates on CFX96 system (Bio-Rad) with protocol: denaturation for 5 min at 94°C, followed by 40 cycles each of denaturation for 20 s at 94°C, annealing for 30 s at 48–60°C and elongation for 20 s at 72°C. The housekeeping genes, 26S and GAPDH were used as internal controls for calculating transcript abundance. The significant differences between replicates were statistically evaluated by standard deviation.

3.12 Statistical analysis

Data was recorded in triplicates and repeated thrice for *P. kurroa* and *P. scrophulariiflora* plants grown on MSS and SWM on 10^{th}, 20^{th} and 30^{th} day. Descriptive analysis of the data was performed using SPSS 17.0. Analysis of variance (ANOVA) with comparative Duncan's multiple range tests at 5% was used to determine the significance of differences between replicates. The expression analysis of the selected genes of primary and secondary metabolic pathways in different morphogenetic stages of *P. kurroa* and *P. scrophulariiflora viz.* EX, CI, CM, SP, MS and FD stages was done in triplicates and demonstrated by heat map. The heat map was generated by using GenEx software (V 1.1).

Table 3.6 List of primer sequences used in qRT-PCR based expression analysis

Genes	**Forward Primer (5′-3′)**	**Reverse Primer (5′-3′)**	**Fragment Size (bp)**	**Annealing Temperatures (ºC)**
26S	CACAATGATAGGAAGAGCCGAC	CAAGGGAACGGGCTTGGCAGAATC	500	58
GAPDH	TTGCCATCAATGACCCCTTCA	CGCCCCACTTGATTTTGGA	215	56
HMGS	GATGGTGCAAGAAAAGGCAACTAGA	GGATATTCACTGGCAAGATTGGGCT	110	54
HMGR	CGTTCATCTACCTTCTAGGGTTCTT	GACATAACAACTTCTTCATCGTCCT	100	60
PMK	TGGATGTTGTCGCATCAGCACCTGG	GTAATAGGCAGTCCACTCGCTTCAA	100	58
DXPS	ACATTTAAGTTCAAGTCTGGGAGTG	ATGTGCACTCTCTTCTCTTTTAGGA	110	55.9
DXPR	GGAGGAACTATGACTGGTGTTCTT	CAGGTCATAGTGTACGATTTCCTCT	110	54.9
ISPD	GAGAAAAGTGTATCTGTGCTTCTTAG	AATAACCTGCGGTGTATGCATTTCC	150	56
ISPE	TTCATCTAGATAAGAAGGTGCCAAC	CCTCTACCAGTACAATAAGCAGCTC	110	55
MECPS	ATCTATAGCGGCAAACCTACAC	ACTTTAGAGAGGGATGGAGGG	110	57.1
GS	TGGGTAGATTAGAAGCCAGA	CTGGTGATTTCTACCAGCTC	139	52
G10H	TATCGAGCTTTTCAGTGGAT	GATGTGAGTCCTGTCGATTT	136	52
10-HGO	GGTAGTGTTTATTGGTGCAG	GATCAACTGATCAAGGTCAA	172	54
IS	AATAAGGCCTTGGTTTATCC	TTAGCCTTAGGATCAACTGC	116	49
DAHPS	ACACCATTAAAGCTCCTTGT	TAACAGTCTGAGATCCACCA	171	59
PAL	GCAAGATAGATACGCTCTAA	GTTCCTTGAGACGTCAAT	136	49
HK	ATGCTCCTTACCTACGTTCA	TCCTAACTGAACCCTCAAGA	108	52

PK	AGCTTGTGGCTAAGTACAGG	TCCCCTGAATATGAGACTGT	128	53
ICDH	TCGACATGATAACGTGGATA	TGTTATGACCTTGAGGCTCT	112	52
MDH	CTGATTCTCAAGGAATTTGC	TACCTGCACTTTCAACCTCT	114	51
G6PDH	GAAACCTGAGCATATTCGAG	GTTGTCTGGAACTGTTGGAT	124	52
RBA	GTCAGGGTAAATCATTCCAA	ATCGTTGATGAATAGGCAAC	185	52
ARP	ATGGTCCTCTGTTTGTCAAG	TGCAGAAGATCCTTCAACTT	199	48
ARF7	TTCCTATGGCGTCTATGACT	TGTTGTTGCAGTCTCTGAAG	199	50
HisK	AGAGGAAGTTTGGGATAAGG	AATTGGTGTAGGAACACTGG	157	54
CytO	GAGAGAAAGCTTCGTGAAAA	GTTCCTGTTTTCCATCTTGA	179	50

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Callus induction and shoot regeneration

Different developmental stages passing through different morphogenetic events wherein de-differentiation of leaf and root segments into callus mass followed by re-differentiation of callus into shoot primordia and fully developed plant were obtained using tissue culture techniques. The callus initiation was observed in both leaf and root segments of *P. kurroa* while only leaf segments of *P. scrophulariiflora* responded to callus induction in all the tested media combinations at 25 ± 2 °C and 15 ± 2 °C. This might be attributed to the internal status of plant growth regulators towards different plant species and plant segments under same in vitro conditions. Growth hormones like 2, 4-D, IBA, NAA and BAP have already been reported for callus initiation in *P. kurroa* and *P. scrophulariiflora* [10, 12]. Our results showed creamish and friable calli on MS media supplemented with different concentrations and combinations of 2, 4-D and/or IBA while greenish and friable calli were obtained in both species at 25 ± 2 °C and 15 ± 2 °C on MS media containing TDZ (0.5 mg/L). Callus initiation was not observed in leaf and root segments of both species on MS basal media without any growth hormone at 25 ± 2 °C and 15 ± 2 °C. Leaf and root segments of *P. kurroa* showed callus initiation within 10-12 and 12-15 days at 15 ± 2 °C and 25 ± 2 °C, respectively (Fig. 4.1), while *P. scrophulariiflora* leaf segments showed callus initiation in 14-15 days at 25 ± 2 °C and 15 ± 2 °C (Fig. 4.2). Further, callus mass was observed in next 10-15 days in both species at 25 ± 2 °C and 15 ± 2 °C on MS media supplemented with TDZ (0.5 mg/L). TDZ has been previously reported for callus initiation and plant regeneration in various plants species like *P. kurroa*, *Scutellaria baicalensis* and *Linum usitatissimum* L. by regulating the morphogenetic systems depending upon its exposure time [15, 173-175].

These calli were then transferred to shoot regeneration media containing MS basal media with different concentrations and combinations of IBA, BAP and KN. KN (0.5 mg/L) was found to be best hormone for shoot primordia formation in 15-20 days followed by multiple shoot formation within next 10-15 days in both species at 25 ± 2 °C and 15 ± 2 °C. Lower concentration of KN was found to be better for shoot initiation and multiple shoot formation. Patial et al. [15] have also suggested the use of KN at low concentration for shoot initiation from in vitro grown leaves of *P. kurroa*. These multiple shoots were then transferred to rooting medium *viz.* MS basal medium supplemented with IBA (3 mg/L) and KN (1 mg/L) since rooting is required for plant development. Fully developed plants with rooting were obtained within next 15-20 days. Leaves of fully developed plants of both species were found to be thicker and longer at 15 ± 2 °C compared to 25 ± 2 °C. This could be attributed to the accumulation of hemicelluloses at low temperature which causes thickening of leaves, thereby increasing plant strength [15].

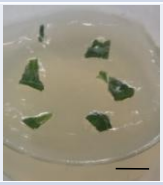
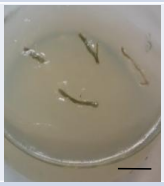
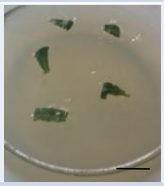
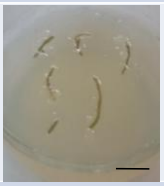
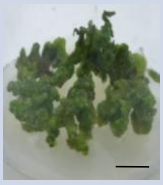
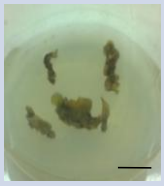
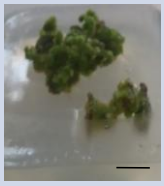
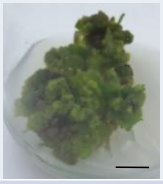
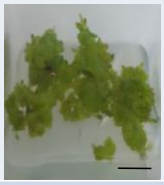
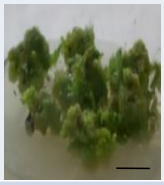
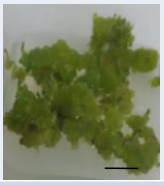
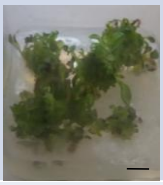
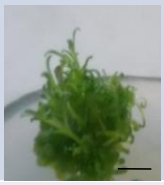
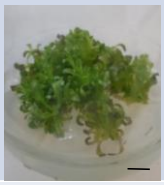
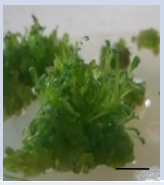
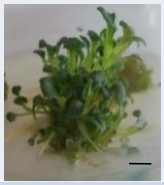
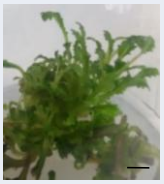
Morphogenetic stage	25˚C		15˚C	
	Leaf explant	Root explant	Leaf explant	Root explant
Explant (EX) (0 day)				
Initiation of callus formation (CI) (1-15 days)				
Callus mass formation (CM) (16-30 days)				
Shoot primordia formation (SP) (31-50 days)				
Multiple shoot formation (MS) (51-65 days)				
Full development (FD) (66-80 days)				

Fig. 4.1 Different developmental stages of *P. kurroa* at 25 ± 2 °C and 15 ± 2 °C on MS media containing different growth hormones: Leaf and root explants on TDZ = 0.5 mg/L represent EX stage; Callus initiation and callus mass formation on TDZ = 0.5 mg/L represent CI and CM stages, respectively; Shoot primordia and multiple shoot formation on KN = 0.5 mg/L represent SP and MS stages, respectively; Shoot elongation, full growth and development on IBA = 3 mg/L and KN = 1 mg/L represent FD stage (Scale bar = 1 cm)

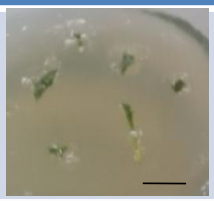
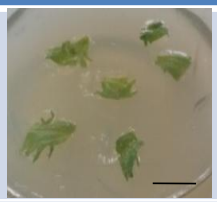
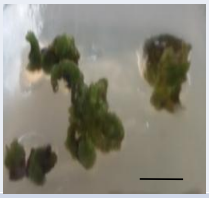
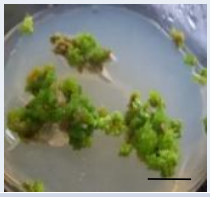
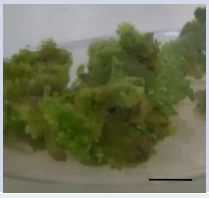
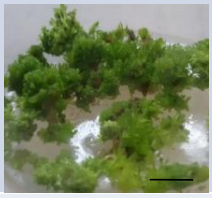
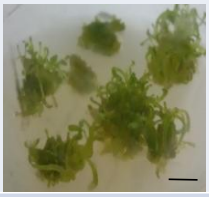
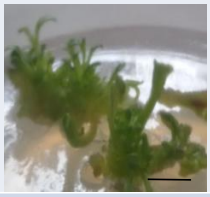
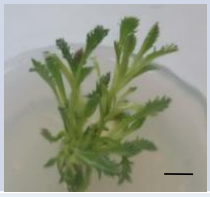
Morphogenetic stage	25˚C	15˚C
Explant (EX) (0 day)		
Initiation of callus formation (CI) (1-15 days)		
Callus mass formation (CM) (16-30 days)		
Shoot primordia formation (SP) (31-50 days)		
Multiple shoot formation (MS) (51-65 days)		
Full development (FD) (66-80 days)		

Fig. 4.2 Different developmental stages of *P. scrophulariiflora* at 25 ± 2 °C and 15 ± 2 °C on MS media containing different growth hormones: Leaf explant on TDZ = 0.5 mg/L represent EX stage; Callus initiation and callus mass formation on TDZ = 0.5 mg/L represent CI and CM stages, respectively; Shoot primordia and multiple shoot formation on KN = 0.5 mg/L represent SP and MS stages, respectively; Shoot elongation, full growth and development on IBA = 3 mg/L and KN = 1 mg/L represent FD stage (Scale bar = 1 cm)

4.2 Expression analysis of primary metabolism genes vis-à-vis shoot development in different morphogenetic stages

P-I biosynthesis occurs in shoots of *P. kurroa* and *P. scrophulariiflora*; therefore, expression analysis of 10 genes *viz.* RBA, ARP, ARF7, HisK, CytO, HK, PK, ICDH, MDH and G6PDH was carried out to understand the molecular basis of shoot regeneration in both plant species. Results revealed that RBA, HisK and CytO genes showed increased expression *viz.* 2-4, 2-5 folds in SP stage; 6-9, 4-6 folds in MS stage; and 7-12, 6-10 folds in FD stage of *P. kurroa* and *P. scrophulariiflora*, respectively (Fig. 4.3). This might be due to the involvement of these genes in shoot development in *P. kurroa* and *P. scrophulariiflora*, since RBA is involved in photosynthetic function, HisK serve as cytokinin receptor and CytO is responsible for cytokinin catabolism, thereby contributing to the regulation of cytokinin-dependent processes [176-178]. Further, HK, PK, ICDH, MDH and G6PDH genes of glycolysis, TCA and pentose phosphate pathways showed an increase of 2-5, 3-7 folds in MS stage and 5-11, 5-13 folds in FD stage compared to CM stage in *P. kurroa* and *P. scrophulariiflora*, respectively (Fig. 4.3). Expression level of selected genes remained unaltered in CI and CM stages of both plant species which could be attributed to the absence of cell programming machinery in callus cultures for biosynthesis of P-I [11]. Overall, these genes showed high transcript abundance in MS and FD stages vis-à-vis shoot development in *P. kurroa* and *P. scrophulariiflora*, thereby regulating the supply of precursors for activation of secondary metabolism genes for P-I biosynthesis [179]. Matt et al. [180] have showed that levels of chlorogenic acid and nicotine reduced with drop in RuBisCO activity leading to decreased levels of primary metabolites, thus hampering the content of secondary metabolites. Lloyd and Zakhleniuk [181] have shown the role of primary metabolites in regulating secondary metabolism by studying the transcript levels of genes encoding enzymes and regulatory proteins involved in primary carbon assimilation in mature rosette leaves of wild-type and mutant Arabidopsis plants. Henkes et al. [182] have also showed that reduction in the oxidative pentose phosphate pathway enzymes affected metabolic flux involved in shikimate and phenylpropanoid pathways.

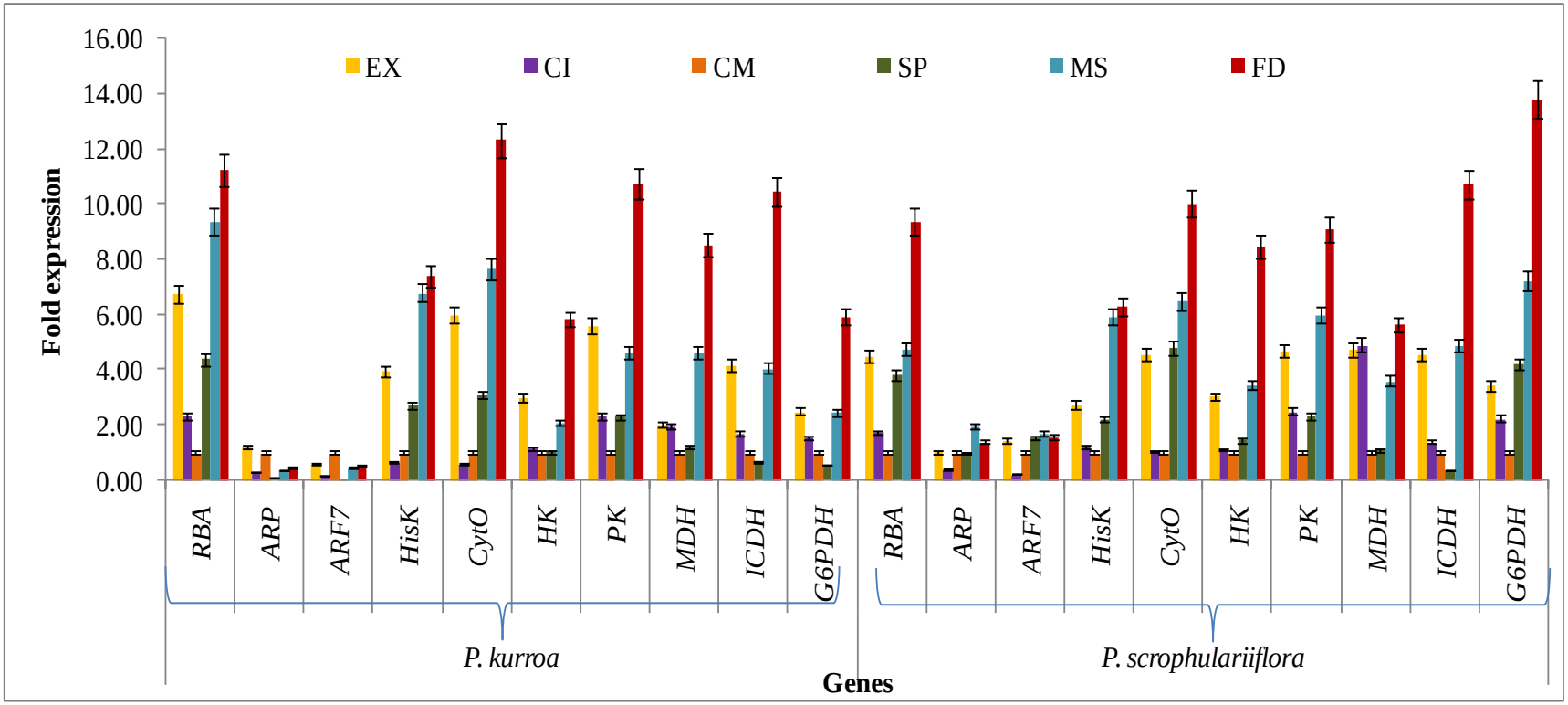


Fig. 4.3 Expression pattern of primary metabolism genes in different morphogenetic stages of *P. kurroa* and *P. scrophulariiflora* obtained from leaf explants at 15 ± 2 °C. These include: Explant (EX), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages. Fold expression of genes was calculated by comparing the transcript abundance of genes in different developmental stages with CM stage. Error bars represent mean±SD for data recorded in triplicates (repeated thrice)

4.3 Expression analysis of secondary metabolic pathway genes vis-à-vis P-I biosynthesis in different morphogenetic stages

P-I production was found to be developmentally regulated during different morphogenetic stages in *P. kurroa* and *P. scrophulariiflora*. It reduced from 2.51 µg/mg FW and 2.12 µg/mg FW to non-detectable level during de-differentiation in CI and CM stages at 15 ± 2 °C obtained from leaf explants of *P. kurroa* and *P. scrophulariiflora*, respectively (Fig. 4.4). With the progress of re-differentiation, levels of P-I increased consistently during SP, MS and FD stages of *P. kurroa* and *P. scrophulariiflora* (Fig. 4.4). Therefore, various genes *viz.* HMGR, PMK, DXPS, ISPD, ISPE, GS, G10H, 10-HGO, IS, DAHPS and PAL of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways were selected and analyzed for modulation of their expression vis-à-vis P-I content at different morphogenetic stages of *P. kurroa* and *P. scrophulariiflora*. Results revealed that genes encoding enzymes HMGR, PMK, DXPS, ISPE, GS, G10H, DAHPS and PAL showed significant modulation of expression in agreement with P-I content at different morphogenetic stages in comparison to CM stage. At day 0, these genes showed 16-47 and 11-32 folds transcript abundance in EX stage, which declined to 0-3 and 1-2 folds by the end of day 50, corresponding to CI and CM stages of *P. kurroa* and *P. scrophulariiflora,* respectively (Fig. 4.5). This might be due to the de-differentiation of leaf explants into callus mass or loss of organogenesis which decreased the expression of genes involved in secondary metabolism leading to non-detectable amount of P-I in CI and CM stages. Conner [183] have also reported low level of alkaloid in *Solanum laciniatum* due to alteration in gene expression level in undifferentiated cells which was attributed to its heterotrophic mode of nutrition. Further, as re-differentiation progressed, HMGR, DXPS, ISPE, G10H and DAHPS showed 2-28 and 2-20 folds high expression during SP stage of *P. kurroa* and *P. scrophulariiflora,* respectively. Formation of shoot primordia might lead to the activation of important regulatory enzymes of all four biosynthetic pathways involved in P-I production. HMGR and DXPS are the rate limiting enzymes of MVA and MEP pathways, which along with ISPE, have been reported to be involved in P-I biosynthesis [22, 25, 115]. G10H and DAHPS have also been reported as key regulatory enzymes for secondary metabolism in Arabidopsis and *Catharanthus roseus* [169-170]. Further, 15-52 and 11-35 folds high expression was observed in HMGR, DXPS, ISPE, G10H and DAHPS during MS stage between days 51-65 compared to CM

stage of *P. kurroa* and *P. scrophulariiflora*, respectively. Up-regulation of HMGR, DXPS and ISPE might be associated with the supply of GPP, which acts as precursor for iridoid biosynthesis [184]. Pandit et al. [22] have also reported the positive correlation of HMGR, DXPS and ISPE enzymes of MVA and MEP pathways with P-I biosynthesis in *P. kurroa*. Higher expression of G10H and DAHPS demonstrated their possible roles in activating iridoid and shikimate/phenylpropanoid pathways [27]. Finally, a drastic increase in expression of genes encoding HMGR, PMK, DXPS, ISPE, GS, G10H, DAHPS and PAL from 27-68 and 13-47 folds vis-à-vis P-I production was observed in FD stage between days 66-80 of *P. kurroa* and *P. scrophulariiflora*, respectively (Fig. 4.5). In addition to genes elevated in MS stage, genes such as PMK, GS and PAL showed high expression in FD stage as compared to CM stage. GS has been reported to initiate monoterpenoid branch of monoterpene indole alkaloid (MIA) pathway in *Catharanthus roseus* [185], while PAL is an important regulatory enzyme of shikimate/phenylpropanoid pathway [186-188]. High expression of these genes in FD stage suggested their possible role in the accumulation of P-I in *P. kurroa* and *P. scrophulariiflora*. Therefore, genes encoding HMGR, PMK, DXPS, ISPE, GS, G10H, DAHPS and PAL of all four pathways were found to be associated with P-I biosynthesis in *P. kurroa* and *P. scrophulariiflora*.

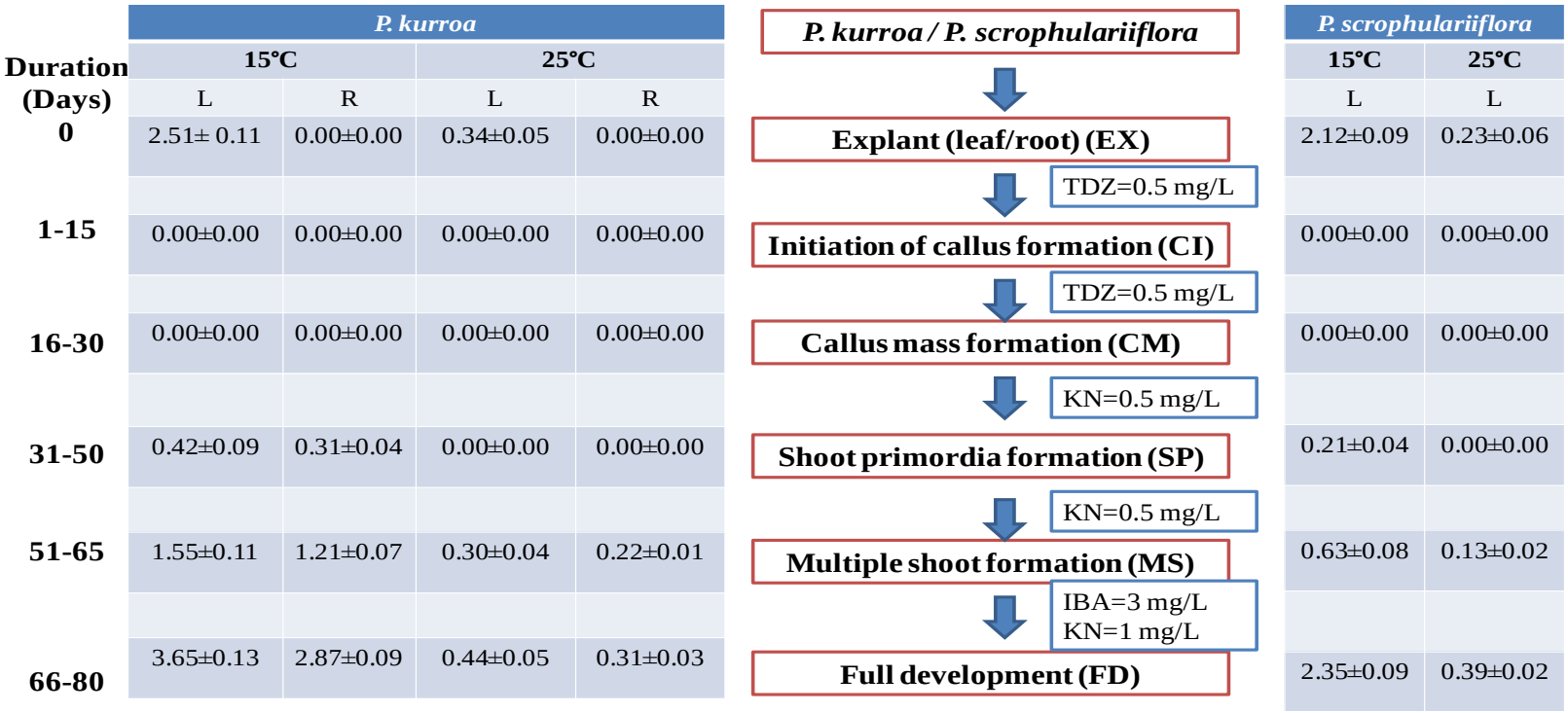


Fig. 4.4 P-I content (µg/mg FW) in different morphogenetic stages of *P. kurroa* and *P. scrophulariiflora* at 15 ± 2 °C and 25 ± 2 °C, where L and R represented leaf and root explants. Data was represented by mean±SD for P-I content estimated in triplicates (repeated thrice)

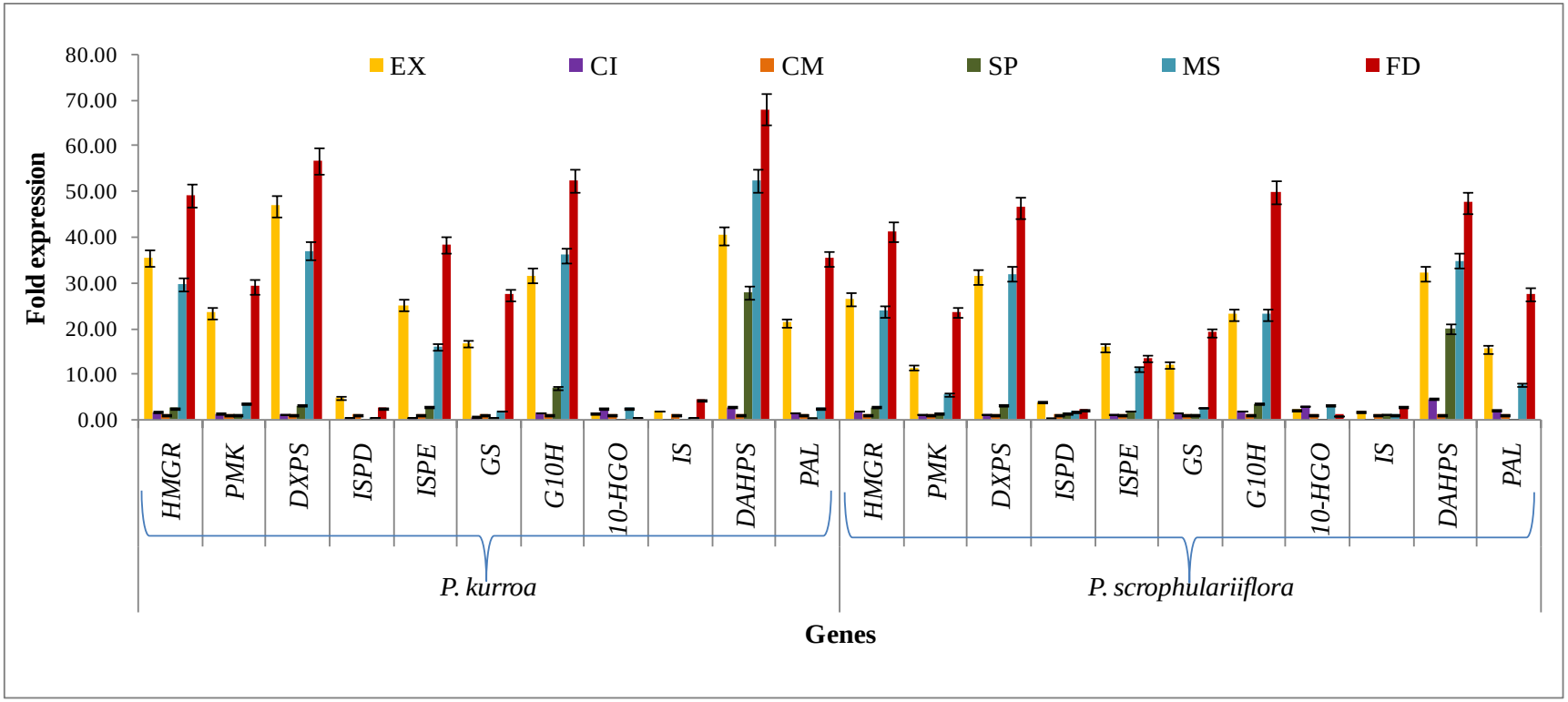


Fig. 4.5 Expression pattern of secondary metabolic pathways genes in different morphogenetic stages of *P. kurroa* and *P. scrophulariiflora* obtained from leaf explants at 15 ± 2 °C. These include: Explant (EX), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages. Fold expression of genes was calculated by comparing the transcript abundance of genes in different developmental stages with CM stage. Error bars represent mean±SD for data recorded in triplicates (repeated thrice)

Overall, multiple genes of secondary metabolic pathways showed higher expression in different developmental stages of *P. kurroa* and *P. scrophulariiflora* compared to those from primary metabolic pathways showing slightly higher expression for P-I biosynthesis. The representative heat maps were generated which highlighted the involvement of secondary metabolic pathways genes such as HMGR, PMK, DXPS, ISPE, GS, G10H, DAHPS and PAL for P-I biosynthesis in FD stages of *P. kurroa* and *P. scrophulariiflora* obtained from leaf explants at 15 ± 2 °C (Fig. 4.6).

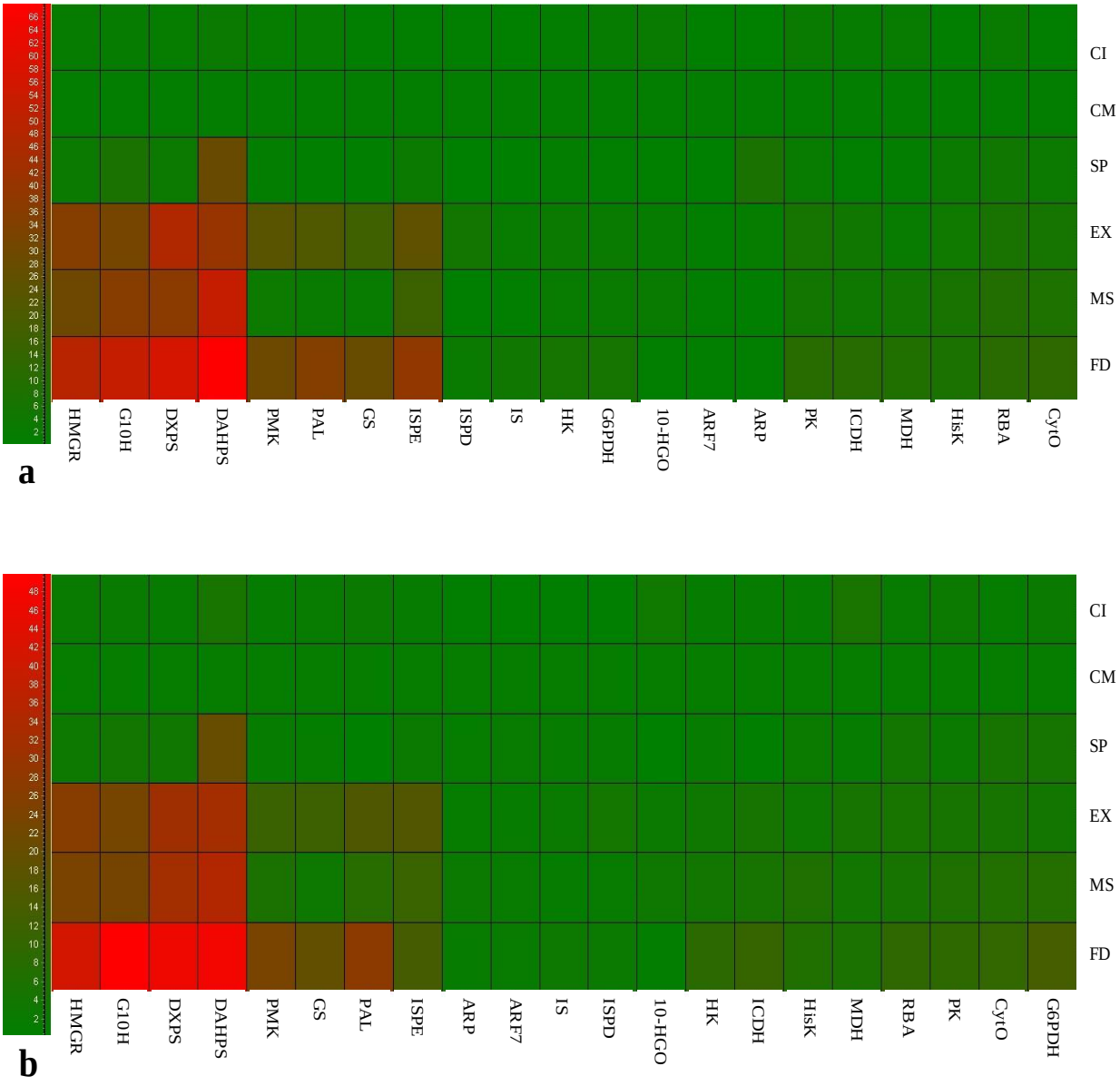


Fig. 4.6 Heat maps demonstrating the differential expression pattern of genes involved in primary and secondary metabolic pathways at different developmental stages of *P. kurroa* (a) and *P. scrophulariiflora* (b) obtained from leaf explants at 15 ± 2 °C. These included: Explant (EX), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages

4.4 Effect of temperature, explant and species on P-I biosynthesis

Secondary metabolism in plants is influenced by various factors such as temperature, explants, species etc [189-191]. Previous studies on *P. kurroa* showed higher P-I content in in vitro grown shoots at 15°C than 25°C while shoots regenerated from leaf explants accumulated higher P-I content compared to stem and root explants [11, 25]. These reports suggested optimal growth conditions for enhanced P-I biosynthesis but mechanism underlying their effects is not clear. Therefore, transcript abundance of P-I pathway genes vis-à-vis temperature, explant and species was studied in different morphogenetic stages of *P. kurroa* and *P. scrophulariiflora*.

Higher P-I contents *viz.* 3.65 µg/mg FW and 2.35 µg/mg FW were observed in *P. kurroa* and *P. scrophulariiflora* shoots, respectively developed from leaf explants at 15 ± 2 °C compared to 25 ± 2 °C (negligible amount) (Fig. 4.4). Seven out of 11 genes *viz.* HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL showed 47-87 and 38-73 folds high expression in fully developed shoots of *P. kurroa* and *P. scrophulariiflora*, respectively originated from leaf explants at 15 ± 2 °C compared to 25 ± 2 °C (Fig. 4.7). These results revealed that development of shoots at low temperature up-regulated the expression of genes involved in secondary metabolism leading to enhanced P-I accumulation in fully developed shoots of both plant species. Hannah et al. [192] also showed that low temperature increased the secondary metabolite production in Arabidopsis by transcriptional up-regulation of genes involved in secondary metabolism.

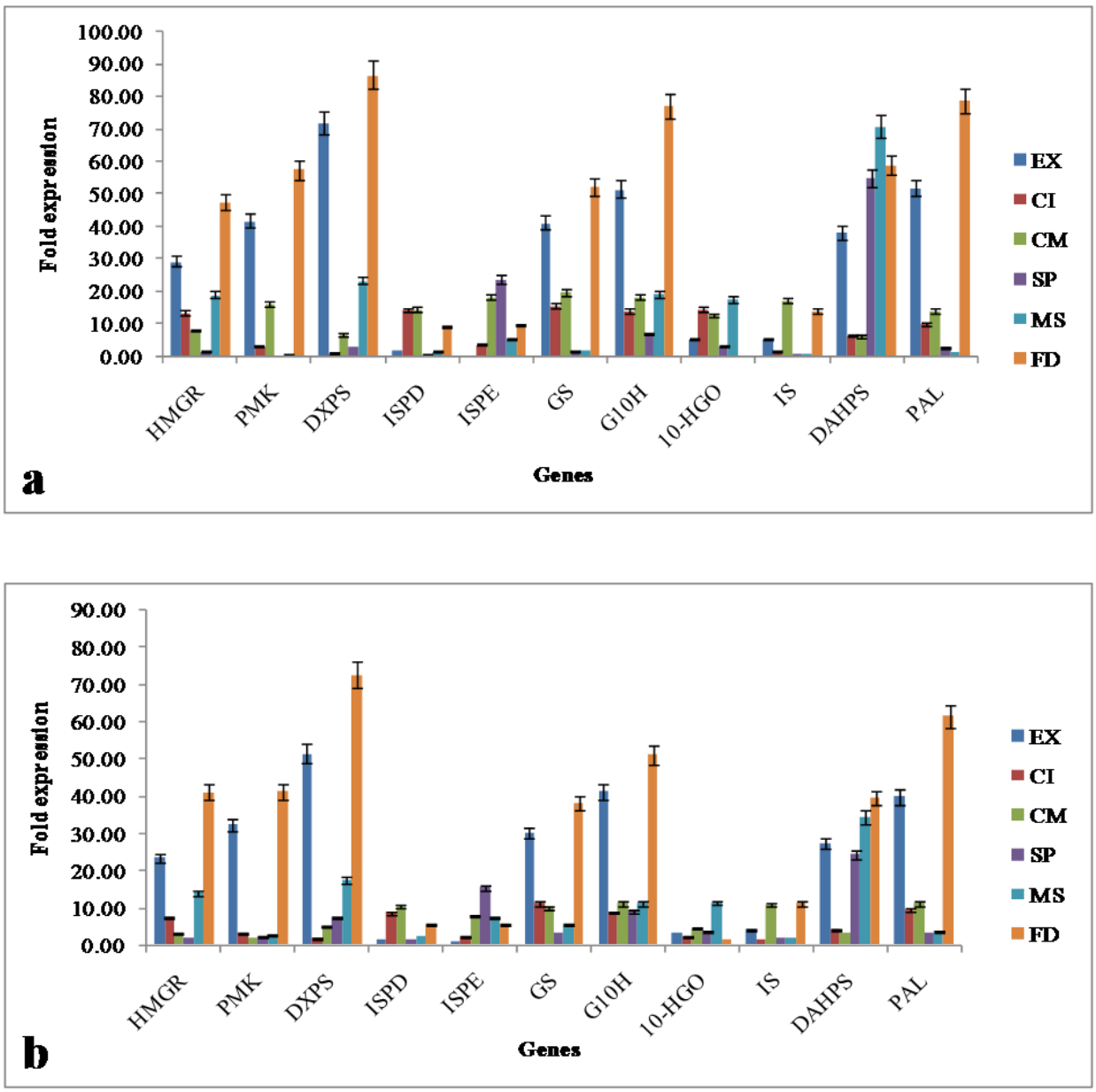


Fig. 4.7 Effect of temperature on transcript levels of selected genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways at different developmental stages of *P. kurroa* (a) and *P. scrophulariiflora* (b) derived from leaf explants at 15 ± 2 °C and 25 ± 2 °C. These included: Explant (EX), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages. Fold expression of genes was calculated by comparing the transcript abundance of genes in different developmental stages at 15 ± 2 °C with 25 ± 2 °C. Error bars represent mean±SD for data recorded in triplicates (repeated thrice)

Plant tissues respond variably from each other for organogenesis on similar combinations of growth regulators [193-195], thereby influencing the metabolite production during shoot regeneration. Our results showed marginal variation in P-I content between shoots derived from different explants *viz.* 3.65 µg/mg FW and 2.87 µg/mg FW P-I content in shoots developed from leaf and root explants, respectively at 15 ± 2 °C (Fig. 4.4). So, to study the effect of explants on P-I production at molecular level, expression analysis was carried out in different morphogenetic stages derived from leaf and root explants at 15 ± 2 °C. Out of 11 genes, 7 genes encoding HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL showed 4-9 and 2-8 folds elevated expression in leaf explants derived CI and CM stages, respectively compared to root explant derived stages which could be correlated to biosynthesis of P-I in leaf tissue (Fig. 4.8). Expression levels remained unaltered in SP, MS and FD stages as same machinery was activated for P-I biosynthesis in the shoots developed from leaf and root explants. Kurz and Constable [196] also suggested that origin of tissue become irrelevant after de-differentiation, and subsequent regenerated plants inherit physiological capability of the source plant to express all the biosynthetic pathways under permissive conditions.

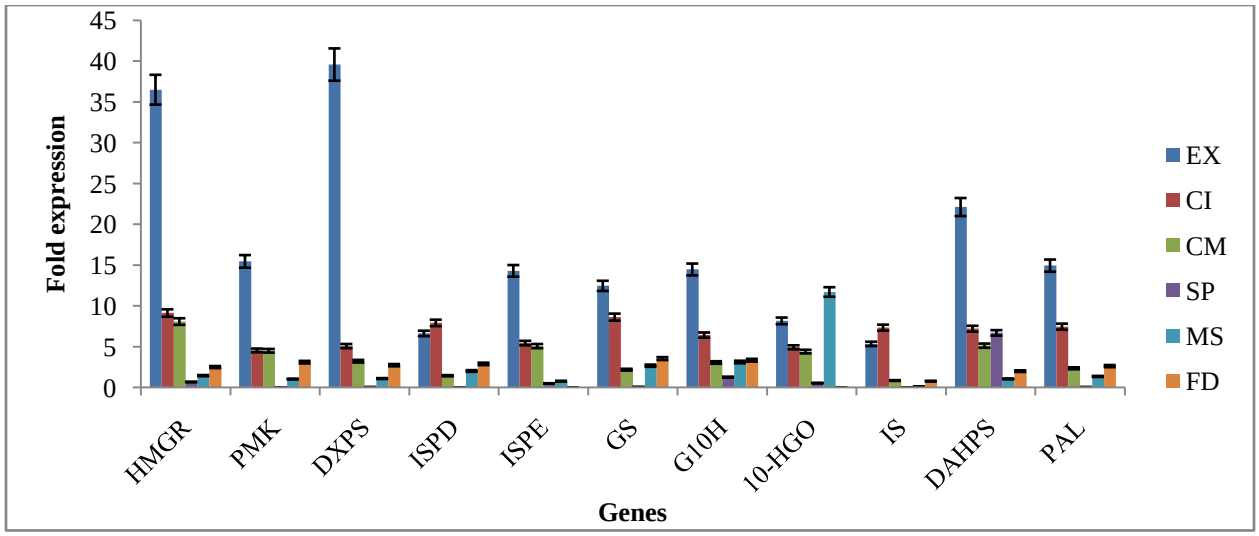


Fig. 4.8 Effect of different explants on transcript levels of selected genes of MVA, MEP, iridoid, and shikimate/phenylpropanoid pathways at different developmental stages of *P. kurroa* at 15 ± 2 °C. These included: Explant (EX), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages. Relative expression of genes was calculated by comparing the transcript abundance of genes from leaf explant derived stages with root explant derived stages. Error bars represent mean±SD for data recorded in triplicates (repeated thrice)

Previous reports on metabolic profiling of important phytochemicals such as podophyllotoxin in *Podophyllum hexandrum* and *P. peltatum*, rutin in *Fagopyrum tataricum* and *F. esculentum*, aconitine in *Aconitum* species have shown variation in their biosynthesis from one species to another [191, 197-198]. Therefore, P-I biosynthesis was also studied between *P. kurroa* and *P. scrophulariiflora*. These two species are rich source of P-I but higher P-I content has been observed in *P. scrophulariiflora* than *P. kurroa* under field conditions [8]. However, our results showed slightly higher P-I content in *P. kurroa* shoots compared to *P. scrophulariiflora* in different developmental stages under in vitro conditions (Fig. 4.4). So, in order to understand the molecular basis of P-I biosynthesis in both species, expression analysis of selected genes involved in secondary metabolism was carried out in different developmental stages obtained from leaf segments of *P. kurroa* and *P. scrophulariiflora* at 15 ± 2 °C. Seven genes *viz.* HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL showed 6-19 folds high expression in FD stage of *P.*

kurroa compared to *P. scrophulariiflora* which was in corroboration with P-I content (Fig. 4.9). Al-Ghazi et al. [199] have also observed stage specific and species specific expression of genes involved in phenylpropanoid and flavonoid pathways for the production of flavonoids, phenylpropanoids, terpenes and waxes in *Gossypium hirsutum* L. and *G. barbadense* L.

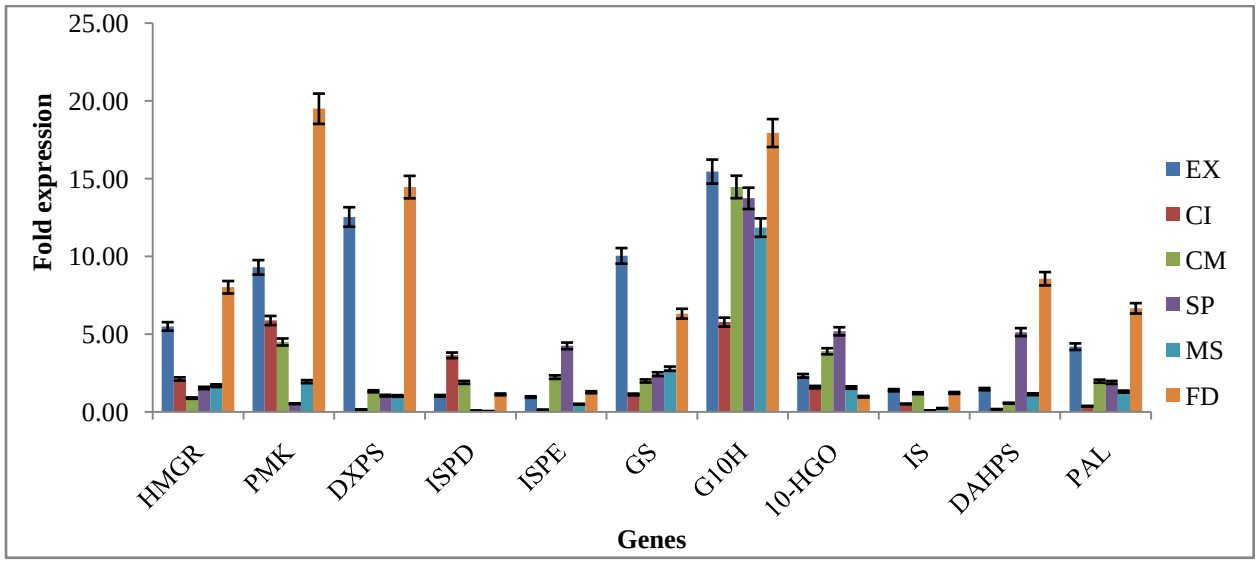


Fig. 4.9 Expression pattern of selected genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways for P-I biosynthesis at different developmental stages of *P. kurroa* and *P. scrophulariiflora* derived from leaf explants at 15 ± 2 °C. These included: Explant (EX), callus initiation (CI), callus mass formation (CM), shoot primordia formation (SP), multiple shoot formation (MS) and fully developed plant (FD) stages. Relative expression of genes was calculated by comparing the transcript abundance of genes in different developmental stages derived from leaf explants of *P. kurroa* with *P. scrophulariiflora*. Error bars represent mean±SD for data recorded in triplicates (repeated thrice)

Overall, effect of temperature and species influenced P-I production by regulating the expression of HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways under in vitro conditions. Therefore, this study has suggested potential gene targets at FD stage for their utilization in genetic improvement of *P. kurroa* and *P. scrophulariiflora*.

4.5 SWE as a biostimulant with MS medium (MSS) for biomass enhancement in *P. kurroa* and *P. scrophulariiflora*

Previous reports have shown the importance of seaweed extracts in improving plant growth and development in different plant species by improving N and S uptake along with their assimilation, basal metabolism and transportation of various nutrients [139]. Our results showed high transcript abundance of genes corresponding to primary and secondary metabolism in fully developed shoots vis-à-vis different morphogenetic stages in *P. kurroa* and *P. scrophulariiflora* under in vitro conditions. Therefore, effect of SWE as a biostimulant was studied for enhancement of shoot biomass in these plant species. MSS media having different concentrations of SWE alone (MSS1-MSS4; Table 3.1) and in combination with growth hormones (MSS5-MSS8; Table 3.2) were tested to evaluate the potential of SWE. MSS3 having SWE (2.0 g/L) showed enhancement of 1.40, 1.55, 1.56 fold in total plant length; 1.28, 2.41, 2.79 fold in shoot biomass; and 1.69, 1.41, 2.67 fold in number of shoots in *P. kurroa* on 10^{th}, 20^{th} and 30^{th} day, respectively while 1.77 and 2.41 fold increment in number of roots was observed on 20^{th} and 30^{th} day, respectively compared to C1 at 15 ± 2 °C (Figs. 4.10 and 4.11). Similarly, MSS3 showed enhancement of 1.36, 1.52, 1.54 fold in total plant length; 1.18, 2.21, 2.61 fold in shoot biomass; and 1.71, 1.86, 2.61 fold in number of shoots in *P. scrophulariiflora* on 10^{th}, 20^{th} and 30^{th} day, respectively while 1.57 and 2.22 fold increment in number of roots was observed on 20^{th} and 30^{th} day compared to C1 at 15 ± 2 °C (Figs. 4.10 and 4.11). Moreover, MSS7 having growth hormones and SWE (2.0 g/L) also improved the plant growth with enhancement of 1.39, 1.50, 1.14 fold in total plant length; 1.40, 1.41, 2.67 fold in shoot biomass; and 1.81, 1.36, 1.47 fold in number of shoots in *P. kurroa*, while an increase of 1.33, 1.47, 1.14 fold in total plant length; 1.31, 1.32, 2.65 fold in shoot biomass; and 1.76, 1.29, 1.37 fold in number of shoots was observed in *P. scrophulariiflora* on 10^{th}, 20^{th} and 30^{th} day, respectively as compared to SM at 15 ± 2 °C (Figs. 4.10 and 4.11). An increment in number of roots by 2.91 and 3.53 fold in *P. kurroa*; 2.73 and 3.36 fold in *P. scrophulariiflora* was observed on 20^{th} and 30^{th} day, respectively compared to SM at 15 ± 2 °C (Fig. 4.10). Hence, MSS having SWE with and without growth hormones enhanced shoot biomass and shoot number in both plant species. This might be due to the presence of various bioactive substances like amino acids, vitamins, cytokinins, gibberellins and betains in seaweed extracts which regulate plant growth and

development [32, 141]. Vinoth et al. [30] have also showed enhanced shoot elongation in *Lycopersicon esculentum* upon seaweed extract treatment under in vitro conditions.

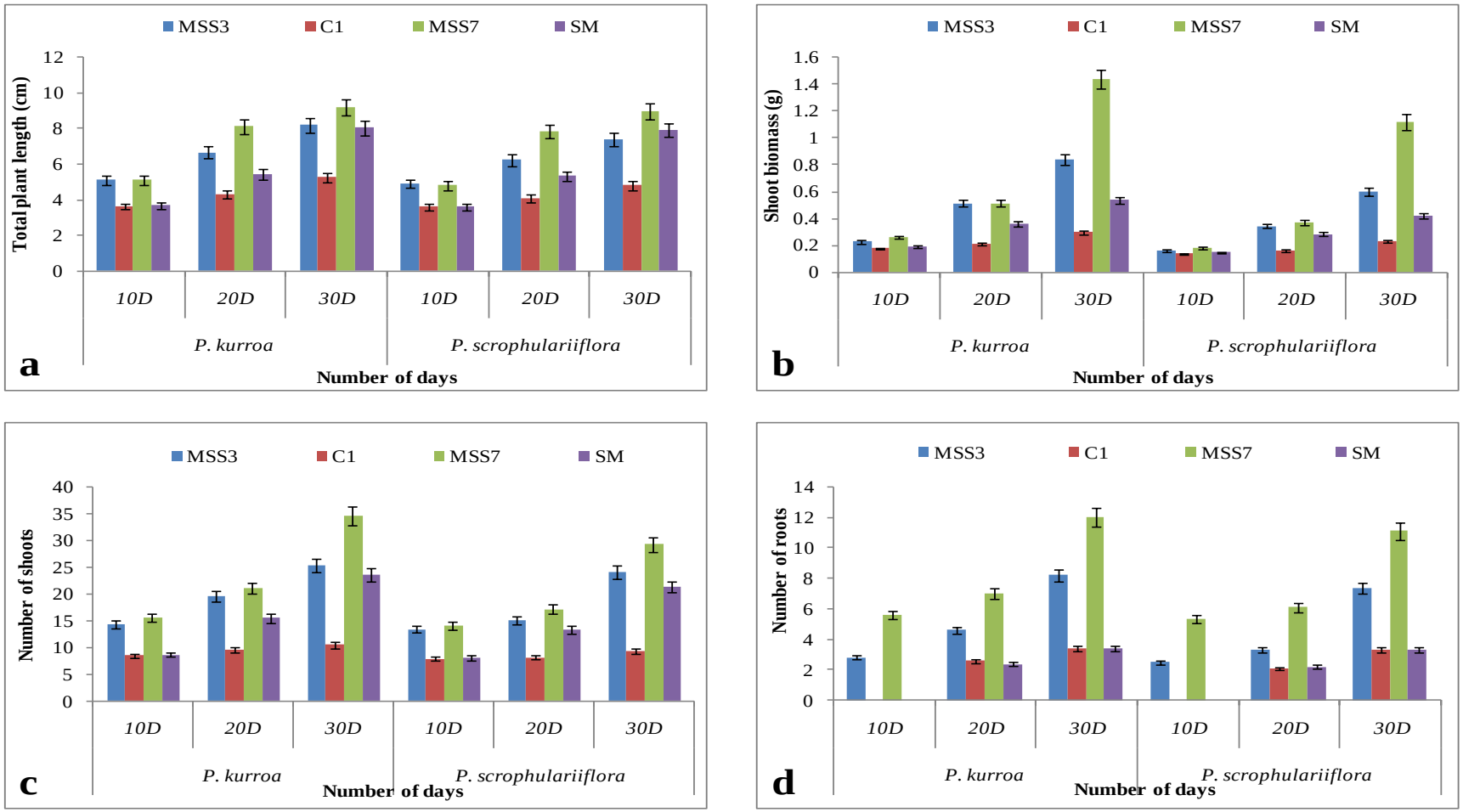


Fig. 4.10 Growth parameters corresponding to total plant length (a), shoot biomass (b), number of shoots (c) and number of roots (d) for *P. kurroa* and *P. scrophulariiflora* plants grown on MSS3 (MS + SWE + sucrose + agar), C1 (MS + sucrose + agar), MSS7 (MS + SWE + sucrose + growth hormones + agar) and SM (MS + sucrose + growth hormones + agar) at 15 ± 2 ºC. Error bars represent mean±SD for data recorded in triplicates (repeated thrice)

	Without growth hormones		With growth hormones	
	MSS3	**C1**	**MSS7**	**SM**
P. kurroa				
P. scrophulariiflora				

Fig. 4.11 *P. kurroa* and *P. scrophulariiflora* plants grown on MSS3 (MS + SWE + sucrose + agar), C1 (MS + sucrose + agar), MSS7 (MS + SWE + sucrose + growth hormones + agar) and SM (MS + sucrose + growth hormones + agar) at 15 ± 2 ºC (Scale bar = 1 cm)

Further, on comparison of SWE with Biovita, MSS7 containing SWE showed increment in shoot biomass with 1.23, 1.28, 1.37 fold in *P. kurroa* and 1.06, 1.07, 1.10 fold in *P. scrophulariiflora* on 10th, 20th and 30th day, respectively at 15 ± 2 °C as compared to MSB1 (Fig. 4.12). Extracts obtained from *Kappaphycus alvarezii* have been reported to improve plant growth by regulating phytohormone signalling and defence responsive genes in *Lycopersicon esculentum* [200].

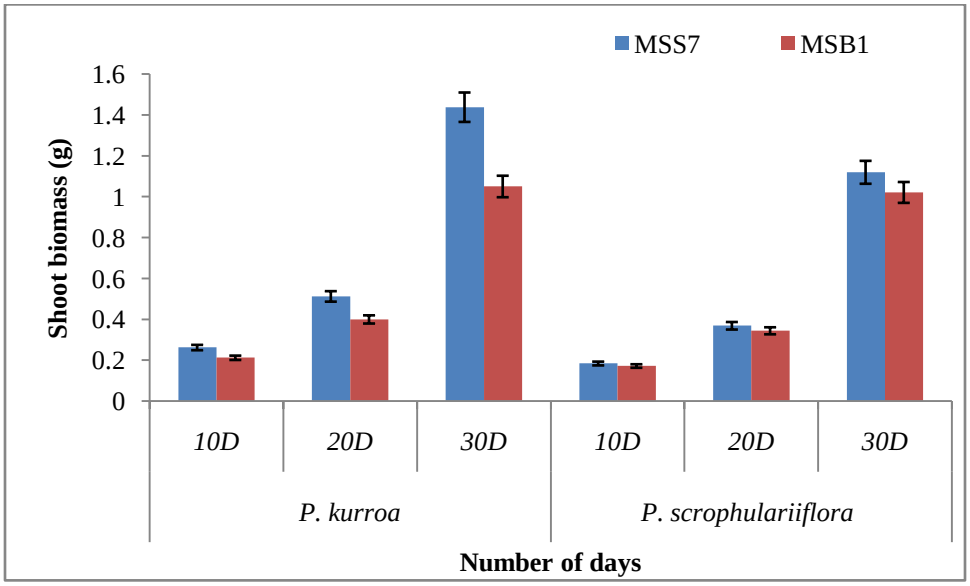


Fig. 4.12 Shoot biomass of *P. kurroa* and *P. scrophulariiflora* plants grown on MSS7 and MSB1 at 15 ± 2 °C. Error bars represent mean±SD for data recorded in triplicates (repeated thrice)

All media combinations from MSS1 to MSS8 were tested on two incubation temperatures for enhancement of shoot biomass in *P. kurroa* and *P. scrophulariiflora*. Higher shoot biomass was observed in *P. kurroa* (1.25 fold) and *P. scrophulariiflora* (1.23 fold) plants grown at 15 ± 2 °C as compared to 25 ± 2 °C after 30 days, which was attributed to increased leaf size at 15 ± 2 °C (Figs. 4.13 and 4.14). However, number of shoots and roots remained same in both species at 15 ± 2 °C and 25 ± 2 °C (Fig. 4.10; Tables A2 and A3), which was in accordance with the findings of Sood and Chauhan [9].

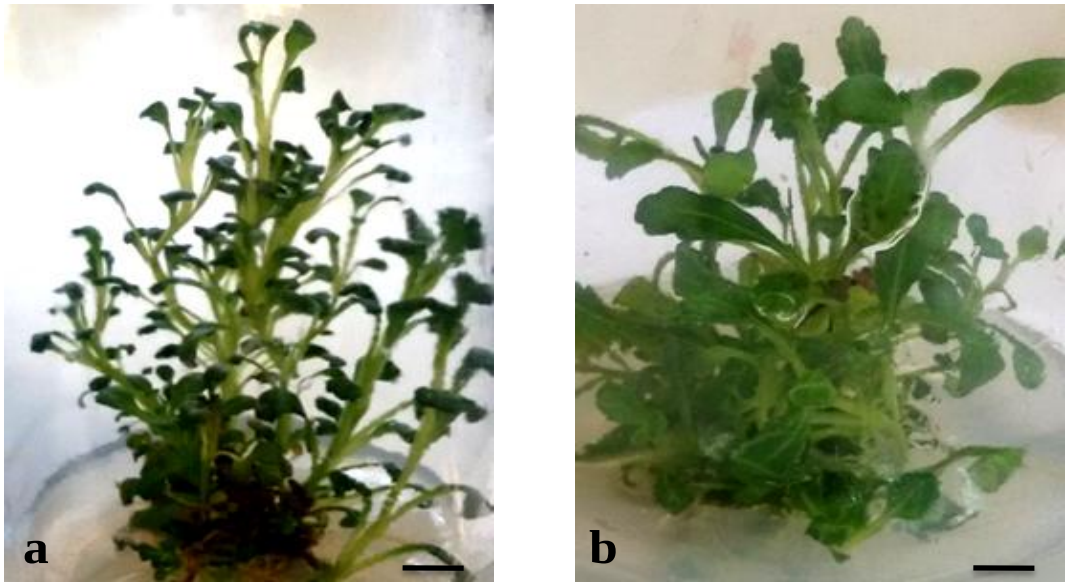


Fig. 4.13 *P. kurroa* shoots grown on MSS7 at 25 ± 2 ºC (a) and 15 ± 2 ºC (b) (Scale bar = 1 cm)

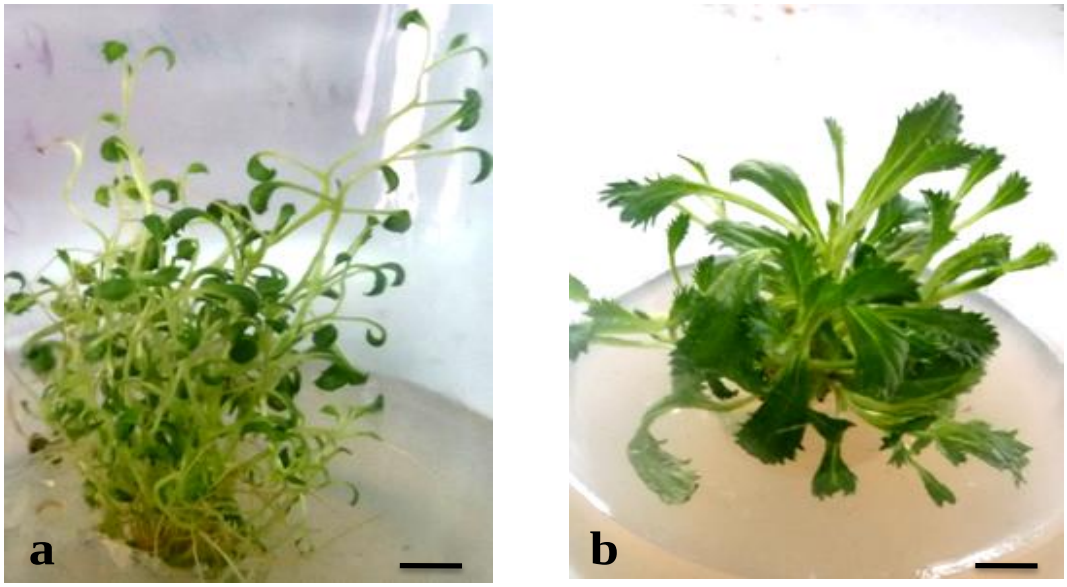


Fig. 4.14 *P. scrophulariiflora* shoots grown on MSS7 at 25 ± 2 ºC (a) and 15 ± 2 ºC (b) (Scale bar = 1 cm)

4.6 SWE as a medium (SWM) alternative to MS medium for micropropagation of *P. kurroa* and *P. scrophulariiflora*

Seaweed extracts contain various macronutrients, micronutrients, vitamins and growth hormones which can maintain and support the plant growth under in vitro conditions; therefore, potential of SWE as a medium alternative to nutrient medium was estimated. Out of 6 different tested concentrations of SWE (0.01, 0.1, 1.0, 2.0, 3.0, 5.0 g/L), 2 g/L showed highest shoot biomass in *P. kurroa* and *P. scrophulariiflora* at 15 ± 2 °C and 25 ± 2 °C. Thus, a comparative study was carried out by replacing MS medium with optimized concentration of SWE (2 g/L) in different media combinations to study the potential of SWM as an alternative to MS medium for the micropropagation of *P. kurroa* and *P. scrophulariiflora*. Various growth parameters such as total plant length, shoot biomass, number of shoots and roots were analyzed in both solid (with agar) and liquid (without agar) media. Plants grown on liquid SWM could not survive after 20 days due to stressful conditions caused by water logging of the apoplast causing hypoxia, thereby leading to death of tissues [201]. However, better growth was observed on solid SWM. SWM4 (SWE + sucrose + agar) was found to be best among different media combinations for growth of *P. kurroa* and *P. scrophulariiflora* plants. It showed comparable shoot number with MSM8 (MS + sucrose + growth hormones + agar) till 20th day; thereafter, shoot multiplication showed better results in MSM8, which was due to faster nutrient depletion in SWM4 as compared to MSM8. Subculturing same plants on SWM4 after 20 days exhibited similar growth as obtained with MSM8 at 15 ± 2 °C (Fig. 4.15). Plants grown on SWM4 showed enhancement of 2.08, 1.82, 2.25 fold in total plant length; 1.21, 1.65, 2.00 fold in shoot biomass; and 1.14, 1.42, 2.12 fold in number of shoots in *P. kurroa*, while an increase of 2.08, 1.85, 2.13 fold in total plant length; 1.09, 1.69, 1.84 fold in shoot biomass; and 1.09, 1.60, 2.23 fold in number of shoots in *P. scrophulariiflora* was observed on 10th, 20th and 30th day, respectively as compared to control MSM4 at 15 ± 2 °C (Fig. 4.15). Early rooting (10th day) in SWM4 with enhancement of 2.15, 3.35 fold in *P. kurroa* and 2.28, 3.24 fold in *P. scrophulariiflora* in number of roots was observed on 20th and 30th day, respectively at 15 ± 2 ºC compared to control MSM4 (Fig. 4.15). These results suggested that MS medium requires additional supply of growth hormones as compared to SWM since optimal growth was observed in SWM4 in both species than MSM4. Similar findings have been observed in *P. kurroa* and *P. scrophulariiflora* plants grown at 25 ± 2 °C (Tables A4 and A5). Findings of Hurtado et al. [202] have shown the use of commercially

available seaweed extract based medium with and without growth hormones for regeneration of *Kappaphycus* varieties using tissue culture techniques, which was analogous to current study in *P. kurroa* and *P. scrophulariiflora*. Seaweed extract has also been found to promote growth of shoots and roots in *A. thaliana* using DR5: GUS assay, which implied that commercially available seaweed extracts modulate the concentration and localization of auxins for enhanced plant growth [29].

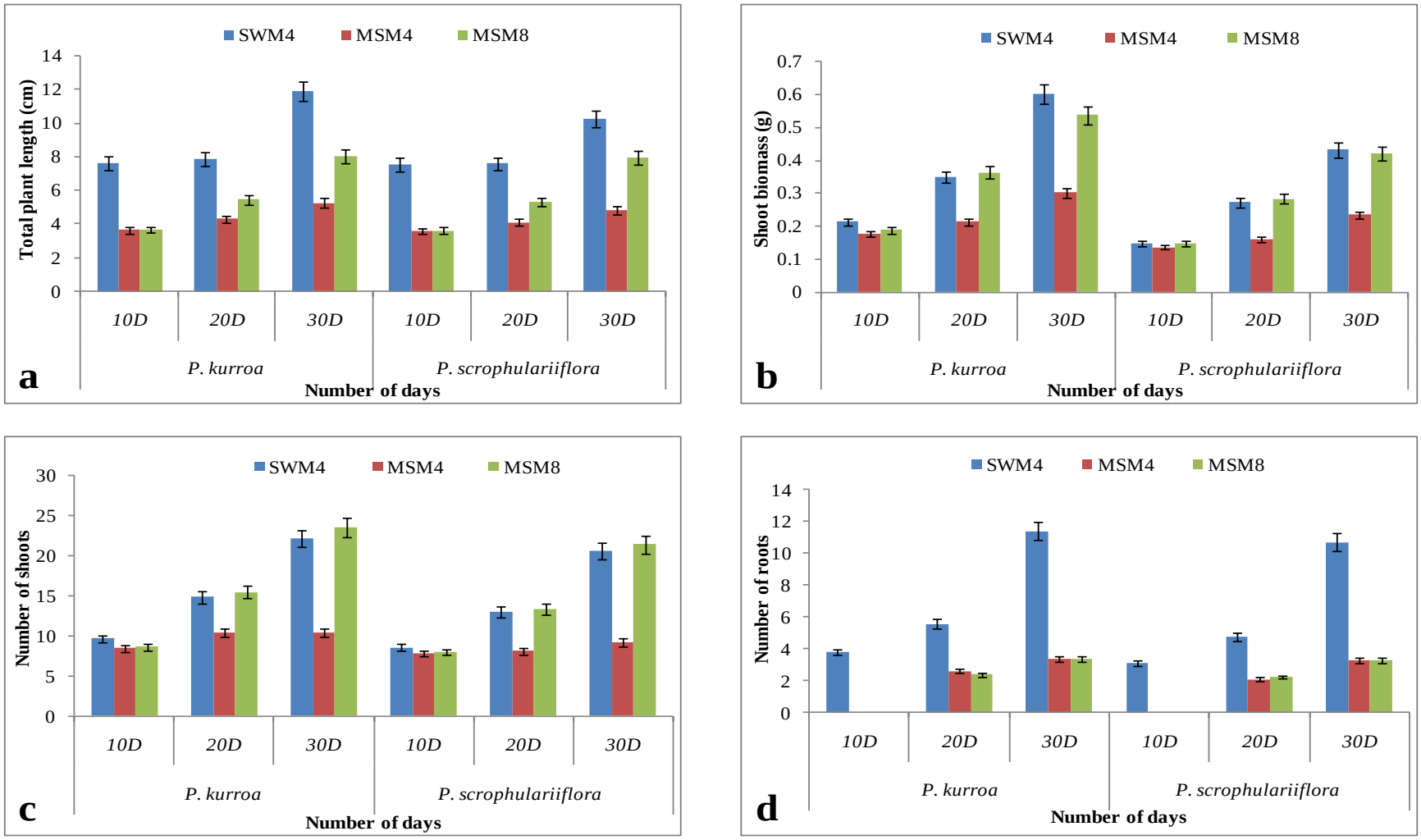


Fig. 4.15 Growth parameters corresponding to total plant length (a), shoot biomass (b), number of shoots (c) and number of roots (d) for *P. kurroa* and *P. scrophulariiflora* plants grown on SWM4 (SWE + sucrose + agar), MSM4 (MS + sucrose + agar) and MSM8 (MS + sucrose + growth hormones + agar) at 15 ± 2 ºC. Error bars represent mean±SD for data recorded in triplicates (repeated thrice)

4.7 Hardening

Well rooted in vitro grown plantlets of *P. kurroa* and *P. scrophulariiflora* were transferred to pots containing mixture of sand:soil:vermiculite (1:1:1) in the greenhouse for hardening maintained at 25 ± 2 ºC, 80–90 % of relative humidity with light irradiance of 100–120 µmol m^{-2} s^{-1} under natural light conditions (Fig. 4.16). *P. kurroa* plants showed 80% survival rate while *P. scrophulariiflora* showed 75% survival rate in green house conditions.

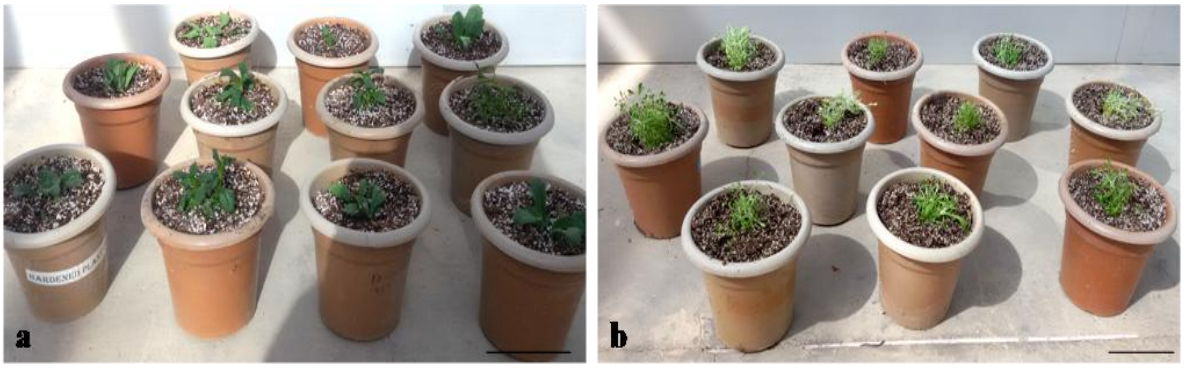


Fig. 4.16 Hardening of *P. kurroa* (a) and *P. scrophulariiflora* (b) plantlets in greenhouse (scale bar = 8 cm)

4.8 Effect of SWE on P-I production in *P. kurroa* and *P. scrophulariiflora*

HPLC analysis of one month old *P. kurroa* and *P. scrophulariiflora* shoots grown on MSS (MS + sucrose + 2 g/L SWE + growth hormones + agar), SWM (SWE + sucrose + agar) and SM (MS + sucrose + growth hormones + agar) media at 25 ± 2 °C and 15 ± 2 °C was done to study the effect of SWE as a biostimulant and as a medium on P-I production in both plant species. Our results showed enhanced P-I content in both incubation temperatures. *P. kurroa* plants grown on MSS showed highest P-I accumulation with increase of 3.84 and 2.62 fold followed by SWM with 3.02 and 2.48 fold as compared to plants grown on SM at 25 ± 2 °C and 15 ± 2 °C, respectively (Figs. 4.17 and 4.18). Similarly, *P. scrophulariiflora* plants showed increment of 3.26 and 2.12 fold on MSS followed by 2.50 and 2.01 fold on SWM as compared to control at 25 ± 2 °C and 15 ± 2 °C, respectively (Figs. 4.17 and 4.18). These results were found to be in conjunction with the previous reports where seaweed extract treatment increased the total

phenolic and flavonoid contents in *Brassica oleraceae* and *Kappaphycus* Doty [31, 165]. Thus, SWE can be utilized for enhancing P-I production in *P. kurroa* and *P. scrophulariiflora* plants for development of economically viable strategies to meet increasing industrial demands.

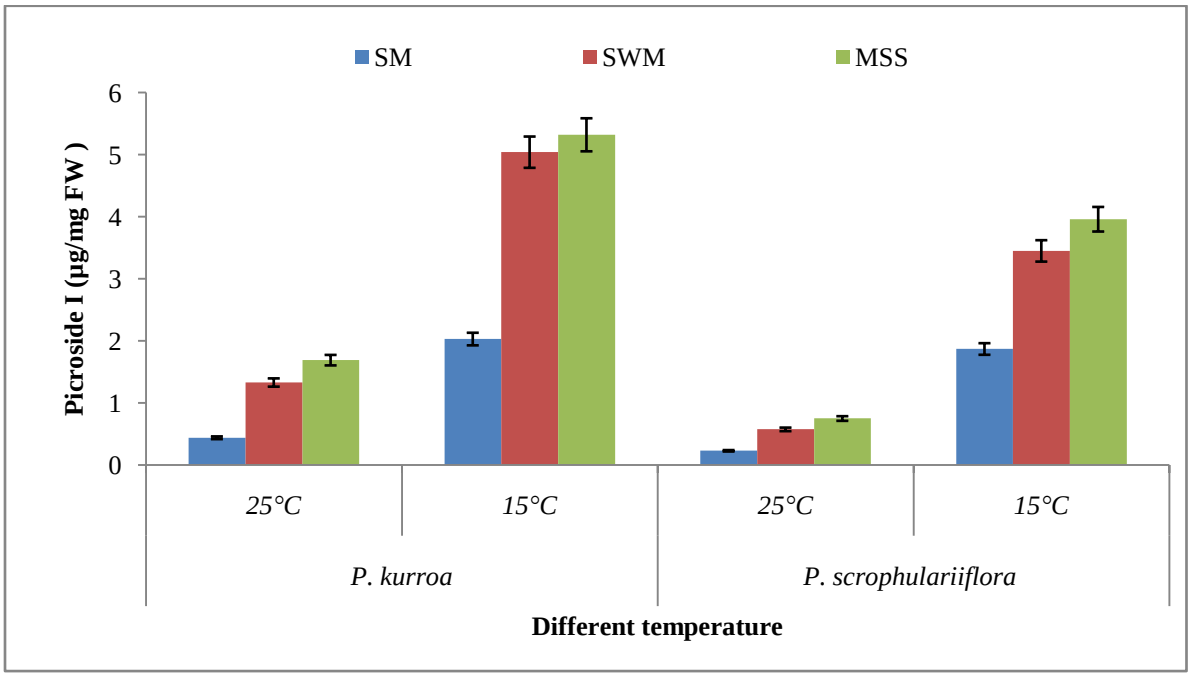


Fig. 4.17 P-I content in *P. kurroa* and *P. scrophulariiflora* plants grown on MSS (MS + sucrose + SWE + growth hormones + agar), SWM (SWE + sucrose + agar) and SM (MS + sucrose + growth hormones + agar) media at 25 ± 2 °C and 15 ± 2 °C. Error bars represent mean±SD for data recorded in triplicates (repeated thrice)

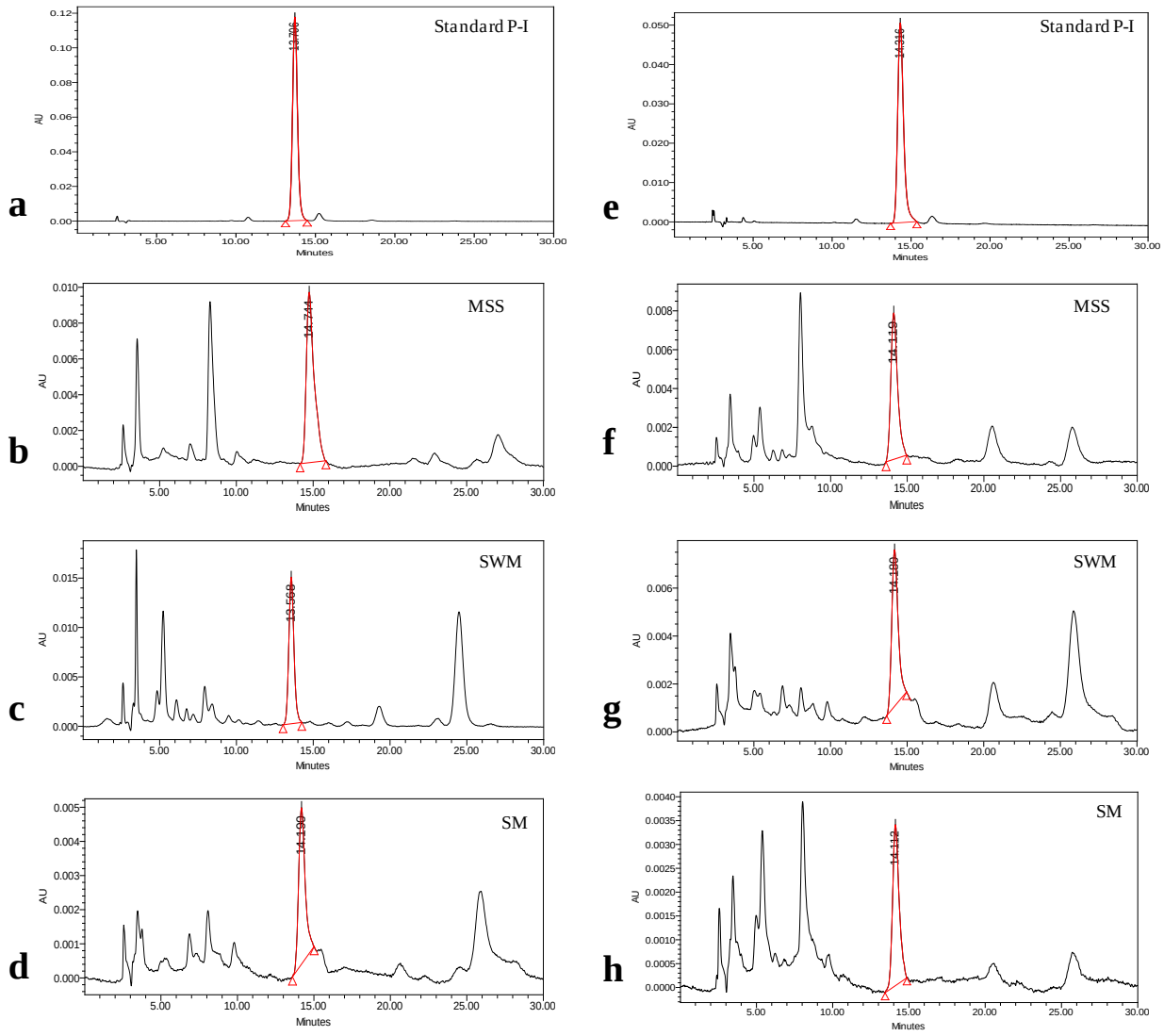


Fig. 4.18 HPLC chromatograms for P-I standards (a, e), *P. kurroa* shoots (b, c, d) and *P. scrophulariiflora* shoots (f, g, h) grown on MSS (MS + sucrose + SWE + growth hormones + agar), SWM (SWE + sucrose + agar) and SM (MS + sucrose + growth hormones + agar) media at 15 ± 2 °C

4.9 Cost estimation

The cost estimation was done by taking single shoot apex in each jar (50 ml media). The cost of MSS, SWM and SM media was estimated to be Rs. 12.72, 7.66 and 12.21 per jar, respectively.

Their components included- MS medium (HiMedia) Rs. 100/L, sucrose (HiMedia) Rs. 80.7/L, agar (HiMedia) Rs. 62.64/L, growth hormones (HiMedia) Rs. 1.05/L and SWE (Sea6 Energy) Rs. 10/L. Number of *P. kurroa* shoots formed in 50 ml of MSS, SWM and SM media were found to be 34.60, 22.10 and 23.51, respectively. Therefore, cost of growing one shoot was found to be Rs. 0.37, 0.34 and 0.52 in MSS, SWM and SM media, respectively. Similarly, cost of growing one shoot of *P. scrophulariiflora* was found to be Rs. 0.44, 0.37, 0.57 on MSS, SWM and SM media, respectively. Our results revealed that media cost per *P. kurroa* shoot was 1.40 and 1.53 fold lower in MSS and SWM, respectively as compared to SM (Table 4.1). Similarly, media cost per *P. scrophulariiflora* shoot was 1.29 and 1.54 fold lower in MSS and SWM, respectively as compared to SM (Table 4.1). Hence, media cost per *P. kurroa* shoot was found to be 1.18 and 1.08 fold lower in MSS and SWM, respectively as compared to *P. scrophulariiflora* shoot grown on same media combinations. These statistics demonstrated that SWE as a biostimulant and as an alternative medium offered low cost multiplication of *P. kurroa* and *P. scrophulariiflora* plants for P-I production. Also, micropropagation of *P. kurroa* on these media combinations was found to be economical as compared to *P. scrophulariiflora.*

Table 4.1 Cost estimation of in vitro micropropagation of *P. kurroa* and *P. scrophulariiflora* plants grown on MSS, SWM and SM media

	MSS	**SWM**	**SM**
Media composition	MS + sucrose + SWE + growth hormones + agar	SWE + sucrose + agar	MS + sucrose + growth hormones + agar
Media cost per liter (Rs.)	254.39	153.34	244.39
Media cost per jar (50 ml) (Rs.)	12.72	7.66	12.21
Number of *P. kurroa* shoots per jar (50 ml)	34.6	22.10	23.51
Media cost/*P. kurroa* shoot (Rs.)	0.37	0.34	0.52
Number of *P. scrophulariiflora* shoots per jar (50 ml)	29.18	20.54	21.30
Media cost/*P. scrophulariiflora* shoot (Rs.)	0.44	0.37	0.57

4.10 Effect of different elicitors on shoot biomass and P-I content

Various elicitors such as ABA, salicylic acid, MeJa, SNP, hydrogen peroxide, yeast extract, chitin, chitosan, etc. have been investigated for plant based secondary metabolite production in different plant species. Hence, for the present study, various concentrations (50, 100 and 200 µM) of different elicitors *viz.* SNP, ABA and MeJa were screened for studying their effect on shoot biomass and P-I content in *P. kurroa* and *P. scrophulariiflora* plants grown under in vitro conditions. Out of tested different concentrations of SNP, highest enhancement with 1.30, 1.21 fold in shoot biomass and 1.35, 1.28 fold in P-I content was observed in *P. kurroa* and *P. scrophulariiflora*, respectively at 100 µM concentration as compared to untreated control (Figs. 4.19a and 4.19b). Filippou et al. [203] have also showed increase in proline and polyamine

accumulation upon administration of SNP in *Medicago trunculata* plants. Further, ABA showed highest enhancement in P-I content with 2.01 fold in *P. kurroa* and 1.92 fold in *P. scrophulariiflora* at 50 µM concentration (Fig. 4.19b), while no increase was observed in shoot biomass of both species at all tested concentrations as compared to untreated control (Fig. 4.19a). This could be correlated to enhanced production of reserpine by ABA treatment in whole plant culture of *Rauwolfia serpentina* L. [204]. On the contrary, MeJa showed highest enhancement with 1.56, 1.47 fold in shoot biomass, while 0.51, 0.45 fold decrease was observed in P-I content of *P. kurroa* and *P. scrophulariiflora*, respectively at 100 µM concentration as compared to untreated control (Figs. 4.19a and 4.19b). Singh and others [36] have also showed decreased P-I content after MeJa treatment in field grown leaves of *P. kurroa* plants. Thus, it was evident from the results that SNP and ABA significantly increased the P-I content while significant decrease was observed with MeJa treatment.

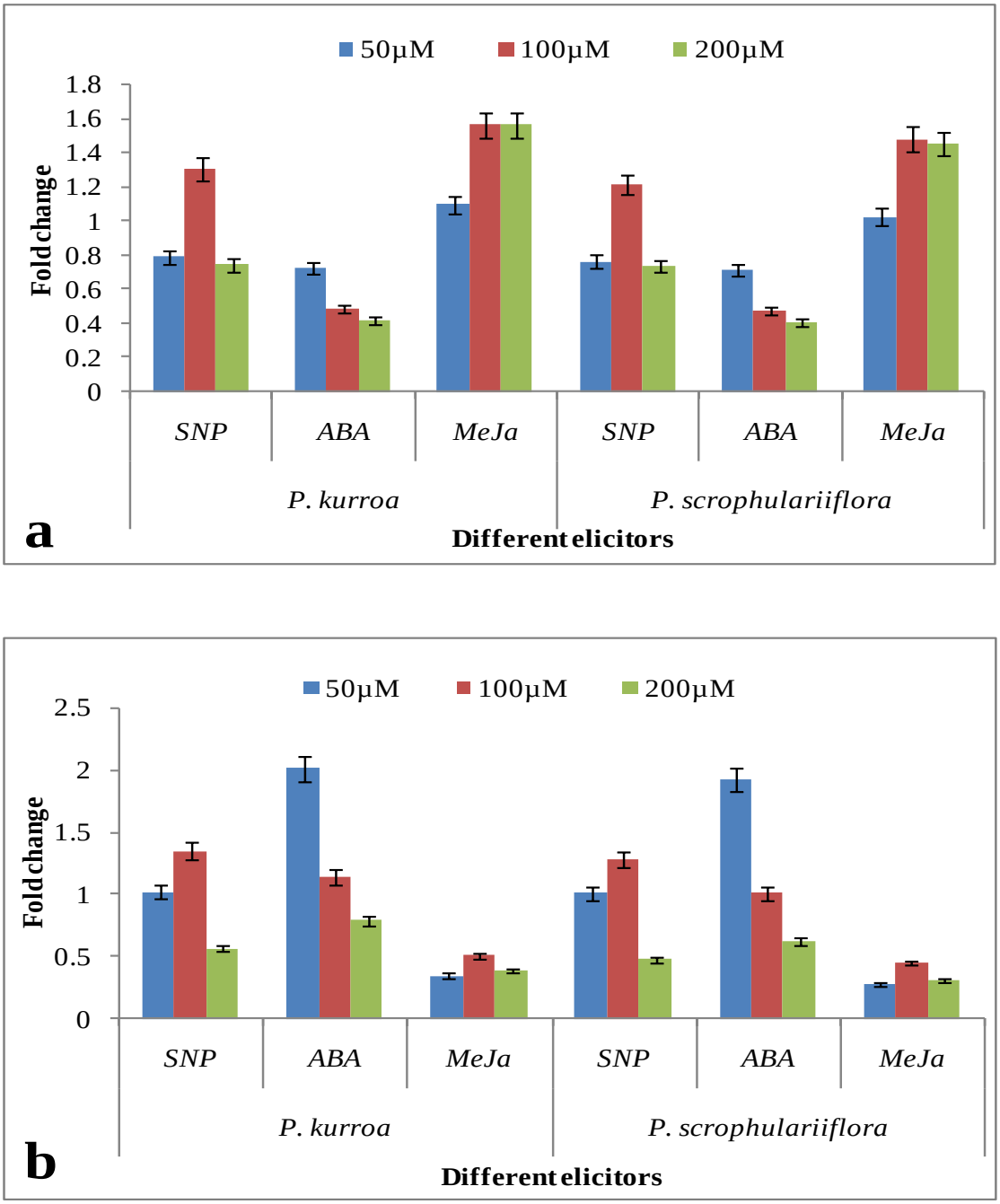


Fig. 4.19 Screening for optimum concentrations of different elicitors. The optimum concentrations of SNP, ABA and MeJa were selected by observing their effect in fold change of shoot biomass (a), and P-I content (b) in *P. kurroa* and *P. scrophulariiflora* plants as compared to their untreated controls. The error bars represent mean±SD of data recorded in triplicates

Further, the effect of SWE was compared with SNP, ABA and MeJa under in vitro conditions. Total shoot biomass and P-I content were analyzed in one month old shoot samples of *P. kurroa* and *P. scrophulariiflora*. Treatment with SWE (2 g/L) showed highest shoot biomass and P-I content in *P. kurroa* and *P. scrophulariiflora* plants as compared to SNP, ABA and MeJa (Fig. 4.20). SWE showed highest enhancement in shoot biomass with 2.66 fold and P-I content with 2.62 fold, while an increase in shoot biomass with 1.30, 0.72, 1.56 fold and P-I content with 1.35, 2.01, 0.51 fold was observed in *P. kurroa* upon treatment with SNP, ABA and MeJa, respectively as compared to untreated control. Similarly, SWE showed increase in shoot biomass with 2.64 fold and P-I with 2.12 fold, while an increase in shoot biomass with 1.21, 0.71, 1.48 fold and P-I content with 1.28, 1.92, 0.45 fold was observed in *P. scrophulariiflora* upon application of SNP, ABA and MeJa, respectively as compared to untreated control. This might be due to the presence of several components in seaweed extracts besides macro- and micro-nutrients *viz.* phytohormones, amino acids, vitamins, sterols, betaines, oligosaccharides and trace minerals [32, 141]. The biostimulation activity of seaweed extracts is mainly associated with bioactive compounds present in it which modulate the expression of different genes coding for proteins involved in plant phytohormone biosynthesis needed for plant growth and development [139]. Wally et al. [205] have also showed that seaweed extract modulated endogenous phytohormones by regulating the hormone biosynthetic genes, thereby leading to growth enhancement in Arabidopsis. Similarly, Fan et al. [153] have reported enhanced total soluble protein content, antioxidant capacity, phenolic and flavonoid contents in spinach attributed to increased transcript abundance of key enzymes involved in nitrogen metabolism, betaine synthesis and endogenous plant hormone activities by seaweed extract treatment. Improved levels of phenolics and flavonoids in *Brassica oleraceae* and *Kappaphycus* Doty upon seaweed extract treatment have also been observed [31, 165].

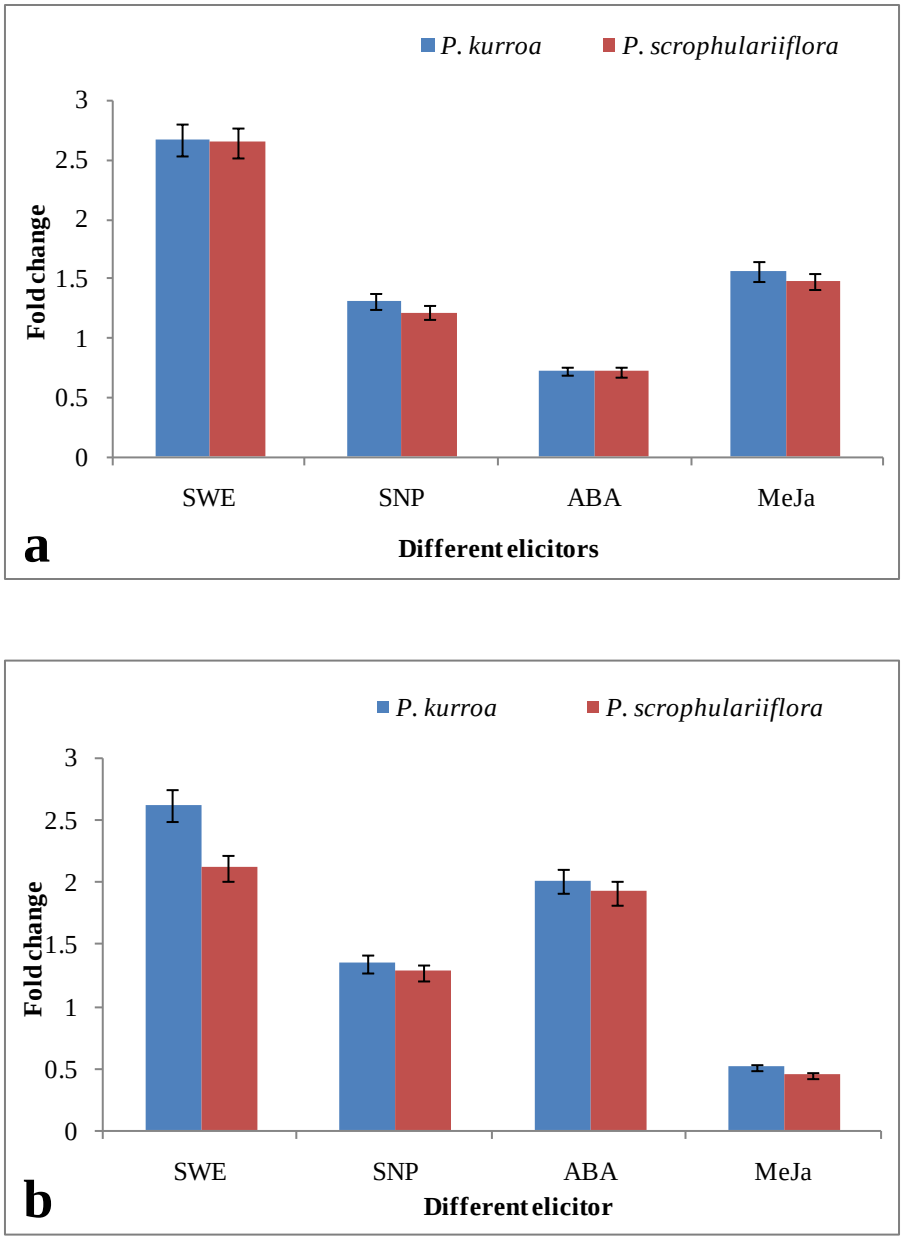


Fig. 4.20 Comparative analysis of SWE with optimum concentrations of SNP, ABA and MeJa by studying their effect in fold change of shoot biomass (a) and P-I content (b) in *P. kurroa* and *P. scrophulariiflora* plants as compared to their untreated controls. The error bars represent mean±SD of data recorded in triplicates

4.11 Gene expression analysis vis-à-vis different elicitors

Elicitor treatment stimulates the production of secondary metabolites in plants. Hence, to observe the molecular basis of modulations in P-I production, various genes *viz.* HMGS, HMGR, PMK, DXPS, DXPR, ISPD, ISPE, MECPS, G10H and PAL of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways were analyzed for their expression vis-à-vis P-I content in *P. kurroa* and *P. scrophulariiflora* shoots treated with optimum concentrations of different elicitors. The expression of genes encoding HMGS, HMGR and PMK enzymes of MVA pathway showed increase with 2-5 and 2-4 folds upon SWE treatment in *P. kurroa* and *P. scrophulariiflora,* respectively as compared to untreated controls (Figs. 4.21 and 4.22). On the other hand, 2 genes of MEP pathway *viz.* DXPS and ISPD showed 3-5 and 2-4 folds high expression in *P. kurroa* and *P. scrophulariiflora,* respectively (Figs. 4.21 and 4.22). Fan et al. [153] have also showed seaweed extract induced systemic physiological responses including elicitation of genes corresponding to phenylpropanoid and flavanoid biosynthetic pathways.

SNP treatment showed 2-3 fold high expression in HMGR, PMK, DXPS, ISPD, ISPE and MECPS genes in *P. kurroa* and *P. scrophulariiflora* as compared to untreated controls (Figs. 4.21 and 4.22). The treatment with MeJa enhanced the expression of PMK, ISPD and ISPE genes with 2-3 folds in both species, while slight modulation was observed in other studied genes of MVA/MEP pathways as compared to untreated controls. Surprisingly, administration of ABA did not increase the expression of all selected genes of MVA and MEP pathways in *P. kurroa* and *P. scrophulariiflora* (Figs. 4.21 and 4.22). This indicated that ABA does not modulated MVA/MEP pathways for enhanced production of P-I in both plant species which was in agreement with the findings of Singh et al. [36]. It was evident from the results that SWE and SNP modulated MVA/MEP pathways as compared to other elicitors which suggested their involvement in enhanced production of P-I. Previous reports have also shown that SNP is a potential NO donor which regulates the plant metabolic pathways [203, 206].

Further, the expression of gene encoding G10H enzyme of iridoid pathway showed increase with 2-3 folds upon administration of SWE, SNP and MeJa, respectively as compared to untreated control in both plant species (Figs. 4.21 and 4.22). Thus, results indicated that enhanced

availability of iridoid precursor upon application of SWE and SNP increase the expression of G10H gene, thereby resulting in P-I elicitation. It has been reported that G10H gene is a potential candidate for regulation of seco-iridoids in *C. roseus* [207-208]. Our results also suggested that SWE and SNP treatments increased P-I content and G10H gene expression in *P. kurroa* and *P. scrophulariiflora* shoots, thereby demonstrating the plausible role of G10H gene in regulation of iridoids production in both plant species. On the other hand, MeJa treatment might benefit seco-iridoid biosynthesis than iridoid biosynthesis as indicated by P-I content and G10H expression in *P. kurroa* plants [208]. An increase in expression of gene encoding PAL enzyme of shikimate/phenylpropanoid pathway was observed with 5.27, 4.87 fold in SWE and 2.73, 2.61 fold in ABA treated plants of *P. kurroa* and *P. scrophulariiflora,* respectively as compared to untreated controls (Figs. 4.21 and 4.22). This suggested that ABA stimulated shikimate/phenylpropanoid pathway while SWE modulated all the integrating pathways for enhancing P-I content. Jiang and Joyce [209] have also demonstrated that exogenous application of ABA up-regulated the PAL activity, thereby increasing the phenolic content in strawberry fruit.

Overall, SWE showed maximum increase in P-I content as compared to other studied elicitors by modulating all the integrating pathways of P-I biosynthesis in *P. kurroa* and *P. scrophulariiflora*. While ABA modulated only shikimate/phenylpropanoid pathway for increased P-I content, SNP targeted MVA/MEP pathways in conjunction with iridoid pathway to increase the P-I content. Lastly, MeJa treatment decreased P-I content which might be due to the redirection of flux towards seco-iridoids as compared to iridoids in *P. kurroa* and *P. scrophulariiflora*.

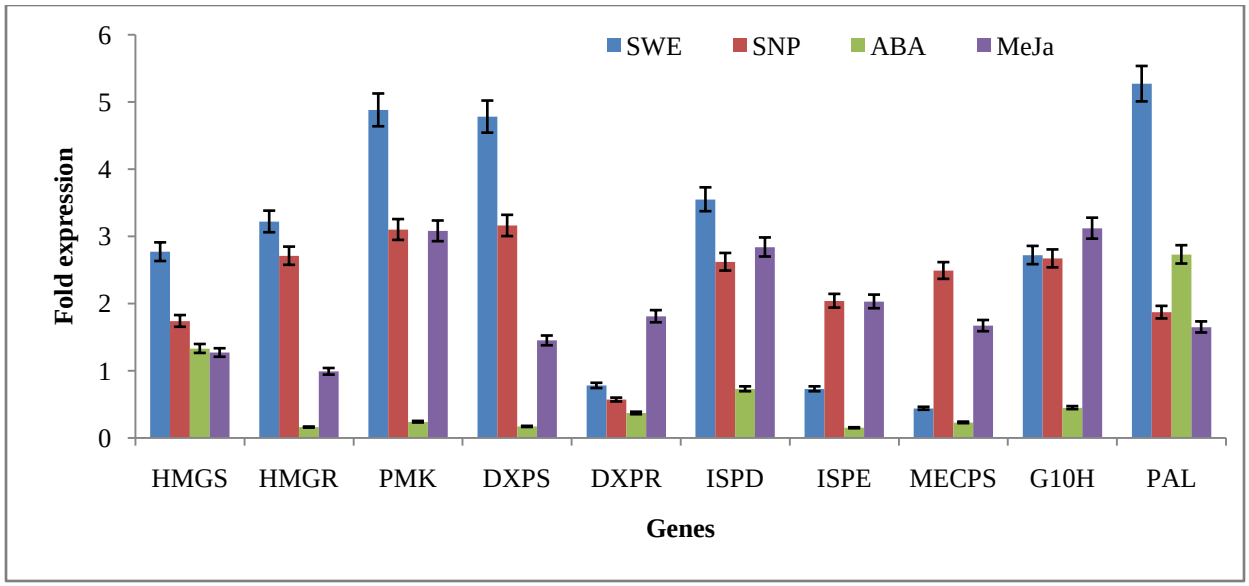


Fig. 4.21 Expression analysis of selected genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways in *P. kurroa* plants treated with different elicitors as compared to untreated control. The error bars represent mean±SD of data recorded in triplicates

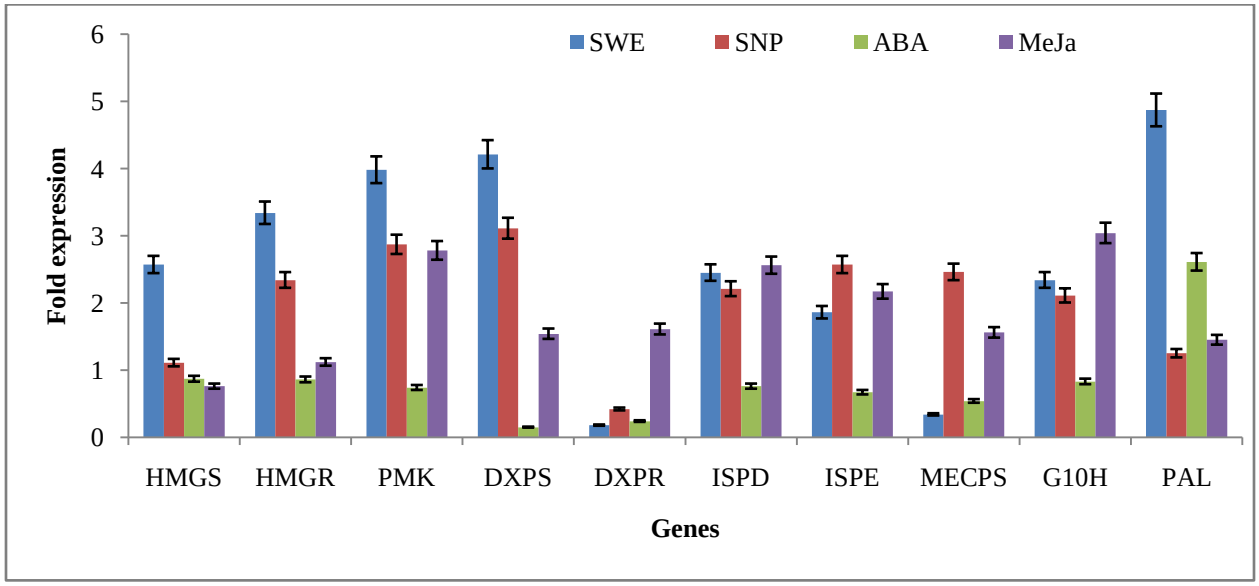


Fig. 4.22 Expression analysis of selected genes of MVA, MEP, iridoid and shikimate/phenylpropanoid pathways in *P. scrophulariiflora* plants treated with different elicitors as compared to untreated control. The error bars represent mean±SD of data recorded in triplicates

SUMMARY

Understanding the role of different morphogenetic stages and potential of seaweed extract along with molecular basis of P-I biosynthesis under in vitro conditions can aid in improving shoot biomass and P-I production in *P. kurroa* and *P. scrophulariiflora.* The current study has therefore, suggested that P-I biosynthesis was developmentally regulated during different stages of differentiation in *P. kurroa* and *P. scrophulariiflora*. Expression analysis of multiple genes of primary and secondary metabolic pathways at different morphogenetic stages of *P. kurroa* and *P. scrophulariiflora* confirmed their involvement in P-I biosynthesis vis-à-vis shoot development. Temperature influenced P-I production by regulating all integrating pathways of secondary metabolism in these plant species. Genes such as HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL showed 47-87 and 38-73 folds high expression in shoots of FD stage of *P. kurroa* and *P. scrophulariiflora*, respectively originated from leaf explants at 15 ± 2 °C compared to 25 ± 2 °C. These genes also showed 6-19 folds high expression in shoots of FD stage of *P. kurroa* compared to *P. scrophulariiflora* which was in positive correlation with P-I content, thus confirming their role in P-I biosynthesis. Further validation of these genes by gene function approaches will fully ascertain their role in P-I biosynthesis. These findings would be helpful in planning genetic intervention strategies for metabolic engineering of *P. kurroa* and *P. scrophulariiflora* for enhancing P-I production.

Further, for cost effective micropropagation and P-I biosynthesis, effect of SWE as a biostimulant and as a medium alternative to nutrient medium was studied in *P. kurroa* and *P. scrophulariiflora*. SWE as a biostimulant with MS medium enhanced the total plant length, shoot biomass, number of shoots and roots in *P. kurroa* and *P. scrophulariiflora* plants. SWM showed comparable results with SM; therefore, it can be used as an alternative to MS medium for large scale micropropagation of these plant species. MSS and SWM increased the P-I production by 2-4 folds at 15 ± 2 ºC and 25 ± 2 ºC thereby, suggesting that SWE can be used as a novel elicitor for enhanced P-I production under in vitro conditions for *P. kurroa* and *P. scrophulariiflora*. SWE as a biostimulant and as an alternative medium offered cost effective micropropagation of *P. kurroa* and *P. scrophulariiflora* plants for P-I production.

A comparative analysis of SWE with other elicitors *viz.* MeJa, SNP and ABA was carried out to identify an efficient elicitor for enhanced plant growth and P-I content in *P. kurroa* and *P. scrophulariiflora*. SWE showed highest enhancement in shoot biomass with 2.66, 2.64 fold and P-I content 2.62, 2.12 fold in *P. kurroa* and *P. scrophulariiflora*, respectively, while increase in shoot biomass with 0.71–1.56 fold and P-I content with 0.45-2.01 fold was observed in these plant species on treatment with SNP, ABA and MeJa as compared to untreated control. The molecular dissection of SWE induced P-I biosynthesis demonstrated the up-regulation of HMGS, HMGR, PMK, DXPS, ISPD, G10H and PAL genes vis-à-vis P-I content in in vitro grown *P. kurroa* and *P. scrophulariiflora* shoots, suggesting modulation of all four integrating pathways of P-I biosynthesis. These findings would benefit the selection of genes for genetic intervention strategies, which can provide the platform for micropropagation of these species with increased P-I content in order to meet the increasing industrial demands.

Overall, the outcome of this study would be helpful in understanding molecular basis of P-I biosynthesis, enhancement of shoot biomass and P-I content in *P. kurroa* and *P. scrophulariiflora* using tissue culture techniques.

APPENDIX

Table A1 Complete biochemical profile of seaweed extract powder (data provided by Sea6 Energy Pvt Ltd)

A.	Macro/Micro-nutrients	
	Element	Concentration (mg/L)
	Potassium (K)	89,117 ± 4693
	Sodium (Na)	15512 ± 556.10
	Sulfur (s)	1601 ± 100.85
	Calcium (Ca)	920 ± 282.84
	Magnesium (Mg)	815.43 ± 84.96
	Nitrogen (N)	443.05
	Phosphorus (P)	220.58 ± 59.01
	Iron (Fe)	68.75 ± 26.52
	Manganese (Mn)	19.25 ± 2.47
	Boron (B)	14.50 ± 2.12
	Copper (Cu)	2 ± 0.71
	Zinc (Zn)	1.30 ± 0.14
	Aresenic (As)	BDL
	Selenium (Se)	BDL
B.	Amino Acids	
	Aspartate	BDL
	Glutamate	1103.85 ± 22.47
	Asparagine	BDL
	Serine	28.64 ± 0.98
	Glutamine	363.35 ± 2.82
	Histidine & Glycine	49.70 ± 3.38

	Threonine	36.98 ± 6.73
	Arginine	1474.76 ± 1.76
	Alanine	50.44 ± 3.32
	Tyrosine	53.8 ± 0.90
	Valine	101.68 ± 2.56
	Methionine	64.02 ± 7.21
	Tryptophan	116.22 ± 8.57
	Phenylalanine	249.97 ± 1.18
	Isoleucine	113.32 ± 5.20
	Leucine	111.67 ± 4.14
	Lysine	43.72 ± 1.33
C.	Plant Growth Regulators (PGR)	
	Total Auxins	216.58 ± 20
	Total Cytokinins	19.53 ± 3.90
D.	Physical Properties	
	Solubility in water	Completely soluble
	pH	5.64
	Viscosity	1.8
	Specific gravity	1.16
*BDL-Below Detectable limit		

Table A2 Growth parameters corresponding to total plant length, shoot biomass, number of shoots and roots for *P. kurroa* plants grown on MS media supplemented with SWE, sucrose, growth hormones and agar at 25 ± 2 ºC. ANOVA test shows high significance with Duncan multiple-range test at 5% (p=0.05). Data was recorded in triplicates and repeated thrice on 10th, 20th and 30th day and represented by mean±SD

	Total plant length (cm)			**Shoot biomass (g)**			**Number of shoots per explant**			**No. of roots per explant**		
	10 D	**20 D**	**30 D**	**10 D**	**20 D**	**30 D**	**10 D**	**20 D**	**30 D**	**10 D**	**20 D**	**30 D**
MS S 1	3.03±0.01^{a}	3.53±0.03^{a}	4.36±0.06^{a}	0.14±0.00^{a}	0.17±0.00^{a}	0.23±0.00^{a}	8.40±0.24^{a}	10.60±0.24^{b}	11.20±0.37^{a}	0.00±0.00^{a}	2.40±0.24^{a}	3.40±0.24^{a}
MS S 2	3.27±0.02^{b}	3.87±0.04^{b}	4.55±0.02^{b}	0.15±0.00^{c}	0.18±0.00^{b}	0.25±0.00^{b}	8.60±0.24^{a}	10.60±0.24^{b}	11.60±0.24^{a}	1.20±0.37^{b}	2.60±0.24^{a}	3.60±0.24^{a}
MS S 3	4.26±0.02^{c}	5.56±0.02^{f}	6.86±0.02^{e}	0.18±0.00^{e}	0.41±0.00^{g}	0.67±0.00^{e}	14.20±0.20^{d}	19.60±0.24^{e}	25.20±0.24^{d}	2.80±0.20^{d}	4.60±0.24^{b}	8.20±0.20^{c}
MS S 4	3.28±0.04^{b}	3.96±0.02^{b}	4.70±0.01^{c}	0.15±0.00^{d}	0.20±0.00^{c}	0.25±0.00^{a}	8.60±0.24^{a}	11.80±0.37^{c}	13.60±0.40^{b}	1.40±0.24^{b}	2.60±0.24^{a}	3.80±0.37^{a}
C1	3.04±0.01^{a}	3.59±0.02^{a}	4.40±0.04^{a}	0.14±0.00^{a}	0.17±0.00^{a}	0.24±0.00^{a}	8.40±0.24^{a}	9.60±0.24^{a}	10.40±0.24^{a}	0.00±0.00^{a}	2.60±0.24^{a}	3.40±0.24^{a}
MS S 5	3.09±0.00^{a}	4.83±0.06^{d}	6.85±0.06^{e}	0.16±0.00^{c}	0.29±0.00^{d}	0.61±0.00^{d}	9.40±0.24^{b}	15.40±0.50^{d}	23.20±0.37^{c}	0.00±0.00^{a}	2.60±0.24^{a}	4.60±0.24^{b}
MS S 6	3.30±0.07^{b}	4.96±0.03^{e}	7.00±0.03^{e}	0.17±0.00^{d}	0.29±0.00^{e}	0.68±0.00^{d}	10.40±0.24^{c}	15.20±0.37^{d}	24.20±0.37^{cd}	2.00±0.31^{c}	3.00±0.31^{a}	4.60±0.24^{b}
MS S 7	4.28±0.12^{c}	6.79±0.07^{g}	7.68±0.07^{f}	0.21±0.00^{f}	0.41±0.00^{h}	1.15±0.00^{f}	15.60±0.50^{e}	21.00±0.31^{f}	34.60±0.81^{e}	5.60±0.24^{e}	7.00±0.31^{c}	12.00±0.31^{d}
MS S 8	3.31±0.02^{b}	4.61±0.02^{c}	6.86±0.06^{e}	0.17±0.00^{d}	0.31±0.00^{f}	0.58±0.00^{c}	11.00±0.31^{c}	15.80±0.37^{d}	25.40±0.68^{d}	0.00±0.00^{a}	2.40±0.24^{a}	3.60±0.24^{a}
SM	3.06±0.00^{a}	4.54±0.06^{c}	6.71±0.01^{d}	0.15±0.00^{b}	0.29±0.00^{d}	0.43±0.00^{cd}	8.60±0.24^{a}	15.40±0.24^{d}	23.51±0.24^{c}	0.00±0.00^{a}	2.40±0.24^{a}	3.40±0.24^{a}

Table A3 Growth parameters corresponding to total plant length, shoot biomass, number of shoots and roots for *P. scrophulariiflora* plants grown on MS media supplemented with SWE, sucrose, growth hormones and agar at 25 ± 2 ºC. ANOVA test shows high significance with Duncan multiple-range test at 5% (p=0.05). Data was recorded in triplicates and repeated thrice on 10th, 20th and 30th day and represented by mean±SD

	Total plant length (cm)			**Shoot biomass (g)**			**Number of shoots per explant**			**No. of roots per explant**		
	10 D	**20 D**	**30 D**	**10 D**	**20 D**	**30 D**	**10 D**	**20 D**	**30 D**	**10 D**	**20 D**	**30 D**
MS S 1	3.02±0.06^{a}	3.45±0.04^{a}	4.16±0.07^{a}	0.12±0.01^{a}	0.15±0.00^{a}	0.21±0.00^{a}	8.32±0.21^{a}	9.86±0.45^{b}	10.12±0.45^{a}	0.00±0.00^{a}	2.10±0.34^{a}	3.20±0.42^{a}
MS S 2	3.11±0.06^{b}	3.67±0.05^{b}	4.35±0.06^{b}	0.12±0.03^{c}	0.17±0.00^{b}	0.23±0.00^{b}	8.56±0.22^{a}	10.46±0.56^{b}	11.34±0.64^{a}	1.11±0.33^{b}	2.30±0.53^{a}	3.40±0.32^{a}
MS S 3	4.11±0.07^{d}	5.21±0.05^{f}	6.18±0.06^{e}	0.13±0.03^{e}	0.28±0.03^{g}	0.49±0.20^{e}	13.37±0.21^{d}	15.08±0.74^{e}	24.04±0.45^{d}	2.50±0.34^{d}	3.30±0.22^{b}	7.34±0.42^{c}
MS S 4	3.18±0.05^{b}	3.79±0.05^{b}	4.56±0.07^{c}	0.13±0.00^{d}	0.19±0.00^{c}	0.24±0.00^{a}	8.59±0.21^{a}	10.38±0.54^{c}	12.86±0.45^{b}	1.30±0.44^{b}	2.40±0.34^{a}	3.45±0.23^{a}
C1	3.01±0.23^{b}	3.42±0.05^{b}	4.01±0.07^{a}	0.13±0.10^{a}	0.13±0.03^{a}	0.19±0.20^{a}	7.82±0.45^{a}	8.11±0.45^{a}	9.21±0.54^{a}	0.00±0.00^{a}	2.10±0.56^{a}	3.30±0.23^{a}
MS S 5	3.05±0.03^{a}	4.53±0.08^{d}	6.72±0.08^{e}	0.13±0.00^{c}	0.25±0.00^{d}	0.56±0.00^{d}	9.14±0.56^{b}	14.40±0.45^{d}	22.80±0.35^{c}	0.00±0.00^{a}	2.50±0.65^{a}	4.21±0.45^{b}
MS S 6	3.21±0.04^{b}	4.79±0.08^{e}	6.91±0.08^{e}	0.14±0.00^{d}	0.27±0.00^{e}	0.61±0.00^{d}	10.24±0.67^{c}	15.02±0.64^{d}	23.72±0.55^{cd}	1.70±0.11^{c}	2.90±0.56^{a}	4.30±0.56^{b}
MS S 7	4.02±0.34^{d}	6.54±0.21^{f}	7.51±0.19^{f}	0.15±0.02^{f}	0.30±0.04^{h}	0.91±0.05^{f}	14.09±0.76^{f}	17.12±0.54^{f}	29.18±0.45^{e}	5.30±0.34^{e}	6.10±0.45^{c}	11.10±0.34^{d}
MS S 8	3.26±0.23^{c}	4.87±0.23^{c}	6.98±0.02^{e}	0.14±0.00^{d}	0.29±0.00^{f}	0.66±0.00^{c}	10.23±0.45^{c}	15.70±0.64^{d}	24.84±0.38^{d}	0.00±0.00^{a}	2.30±0.34^{a}	3.54±0.56^{a}
SM	3.03±0.21^{a}	4.45±0.23^{c}	6.61±0.03^{cd}	0.12±0.02^{b}	0.23±0.05^{d}	0.34±0.08^{cd}	8.01±0.67^{b}	13.31±0.74^{d}	21.30±0.46^{c}	0.00±0.00^{a}	2.23±0.23^{a}	3.30±0.56^{a}

Table A4 Growth parameters corresponding to total plant length, shoot biomass, number of shoots and roots for *P. kurroa* plants grown on novel SWM and MS media at 25 ± 2 ºC. ANOVA test shows high significance with Duncan multiple-range test at 5% (p=0.05). Data was recorded in triplicates and repeated thrice on 10th, 20th and 30th day and represented by mean±SD

	Total plant length (cm)			**Shoot biomass (g)**			**No. of shoots per explant**			**No. of roots per explant**		
	10 D	**20 D**	**30 D**	**10 D**	**20 D**	**30 D**	**10 D**	**20 D**	**30 D**	**10 D**	**20 D**	**30 D**
SWM 1	3.13±0.03 d	0.00±0.00 a	0.00±0.00 a	0.14±0.02 b	0.00±0.00 a	0.00±0.00 a	8.60±0.24 ab	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a
SWM 2	4.64±0.01 h	5.85±0.02 h	8.62±0.10 g	0.14±0.00 bc def	0.26±0.00 d	0.41±0.00 d	9.40±0.24 bc	13.80±0.37 gh	20.16±0.51 f	3.60±0.24 d	5.40±0.24 e	9.60±0.51 d
SWM 3	3.55±0.01 g	4.15±0.03 f	0.00±0.00 a	0.22±0.00 i	0.44±0.00 g	0.00±0.00 g	9.20±0.37 abc	10.00±0.32 de	0.00±0.00 a	2.60±0.20 b	3.00±0.32 bd	0.00±0.00 a
SWM 4	6.35±0.01 j	6.55±0.02 j	9.92±0.13 h	0.17±0.00 h	0.28±0.00 e	0.48±0.00 e	9.60±0.24 c	14.80±0.37 ij	22.1±0.58 f	3.80±0.20 d	5.60±0.24 e	11.40±0.51 e
SWM 5	3.20±0.03 e	0.00±0.00 a	0.00±0.00 a	0.16±0.00 cd efg	0.00±0.00 a	0.00±0.00 a	8.60±0.24 ab	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a
SWM 6	3.19±0.01 e	3.29±0.03 b	3.51±0.13 b	0.17±0.00 ef g	0.19±0.00 c	0.21±0.00 b	9.20±0.20 abc	11.20±0.37 f	15.10±0.37 c	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a
SWM 7	3.29±0.01 f	3.88±0.01 e	0.00±0.00 a	0.17±0.00 g	0.29±0.00 e	0.00±0.00 a	8.60±0.24 ab	9.60±0.24 c d	0.00±0.00 a	0.00±0.00 a	2.40±0.24 cd	0.00±0.00 a
SWM 8	4.96±0.02 i	6.11±0.02 i	7.31±0.02 f	0.16±0.00 fg	0.28±0.00 d	0.43±0.00 d	8.60±0.24 ab	14.40±0.24 hi	20.45±0.24 e	3.20±0.20 c	5.40±0.24 e	9.40±0.24 d
MSM 1	2.89±0.02 a	0.00±0.00 a	0.00±0.00 a	0.13±0.00 a	0.00±0.00 a	0.00±0.00 a	8.40±0.24 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a
MSM 2	2.99±0.02 b	3.23±0.09 b	0.00±0.00 a	0.13±0.00 a	0.15±0.00 b	0.00±0.00 a	8.40±0.24 a	8.80±0.20 b	0.00±0.00 a	0.00±0.00 a	0.60±0.24 a	0.00±0.00 a
MSM 3	3.04±0.02 bc	3.20±0.03 b	3.98±0.02 c	0.19±0.00 h	0.28±0.00 d	0.61±0.00 f	8.40±0.24 a	9.20±0.20 b c	15.60±0.24 d	0.00±0.00 a	1.40±0.24 b	2.40±0.24 b

MS M 4	3.01±0.01 bc	3.68±0.01 d	4.38±0.08 d	0.14±0.00 bc	0.17±0.00 c	0.24±0.00 c	8.40±0.24 a	10.40±0.24 e	10.40±0.24 b	0.00±0.00 a	2.20±0.20 c	2.80±0.20 bc
MS M 5	3.02±0.02 bc	0.00±0.00 a	0.00±0.00 a	0.15±0.00 bc d	0.00±0.00 a	0.00±0.00 a	8.60±0.24 ab	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a
MS M 6	3.07±0.01 c	3.55±0.02 c	0.00±0.00 a	0.16±0.00 de fg	0.17±0.00 c	0.00±0.00 a	8.60±0.24 ab	10.60±0.24 ef	0.00±0.00 a	0.00±0.00 a	1.20±0.20 b	0.00±0.00 a
MS M 7	3.19±0.01 e	3.80±0.01 de	4.08±0.04 c	0.27±0.00 j	0.61±0.00 h	1.19±0.02 h	10.40±0.24 d	13.60±0.51 g	24.40±0.51 h	0.00±0.00 a	1.40±0.24 b	2.20±0.20 b
MS M 8	3.06±0.01 c	4.36±0.05 g	6.51±0.11 e	0.15±0.00 bc de	0.29±0.00 f	0.43±0.00 g	10.60±0.24 d	15.20±0.20 f	23.40±0.51 g	0.00±0.00 a	2.40±0.24 cd	3.40±0.24 c

Table A5 Growth parameters corresponding to total plant length, shoot biomass, number of shoots and roots for *P. scrophulariiflora* plants grown on novel SWM and MS media at 25 ± 2 ºC. ANOVA test shows high significance with Duncan multiple-range test at 5% (p=0.05). Data was recorded in triplicates and repeated thrice on 10^{th}, 20^{th} and 30^{th} day and represented by mean±SD

	Total plant length (cm)			**Shoot biomass (g)**			**No. of shoots per explant**			**No. of roots per explant**		
	10 D	**20 D**	**30 D**	**10 D**	**20 D**	**30 D**	**10 D**	**20 D**	**30 D**	**10 D**	**20 D**	**30 D**
SWM 1	3.11±0.13^{c}	0.00±0.00^{a}	0.00±0.00^{a}	0.11±0.02^{b}	0.00±0.00^{a}	0.00±0.00^{a}	8.46±0.28^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
SWM 2	4.54±0.04^{h}	5.71±0.05^{h}	8.56±0.10^{g}	0.12±0.00^{b}	0.19±0.00^{d}	0.32±0.00^{d}	9.22±0.25^{b}	11.89±0.37^{gh}	20.16±0.51^{f}	3.10±0.24^{d}	5.14±0.24^{e}	9.26±0.51^{d}
SWM 3	3.43±0.03^{g}	4.08±0.04^{f}	0.00±0.00^{a}	0.18±0.00^{i}	0.32±0.00^{g}	0.00±0.00^{g}	9.19±0.37^{b}	9.82±0.37^{d}	0.00±0.00^{a}	2.16±0.20^{b}	2.98±0.32^{bd}	0.00±0.00^{a}
SWM 4	6.31±0.04^{j}	6.49±0.05^{j}	9.87±0.21^{h}	0.12±0.00^{h}	0.22±0.00^{e}	0.35±0.00^{e}	8.52±0.28^{d}	12.98±0.41^{j}	20.54±0.61^{f}	3.12±0.26^{d}	4.78±0.27^{e}	10.71±0.52^{e}
SWM 5	3.11±0.04^{e}	0.00±0.00^{a}	0.00±0.00^{a}	0.11±0.00$^{cd efg}$	0.00±0.00^{a}	0.00±0.00^{a}	8.41±0.24$^{a b}$	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
SWM 6	3.12±0.01^{e}	3.21±0.03^{b}	3.43±0.15^{b}	0.13±0.00$^{ef g}$	0.15±0.00^{c}	0.19±0.00^{b}	9.18±0.22^{a}	10.26±0.34^{f}	14.11±0.34^{c}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
SWM 7	3.21±0.01^{f}	3.76±0.04^{e}	0.00±0.00^{a}	0.15±0.00^{g}	0.21±0.00^{e}	0.00±0.00^{a}	8.26±0.25^{a}	9.51±0.24$^{c d}$	0.00±0.00^{a}	0.00±0.00^{a}	2.04±0.24^{cd}	0.00±0.00^{a}
SWM 8	4.87±0.02^{i}	6.03±0.01^{i}	7.12±0.02^{f}	0.12±0.00^{fg}	0.21±0.00^{d}	0.31±0.00^{d}	8.56±0.25^{a}	13.01±0.24^{hi}	19.65±0.23^{e}	3.02±0.20^{c}	5.14±0.24^{e}	9.14±0.24^{d}
MSM 1	2.76±0.03^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.10±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	8.11±0.25^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
MSM 2	2.87±0.02^{b}	3.13±0.09^{b}	0.00±0.00^{a}	0.11±0.00^{a}	0.12±0.00^{b}	0.00±0.00^{a}	8.32±0.24^{a}	8.41±0.20^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.40±0.24^{a}	0.00±0.00^{a}
MSM 3	3.01±0.01^{bc}	3.12±0.02^{b}	3.91±0.02^{c}	0.15±0.00^{h}	0.21±0.00^{d}	035±0.00^{f}	8.12±0.24^{a}	9.01±0.23$^{b c}$	14.20±0.25^{d}	0.00±0.00^{a}	1.10±0.24^{b}	2.10±0.24^{b}

MS M 4	2.97±0.04 bc	3.57±0.02 d	4.21±0.01 c	0.11±0.00 bc	0.13±0.00 c	0.19±0.00 c	7.82±0.28 a	8.11±0.27 e	9.21±0.21 b	0.00±0.00 a	2.10±0.22 c	3.30±0.22 bc
MS M 5	2.95±0.05 bc	0.00±0.00 a	0.00±0.00 a	0.13±0.00 bc d	0.00±0.00 a	0.00±0.00 a	8.440±0.24 ab	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a	0.00±0.00 a
MS M 6	2.97±0.01 c	3.45±0.02 c	0.00±0.00 a	0.11±0.00 de fg	0.12±0.00 c	0.00±0.00 a	8.21±0.24 a b	10.26±0.24 ef	0.00±0.00 a	0.00±0.00 a	1.10±0.20 b	0.00±0.00 a
MS M 7	3.04±0.02 e	3.72±0.01 de	4.01±0.03 c	0.22±0.00 j	0.41±0.00 h	1.02±0.02 h	9.14±0.24 d	12.16±0.51 g	21.14±0.51 h	0.00±0.00 a	1.10±0.24 b	2.12±0.20 b
MS M 8	3.01±0.01 c	4.12±0.05 g	6.42±0.23 e	0.12±0.00 bc de	0.23±0.00 f	0.34±0.00 g	8.01±0.24 d	13.31±0.20 f	21.30±0.51 g	0.00±0.00 a	2.23±0.21 cd	3.30±0.21 c

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LIST OF PUBLICATIONS

Research publications

1. **Sharma N**, Chauhan RS, Sood H (2016). Discerning Picroside-I biosynthesis via molecular dissection of in vitro shoot regeneration in *Picrorhiza kurroa. Plant Cell Reports. 35(8):1601-1615* (IF 2.86)

2. **Sharma N**, Kumar V, Chauhan RS, Sood H (2016). Modulation of Picroside-I biosynthesis in grown elicited shoots of *Picrorhiza kurroa* in vitro. *Journal of Plant Growth Regulation 35(4):965-973* (IF 2.07)

3. **Sharma N**, Chauhan RS, Sood H (2015). Seaweed extract as a novel elicitor and medium for mass propagation and Picroside-I production in an endangered medicinal herb *Picrorhiza kurroa. Plant Cell, Tissue and Organ Culture 122(1):57-65* (IF 2.00)

4. **Sharma N**, Shitiz K, Kalia D, Ahuja JK, Chauhan RS, Sood H (2014). Expression analysis of key genes for Picroside-I production in *in vitro* grown shoots of different genotypes and species of endangered herb *Picrorhiza. International Journal of Basic and Applied Biology 2(1):36-39*

5. Kumar V, **Sharma N**, Sood H, Chauhan RS (2016) Exogenous feeding of immediate precursors reveals synergistic effect on picroside-I biosynthesis in shoot cultures of *Picrorhiza kurroa* Royle ex Benth. *Scientific Reports 6:29750* (IF 4.25)

6. Shitiz K, **Sharma N**, Pal T, Sood H, Chauhan RS (2015). NGS transcriptomes and enzyme inhibitors unravel complexity of picrosides biosynthesis in *Picrorhiza kurroa* Royle ex. Benth. *PLoS One doi:10.1371/journal.pone.0144546* (IF 2.81)

7. Kumar V, **Sharma N**, Shitiz K, Singh TR, Tandon C, Sood H, Chauhan RS (2015). An insight into conflux of metabolic traffic leading to picroside I biosynthesis by tracking molecular time course changes in a medicinal herb, *Picrorhiza kurroa*. *Plant Cell Tissue and Organ Culture 123(2):435-441* (IF 2.00)

International conference publication

1. **Sharma N**, Chauhan RS, Sood H. Seaweed extract an effective alternative of growth hormones for mass propagation and enhanced secondary metabolite production in *Picrorhiza* spp. *Proceedings of International Conference on Emerging Trends in Biotechnology*, Jawaharlal Nehru University, New Delhi, India, November 6-9, 2014