Transcriptome Analysis Reveals Upregulated Secondary Metabolite Pathways in Micropropagated Lawsonia Inermis L.

Dipu Samanta
Dr Kanailal Bhattacharyya College
Debapriya Das
Lady Brabourne College
Sayak Ganguli
St Xavier's College
Debleena Roy (debleenaroy@rediffmail.com)
Lady Brabourne College https://orcid.org/0000-0002-3271-2702

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Abstract

Humans are dependent on plants for medicines, food and raw materials. Among the many plants used, Henna plant (Lawsonia inermis) have been found to be useful, medicinally and commercially. The pigment lawsone (2-hydroxy-1,4-nathaquinone) derived from the plant is the source of Mehendi, used for dying. The plant has a vast repertoire of secondary metabolites, giving the plant medicinal properties like anticarcinogenic, anti-diabetic, anti-inflammatory, immunostimulatory and many more. This creates a huge demand pressure for the plant, which can't be met by traditional farming. There have been reports of low propagation rate from stem cuttings and seed. So, micropropagation remains the only tool, for the large scale vegetative propagation, without exposing the plant to stress, saving the plant from overexploitation. The plant was established in vitro, with hormonal manipulation. The study shows BAP 4 mg/L and combination hormone of BAP 2 mg/L + Kn 2 mg/L to have best results for shoot bud multiplication. Rooting was seen to be significant in MS medium, highlighting the low cost of maintenance of the plant. Karyotype analysis was performed between in vivo and in vitro plant sets, followed by transcriptome analysis. Karyotype analysis, performed to validate the homogeneity between in vivo and in vitro plants, showed a chromosome number of 30. Transcriptome analysis through the TRAPID platform showed significant difference between in vivo and in vitro plant's transcript sets. Pathway analysis revealed enriched secondary metabolite pathways of commercial and economic importance in the in vitro generated plantlets as compared to the wild type control.

Key Message

The main objective is in vitro plant regeneration. Comparative transcriptome analysis between in vivo and in vitro plant sets, highlights, how micropropagation enhances secondary metabolic pathways, further manipulated by industry.

Introduction

Plants have been intricately associated with human beings, being the source of food, medicines and raw materials. India with its natural resources can be the ethnomedicinal hub of the world (Ahmad and Beg 2001). According to the records of World Health Organization, 2003, it was revealed that, nearly 80% of the population of developing countries, cannot produce their required medication and primarily depend on plant-based medicines (Chaudhary, et al. 2010). Among the many medicinal plants used, *Lawsonia inermis* L., or Henna, has been reported to be useful both medicinally and commercially (Ponugoti, 2018). *Lawsonia inermis* L. leaves are the source of the dye henna, used to dye skin and fabrics. The main component of the dye is, lawsone (2-hydroxy-1, 4-napthoquinone), derived mainly from the leaves of the plant. Large scale production of Henna plant in India is confined to the states of Punjab, Gujarat, Rajasthan and Madhya Pradesh (Phirke and Saha 2013). It is a perennial tall shrub, with white fragrant flowers (Fig. 1f); fruits are small with brownish capsules, carrying usually 30-50 hard, triangular yellowish brown seeds. The loss of cultivable land due to growing urbanization, has put immense pressure on farmers. The global rise in temperature, with the uncertainty of rainfall have forced farmers to shift their

focus on specific strategic crops for commercial gains, leading to a loss of cultivation of native crops, which could survive in harsh environmental conditions. Another problem is low availability of water (due to global warming) for the farmers to grow newer crops. These grave circumstances eventually results in farmer's suicide, an unavoidable consequence of climate change (Poornaprajna Belur, 2014). Sarhadi, et al. (2016), reported that Henna plants could tolerate a water scarcity of about 50%, with negligible effects on morphological characteristics of the plant, making this plant fit to be grown in elevated temperatures, with low water requirements. These problems can be easily solved with tissue culture techniques where hormonal manipulations can lead to large scale propagation of the plant. Tissue culture also provides various avenues wherein the plant's metabolic pathways can be manipulated, resulting in *in vitro* plants with increased secondary metabolic content. Previous studies have shown similar results in *Mentha*, which when grown in MS media, supplemented with indole-butyric acid (Lyczko et al. 2020) showed higher accumulation of odor active and volatile compounds.

Apart from commercial usage, medicinally the plant has several important properties, like anti-carcinogenic, anti-diabetic, antimicrobial, and many more. Leaf chloroform extracts have been reported to slow down the tumor growth of the breast cancer and colon cancer cell lines (Endrini et al. 2007). A significant increase in free radical scavenging activity was seen in serum and tissue homogenates of rats administered with Henna extracts (Al-Damegh 2014). This antioxidant activity can be attributed to the high phenol content of the leaves of Henna. Mice treated with *Lawsonia* extracts showed low levels of glucose, cholesterol, triacylglycerol and lipoprotein cholesterol in their blood (Ojewunmi, et al. 2014). Henna leaves, extracted in methanol, showed effective results on ulcer in rats, up to above 50% (Basipogu and Syed 2015). After phytochemical screening, a large number of chemical compounds like alkaloids, glycosides, naphthaquninones, terpenoids, tannins, phenolic compounds and carbohydrates were identified, which helped to cure ulcers (Sravanthi et al. 2011). The methanolic leaf extracts showed significant CNS depressant and anti-inflammatory activity in a dose dependent manner (Nesa et al. 2014). *Lawsonia inermis* L. petroleum ether seed extracts have shown effects against fourth instar larvae of *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* (Muruges and Daniel 2007). Methanolic extracts of *Lawsonia inermis* L. rich in flavonoids and bicyclic sesquiterpenes, have successfully shown strong toxicity against *Anopheles stephensi* larvae (Khanavi et al. 2013).

Modern high throughput techniques using next generation sequencing allows us to explore and analyse the variability in the functional profiles of plants under different conditions. De novo transcriptome analysis allows for the elucidation of the pathway enrichment and gene expression categorization in a fast and accurate manner (Kundu et al 2019) which can then be validated using chemical identification and quantification studies. In *Solanum* (Pandey et al. 2018), a similar study revealed that, flavonoids, terpenoids, glucosinolates, production increased and pathways leading to their biosynthesis were upregulated under *in vitro* conditions when compared to *in vivo* sets. It was seen that grapevine plants, when subjected to stress, had an increased accumulation of important secondary metabolites, which could be easily detected by transcriptome analysis (Haider et al, 2017). In this work we devised a strategy where pooled *de novo* transcriptome sequencing and analysis was performed to compare the pathway enrichments from *in vivo* and *in vitro* Henna plants. We hypothesized that micropropagation practices

and different media compositions fortified with myriad growth regulator combinations could result in the enrichment of secondary metabolite biosynthesis pathways, which would increase the chances of production of the essential economically important small molecules.

Materials

The seeds of *Lawsonia inermis* L. were collected from the nursery. 50-100 seeds of *Lawsonia inermis* L. were imbibed in water overnight, and placed in soil, devoid of any fertilizers. After 7 days, germination started and within 14 days, all the seeds germinated wholly. After this, the plants were maintained in the medicinal garden of Lady Brabourne College. The herbarium was submitted to CUH (Calcutta University Herbarium) for authentication, and received an accession number of ACC No: 20090.

Methods

Shoot bud multiplication

Apical and axillary buds were transferred to MS basal media. Containing different concentrations of cytokinin, kinetin (Kn), 6- benzylaminopurine (BAP) and 2-isopentenyladenine (2ip) (Table1, 2).

Root induction

The regenerated shoots of 3 month old plants were transferred to different rooting media (Table3).

Acclimatization

In vitro plants with well-developed root and shoot system were transferred to pre acclimatization chamber (PAC) where plantlets were kept in conical flask and were filled with soilrite and soil in the ratio 1:1. The mouth of the flask was sealed with parafilm and three holes of equal sizes (to permit free passage of air). This chamber was sprayed with water and 1/4th MS organic solution after every alternate day. The chamber was kept in a BOD at 26±1ºC under 16 hour photoperiod. After 4 weeks of acclimatization, the plants were moved to the fields.

Karyotype analysis

Root tips were taken for cytological analysis following aceto-orcein squash technique, a modification of technique proposed by Sharma and Sharma, 1987. The root tips were pre-treated at around 7.30 A.M. IST in saturated solution of PDB (for 3 hours at 18˚C to 20˚C). The root tips were then washed thoroughly in distilled water and fixed in 1:3 acetic acid: ethanol for overnight at 18˚C to 20˚C. Root tips were then transferred to 45% acetic acid till they settled at the base of the vial, and then stained in 2% aceto-orcein: 1N HCL 9:1 solution, and kept in room temperature for 1 hour. Finally, the root tips were squashed in 45% acetic acid and were observed under microscope.

Karyotype analysis was carried out by critically examining about 25 clear and well-scattered metaphase plates from different root tips of each plant. The plates showed a count of 30 chromosomes (2n = 30). The chromosomes were classified into different types based on their *I* values and drawn with the help of a drawing prism (Levan et al. 1964). The *I* values were calculated by using the following formula: *i*= Short arm length/Total chromosome length× 100

The chromosomes were arranged into different groups on the basis of their *i*-values.

Library Construction and Illumina sequencing

Total RNA was extracted from 1g, one month to three month old seedlings of *in vivo* and *in vitro* *Lawsonia inermis*, using IlluminaHiseq platform. Qualitative and quantitative estimation of the extracted RNA were done by Bioanalyzer (Agilent). An equimolar amount of RNA samples with an RNA integrity number (RIN) of 7.1 were pooled together for further analysis. Sequencing was carried out using the Illumina platform (https://www.illumina.com/systems/hiseq_systems/hiseq_2000_1000/kits.html). (Ganguli et al. 2016)

RNA-Seq data processing and quality control

Quality control of raw data was performed using FastQC programme (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to generate high quality reads. Raw reads were filtered and adapters and primer sequences, were trimmed, if any (Ganguli et al. 2016).

De novo transcriptome assembly and clustering

Quality controlled IlluminaHiSeq reads of *in vivo* and *in vitro* plant sets were de novo assembled using the Trinity tool to generate contigs. The assembled contigs were processed through Oases 0.2.08 (https://www.ebi.ac.uk/zerbino/oases) pipeline to generate transcripts. Removal of redundant sequences was done with CD-HIT-EST run. Subsequent clustering of non-redundant (nr) sequences was done using CD-HIT v 4.5.4 (http://www.bioinformatics.org/cd-hit/) with a sequence identity threshold of 80%. (Ganguli et al. 2016).

Functional annotation, GO characterization and KEGG analysis

Due to lack of whole genome sequence information in *Lawsonia inermis,* functional annotations of the assembled transcripts were carried out using the EGGNOG database. Functional categorization of the transcripts was performed using TRAPID webserver to segregate the transcripts into respective gene ontologies. Pathway mapping of the annotated transcripts was performed using the KAAS server against the KEGG database (Kyoto Encyclopedia of Genes and Genomes) (http://www.genome.jp/kegg) to identify the enrichment of respective pathways.

Results

In vitro seed germination:

Sterilization of seeds with 0.1% (w/v) $HgCl_2$ for 13 minutes resulted in completely sterilized seeds. Time should be very carefully maintained as more than 13 minutes might lead to lower germination of the seeds (Fig. 1a).

Shoot bud multiplication:

For every hormone set, a total of 20 replicates were prepared. Bud breaks were observed after 7-9 days of inoculation in hormonal media. Data were given up to 2 months, in the time interval of which, rapid shoot bud induction was observed. It was seen that media number A7 (BAP- 4mg/L) responded best to shoot bud induction (Table 1). Among the hormones used, BAP showed better result in shoot bud multiplication, than Kn or 2ip (Table 1). From among the combination media used, D_2 (BAP 2 mg/L + Kn 2 mg/L) showed the best response to shoot induction (Table 2).

The seedlings were grown in MS basal media for about 2-3 months. The axillary buds were transferred into media containing hormones BAP, Kn and 2ip for recording the shoot bud multiplication efficiency. At the end of 2 months, BAP4 media showed the best response with respect to shoot bud multiplication (6 shoot buds), followed by Kinetin. However, the response of 2ip was comparatively poor than BAP by 53.33 % and Kn by 48.14 %. These findings were similar to those reported earlier (Singh et al. 2016), where BAP was the best hormone to induce shooting, showing a response of 95-100%, in *Lawsonia inermis* L (Fig. 1b). Hence, it can be concluded that BAP, alone or in combination with other hormones (Moharanna et al. 2017, Ram and Shekhawat 2011) can efficiently help in shoot regeneration from explants. Also, the length of shoots, grown in BAP, was taller in comparison to other hormones.

Root induction:

An interesting observation was seen when the plants were induced for rooting. *Lawsonia inermis* L. showed highest root number in MS basal media (nearly 31 roots). A comparative rooting was recorded for 3 concentrations of MS media, ½ MS and ¼ MS. This further highlights a positive aspect of the plant, where no hormone is needed to induce rooting, further lowering the cost of regeneration of the plant (Table 3, Fig. 1c).

Acclimatization

The success of acclimatization process was 80%- 90% (Fig. 1d, 1e).

Karyotype analysis

Cytologyand karyotype analysis revealed that the *in vivo* and *in vitro* plants had the same chromosome number (2n= 30 chromosomes), hence proving that the *in vitro* plants are a homogenous clone and no variability is seen after repeated sub-culturing. There are 30 chromosomes in *Lawsonia inermis* L (Fig. 2).

Transcriptome profiling and assembly of the reads

TRAPID is an efficient platform, for de novo transcriptome analysis, having the ability to detect frame shift mutations, phylogenetic and taxonomic position of the sample in question. TRAPID analysis of the transcript sets with EGGNOG as reference database, showed a basic difference in transcript number, and ORF length. A stark contrast was seen in the total number of transcripts for the *in vitro* plant set, showing nearly 2 times more transcripts than *in vivo* plant set (fig 1). Similarity searches revealed that *in vivo* plant set have 39.2%, 33.9% and 12.3% similarity where as *in vitro* plants sets 32.3%, 28.6% and 9.7% similarity to *Populus trichocarpa*, *Vitis vinifera* and *Glycine max* respectively. Both the transcript sets show large transcripts allotted for maintenance of cell and cellular structures.

Gene Ontology Classifications of *in vivo* and *in vitro* transcripts of *Lawsonia inermis*: *In vivo* plant's transcript sets show cell (GO: 0005623; 46.9%), cell part (GO: 0044464; 46.9%) and intracellular (GO: 0005622; 42.6%) as the dominant cellular component localization of the transcripts. Most abundant biological process includes cellular process (GO: 0009987; 27.7%), single organism process (GO: 0044699; 25.1%) and metabolic process (GO: 0008152; 24.8%). Molecular function includes response to stimulus (GO: 0050896; 17%), catalytic activity (GO: 0003824; 15.6%) and biological regulation (GO: 0065007; 11.7%). For *in vitro* plant's transcript sets, the transcripts assigned to each category was seen to be 1.4 times more as compared to the *in vivo* plant set. Cellular components, namely, cell (GO: 0005623; 38.5%), cell part (GO: 0044464; 38.5%) and intracellular component (GO: 0005622; 35.2%) was most abundant. Biological processes included cellular process (GO: 0009987; 22.7%), single organism process (GO: 0044699; 20.9%) and metabolic process (GO: 0008152; 20.5%). Response to stimulus (GO: 0050896; 13.2%), catalytic activity (GO: 0003824; 12.8%) and biological regulation (GO: 0065007; 9.5%) comprised the most abundant molecular function (Table 5, Fig. 3,4).

Pathway enrichment analysis

KEGG analysis revealed 177 pathways potentially enriched for *in vivo* plant and 174 pathways for *in vitro* plants. KEGG analysis revealed a wide range of secondary metabolic pathways like terpenoid backbone synthesis, carotenoid biosynthesis, flavonoid, phenylpropanoid biosynthesis, isoquinoline and alkaloid biosynthesis, glucosinolate biosynthesis, with more genes allocated to the secondary metabolic pathways in *in vitro* plants. Our analysis revealed flavonoid and steroid hormone biosynthesis, to be abundant pathways, expressed in *in vitro* plants. Lawsone (naphthoquinone), the main pigment of the plant, contributing to Mehandi, is the main focus here. Several intermediates of shikimic acid pathway, mevalonic acid pathway, serve as precursors in forming the pigment lawsone (Widham and Rhodes 2016). *In vitro* plants show a more enriched expression of the concerned pathways [terpenoid backbone biosynthesis pathway (7.14%), ubiquinone and other terpenoidquinone biosynthesis pathway (5%), phenylpropanoid biosynthesis pathway (11%), phenylalanine metabolism pathway (16%), and sesquiterpenoid and triperpenoid synthesis pathway (16%)], as compared to *in vivo* plants of *Lawsonia inermis*.A wide variety of signaling pathways were predicted like MAPK (mitogen activated protein kinase) signaling pathway, plant hormone signaling pathway, AMPK (AMP-activated protein kinase) and

sphingolipid signaling pathways, which might help the plant to orchestrate complex biological events like flowering, growth, metabolism and responses to biotic and abiotic stresses (Zhang and Klessig, 2001). Presence of transcripts associated with drug metabolism and xenobiotic metabolism by cytochrome P450 throws light on the ability of plant to detoxify toxic compounds to less toxic forms, seen in *in vitro* plants(Table 6, Fig. 5).

Discussion

The seeds are reported to show non-deep physiological dormancy, which can be reduced by chilling, leaching, priming, GA3 and KNO3 pre-treatment. The germination was also observed to be higher at 35ºC than at lower temperatures, that is 20ºC to 30ºC (Parihar et al. 2016). Seed treatment with 5% NaCl resulted in higher rates of germination than scarification with sand or water soaking.

L. inermis have been high in demand due to medicinal and commercial uses (Ponugoti 2018). The present study describes an attempt to develop an *in vitro* protocol for mass multiplication of *L. inermis* by manipulating the nutrient salts, growth regulators and culture conditions. Rapid shoot multiplication from cotyledonary node regions of *Lawsonia inermis* L*.* (Moharanna et al. 2017) was seen (96.6%), on MS medium, supplemented with 8.88µM Benzyladenine and 2.68µM naphthalene acetic acid. The quality and size of shoots improved on medium with 4.44µM of Benzyladenine. Apart from the cotyledonary nodes, apical shoot buds and meristems were also be used for efficient shoot proliferation (Singh et al. 2012). Rout et al. (2001) used MS medium with 0.25 mg/l 6-benzylaminopurine, 0.25 mg/l Kinetin and 0.5 mg/l ascorbic acid for mass propagation of *Lawsonia inermis* L. It was concluded that axillary meristems served to be better explants in the propagation of *Lawsonia inermis* L*.* Nodes from mature plants could be also used on MS medium with 6-Benzylaminopurine (3.33 µM) (Singh et al. 2012). But with an increase in the amount of benzyladenine or Kinetin, the number of shoots gradually decreased (Singh, et al*,* 2016). An efficient system of regeneration was established using the nodal segments of *Lawsonia inermis* L*.* (Ram and Shekhawat 2011). Murashige and Skoog (MS) medium, with hormonal concentration of 0.25 mg/l 6-Benzylaminopurine, 0.25 mg/l Kinetin, 0.1 mg/l indole-3-acetic acid and 150 mg/l ammonium sulphate showed best response. About 95% of the shoots were rooted successfully in soilrite after treating the shoots with 300 mg/l indole-3-butyric acid and 100 mg/l 2-napthoxy acetic acid. In our analysis, the percentage response to shooting was better (100%), regenerating more healthy shoots with less hormonal combinations [(BAP-4 mg/L: 8 shoots); (BAP 2 mg/L + Kn 2 mg/L: 12 shoots)]. Efficient rooting was seen in MS basal medium, devoid of any hormones. These findings further validate the low cost of maintenance of the plant.

Gene ontology data of the two transcript sets show distinct variation in the number of transcripts. *In vitro* plant set of *Lawsonia inermis* show a higher accumulation of transcripts than *in vivo* plants. A similar increase in the transcript number was observed in infected *Vigna mungo* as compared to the control, non-infected sample (Ganguli et al. 2016). Results show that cellular processes, single cell organism process and metabolic processes are abundant in the plant, indicating the plant's role in operating the intricate

machinery. Response to stimulus can be seen as a dominant function, which might suggest that the plant is prompt in responding to abiotic as well biotic stresses (Fig. 4).

Biochemical assays have shown henna plant to be a rich source of important components, giving the plant the commercial and medicinal importance (Badhai et al. 2013) (Ojewunmi et al. 2014) (Endrini et al. 2007) (Song et al, 2017). These observations can further be validated by the pathway enrichment analysis performed in this study. Phenylpropanoid biosynthesis pathway, along with the enzyme PAL (Phenylalanine ammonialyase) were reported to be instrumental in combating drought stress, as revealed in drought tolerant cultivars of foxtail millet. (Yu et al. 2020) In our analysis we have identified the over representation of phenylpropanoid biosynthesis pathway in *in vitro* plants, nearly 11% more than *in vivo* plants. This enzyme PAL plays an important role in contributing to the phenolics and flavonoid content of the plant. The various important medicinal properties of Henna plants (radical scavenging, antimicrobial, anticancer, antiulcer, anti-diabetic), are due to the high phenolic and flavonoid content, the production of which depends largely on the expression of this particular enzyme. The accumulation of flavonoids can also be correlated to the high pigment content in Henna. Similar observations were reported in the ornamental plant of *Syringa oblate*, with a high accumulation of flavonoids (Zheng, et al. 2015). An enriched expression of flavonoid biosynthesis pathway can also be a result of stress adaptation, reported in wheat subjected to drought conditions (Dalal, et al. 2018). It was interesting to observe that transcript abundance of flavonoid pathway remained the same for *in vivo* and *in vitro* Henna plants, showing that, tissue culture conditions did not affect the flavonoid pathway in any way. Apart from defense and stress, flavonoid and anthocyanins also help in the protection of chlorophyll, as observed in light stress induced leaves of *Cornussto lonifera* (Field et al. 2001). Phenols and terpenoid pathways have also been reported to have been elevated during the successful micropropagation of *Nardostachys jatasamani*, an important endangered medicinal plant (Dhiman et al. 2020). Presence of pathways related to volatile terpenoids, monoterpenoid, limonene and pinene, were seen to be responsible for the scent in *Cinnamomum camphora* (Hao, *et al*, 2020). Our pathway analysis of henna showed an enriched abundance of terpenoid biosynthesis pathway. Comparative transcriptome analysis between *Ocimum basilicum* and *Ocimum sanctum*, reported phenylpropanoid and terpenoid pathways to be abundant (Rastogi et al. 2014). Drought affected white grapes similarly showed a heightened expression of these pathways (Savio et al. 2016). Terpenoids, were also reported to have protective action against diabetic complications, detected in *Zingiber officinale* (Saraswat et al. 2010). In our analysis, we have also observed an enriched expression of pathways related to synthesis of terpenoids, in *in vitro* plants of Henna, about 7.14%. Steroid hormone biosynthesis pathway, exclusively found in *in vitro* Henna plants, can be held responsible to help the plant cope with stress.

We have been able to identify the increased expression of HSP 70, HSP 40/ DnaJ in *Lawsonia* under *in vitro* condition which has been previously reported to be associated with stress responses (Kampinga and Craig 2010). The presence of “ethylene overproduction protein”, “abscisic acid receptor”, “protein ethylene insensitive”, “TIFY 6B-like proteins” and “Ninja-like protein” suggests that the plant is adept in dealing with abiotic stresses, reported by Thireult, *et al*, (2015) in Arabidopsis. The presence of “mate efflux protein” and “TRANSPARENT TESTA 12-like proteins” calls for the xenobiotic transporter activity of the plant, as

previously proposed by Omote et al. (2006), in plants, bacteria and even in mammals. TPR repeat containing thioredoxin also helps plant to adapt to changes in hydration levels and osmolarity. *In vitro* plants can be seen to express exclusive proteins and domains like “thaumatin domain”, “TIFY 10-like”, “SAM domain” and various proteins which help to nullify the effect of free radicals (Damme et al. 2002). The plants also show presence of cellulose synthase, callose synthase and cell wall biogenesis proteins, which indicate the capability of plant to synthesize cell wall elements, aiding in responses to wounding. Apart from a large number of serine threonine protein kinases, aiding in pathogen identification and defense responses, (Afzal et al. 2008) MAPK signaling proteins can be detected, aiding in immunity (Zhang and Klessig 2001). Several transcription factors were seen to be well represented, among *in vivo*and*in vitro* plants of *Lawsonia inermis*, like WRKY, WD40, LURP1 related, calcium calmodulin binding proteins, which has pronounced role in pathogen defense (Pandey and Somssich 2009). Chromatin remodeling proteins found in the plant, like topless related protein, Tubby like F-box protein, histone deacetylase, could affect transcription of the genes (Wang et al. 2015). Chloride, sulphate, magnesium, proton transporters and transition metal transporters, were responsible for maintaining the electrochemical balance in cells, without harming the plant (Hall and William, 2003). Transcript abundance of ABC transporters were also observed, which might help the plant sequester toxic compounds, byproducts of chlorophyll degradation, and other unwanted compounds into vacuoles, as previously reported by Walter et al. (2015), in wheat. Ubiquitin E3 ligases, ubiquitin conjugation factors, found to be more enriched in *in vitro* plants, aiding in hormonal signaling, plant’s response to stress, and also, in regulating the protein content within the plant cell (Sharma et al. 2016).

Gene families encoding various enzymes like, cytochrome P450, phenylalanine ammonium lyase, prephenatedehydratase, tropinonereductase, polyamine oxidase and serine carboxypeptidase, were observed, instrumental in increasing the secondary metabolic pool, aiding in defense responses. Gene families encoding enzymes related to the production of phenols were observed to be very well represented in *in vitro* plants of Henna. This huge array of gene families encoding specific enzymes, further validates the rich secondary metabolic pool for *in vitro* plants. (Table 6, Fig. 5)

Conclusion

Henna plant was successfully regenerated in tissue culture laboratory, using BAP 4 mg/l hormone which resulted in the best shoot bud induction and shoot length. However, a combination of both BAP and Kn, enhanced the response many folds. Efficient induction of root was obtained in MS media, with no additional plant growth regulator supplementation. Tissue culture grown plants, showed a higher accumulation of genes and transcripts, along with important enzymes, transporters and transcription factors which help the plant, cope up with stress elements. Pathway analysis via KEGG, revealed the huge repertoire of important secondary metabolites in the plant. *In vitro* plants of *Lawsonia inermis* show an over representation of terpenoid backbone biosynthesis pathway (7.14%), ubiquinone and other terpenoid quinone biosynthesis pathways (5%), phenylpropanoid biosynthesis pathway (11%), phenylalanine metabolism pathway (16%), and sesquiterpenoid and triterpenoid synthesis pathway (16%), as compared to *in vivo* plants of *Lawsonia inermis*. Richness of the shikimic acid pathway, mevalonic acid pathway

and its intermediates, in *in vitro* grown plants, as seen in this study, can be suggestive of high secondary metabolite content. Comparative transcriptome analysis between *in vivo* and *in vitro* plant sets, thus, throws light on how micropropagation has the potential of enhancing the secondary metabolite pathways, which can be manipulated according to the need of the industry.

Abbreviations

Kinetin (Kn), Murashige and Skoog (MS) 6- benzylaminopurine (BAP) and 2-isopentenyladenine (2ip)

Declarations

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Tables

Table 1 Effect of hormones on shoot bud multiplication:

Media	Growth Regulator (mg/l)	Number of Shoot Buds/ Explant (8 weeks)	Response (% of Bud Breaking 8 weeks)	Shoot Length 8 weeks (cm)
A1	BAP (1.0)	3.8±0.18	75	3.5±0.03
A2	BAP (1.5)	3.6±0.22	80	3.2±0.12
A3	BAP (2.0)	3.8±0.18	80	4.1±0.12
A4	BAP (2.5)	4±0.28	80	3.7±0.07
A5	BAP (3.0)	4±0.28	85	4.2±0.04
A6	BAP (3.5)	4.2±0.18	90	4.2±0.09
A7	**BAP (4.0)**	**7.8±0.22**	**100**	6±0.06
A8	BAP (4.5)	5.6±0.22	90	4.1±0.09
A9	BAP (5.0)	4±0.28	90	4.0±0.05
B1	Kn (1.0)	2.9±0.18	75	3.2±0.21
B2	Kn (1.5)	2.6±0.20	75	3.2±0.10
B3	Kn (2.0)	3.8±0.18	75	3.5±0.08
B4	Kn (2.5)	2.6±0.22	75	3.3±0.12
B5	Kn (3.0)	3.2±0.30	75	3.9±0.18
B6	Kn (3.5)	3.6±0.22	80	2.9±0.09
B7	**Kn (4.0)**	**4.4±0.22**	**100**	5.4±0.21
B8	Kn (4.5)	4.4±0.22	90	4.5±0.17
B9	Kn (5.0)	3.2±0.18	90	3.2±0.08
C1	2ip (1.0)	1.8±0.18	60	2.1±0.04
C2	2ip (1.5)	1.8±0.18	75	2.3±0.05
C3	2ip (2.0)	2.8±0.18	70	2.4±0.10
C4	2ip(2.5)	2.6±0.22	75	2.4±0.04
C5	2ip(3.0)	2.6±0.22	75	2.5±0.10
C6	2ip(3.5)	2.8±0.18	75	2.5±0.03
C7	**2ip(4.0)**	**3.8±0.18**	**75**	2.7±0.10
C8	2ip (4.5)	3.8±0.28	70	3.2±0.02
C9	2ip (5.0)	2.8±0.18	65	3.1±0.05

Table 2 Effect of BAP+ Kn on shoot bud multiplication

Media	Growth Regulator (mg/l)	Number of Shoot Buds/ Explant (8 weeks)	Response (% Bud Break after 8 weeks)	Length of shoot (8 weeks) (cm)
D1	BAP (1) + Kn (3)	8.7±0.18	95	5.7±0.08
D2	**BAP (2) + Kn (2)**	**11.7±0.26**	**100**	**8.1±0.10**
D3	BAP (3) + Kn (1)	9.6±0.22	95	6±0.03

Table 3 Root induction in *Lawsonia inermis*L.

Media	Growth Regulator (mg/l)	No.of roots/ Explant (2 weeks)	Root length (2 weeks) (cm)	No.of roots/ Explant (4 weeks)	Root length (4 weeks) (cm)
R_0	**MS Basal media**	**3.2±1.36**	**0.38±0.17**	**24.4±3.79**	**3.74±0.19**
R_1	½ MS media	3±1.47	0.16±0.06	5.2±1.43	1.24±0.29
R_2	¼ MS media	1.2±0.72	0.11±0.07	3.6±1.51	0.34±0.17

Table 4 Karyotype analysis in *Lawsonia inermis* L.**

Chromosome no.	Chromosome type	Short arm length (μm)	Total arm length (μm)	i-value	Secondary constriction	Constriction type
1.	A	0.635, 1.905	4.445	14.28, 42.85	Present	Sm, m
2.	A	0.635, 1.905	4.445	14.28, 42.85	Present	Sm, m
3.	B	1.905	4.445	42.85	Absence	m
4.	B	1.905	4.445	42.85	Absence	m
5.	C	1.27	3.81	33.33	Absence	Sm
6.	C	1.27	3.81	33.33	Absence	Sm
7.	C	1.27	3.81	33.33	Absence	Sm
8.	C	1.27	3.81	33.33	Absence	Sm
9.	C	1.27	3.81	33.33	Absence	Sm
10.	C	1.27	3.81	33.33	Absence	Sm
11.	C	1.27	3.81	33.33	Absence	Sm
12.	C	1.27	3.81	33.33	Absence	Sm
13.	C	1.27	3.81	33.33	Absence	Sm
14.	C	1.27	3.81	33.33	Absence	Sm
15.	C	1.27	3.81	33.33	Absence	Sm
16.	C	1.27	3.81	33.33	Absence	Sm
17.	C	1.27	3.81	33.33	Absence	Sm
18.	C	1.27	3.81	33.33	Absence	Sm
19.	C	1.27	3.81	33.33	Absence	Sm
20.	C	1.27	3.81	33.33	Absence	Sm
21.	D	1.27	2.54	50.00	Absence	M
22.	D	1.27	2.54	50.00	Absence	M
23.	D	1.27	2.54	50.00	Absence	M
24.	D	1.27	2.54	50.00	Absence	M
25.	D	1.27	2.54	50.00	Absence	M
26.	D	1.27	2.54	50.00	Absence	M

27.	D	1.27	2.54	50.00	Absence	M
28.	D	1.27	2.54	50.00	Absence	M
29.	D	1.27	2.54	50.00	Absence	M
30.	D	1.27	2.54	50.00	Absence	M

(**No changes found in karyotype of *in vivo* and *in vitro Lawsonia inermis* L.)

Table 5 Transcriptome profiling and assembly of the reads.

Parameters	*In vivo* plant	*In vitro* plant
Total number of raw reads	175534	210765
Number of reads used	171100	195356
Transcripts generated	30866	53426
Maximum transcript length (bp)	5496	5496
Minimum transcript length (bp)	300	300
Average transcript length (bp)	1217.8	803.4
Transcripts ≥500 bp	24184	28150
Transcripts ≥1kbp	14812	12772
N50 size (bp)	400bp	400bp
GC content (%)	48%	48%

Table 6 Top 10 secondary metabolic pathways of *in vivo* and *in vitro* plants along with their pathway ID and gene numbers.

Sl. No.	KEGG Pathways	Pathway ID	Number of genes (*in vivo*)	Number of genes (*in vitro*)
1.	Terpenoid backbone biosynthesis	00900	28	30
2.	Phenylalanine, tyrosine and tryptophan biosynthesis	00400	22	22
3.	Ubiquinone and other terpenoid-quinone biosynthesis	00130	20	21
4.	Carotenoid biosynthesis	00940	18	20
5.	Phenylpropanoid biosynthesis	00906	18	18
6.	Phenylalanine metabolism	00360	12	14
7.	Flavonoid biosynthesis	00941	10	10
8.	Isoquinoline alkaloid biosynthesis	00950	8	8
9.	Tropane, piperidine and pyridine alkaloid biosynthesis	00960	8	8
10.	Monoterpenoid biosynthesis	00902	6	5

Figures

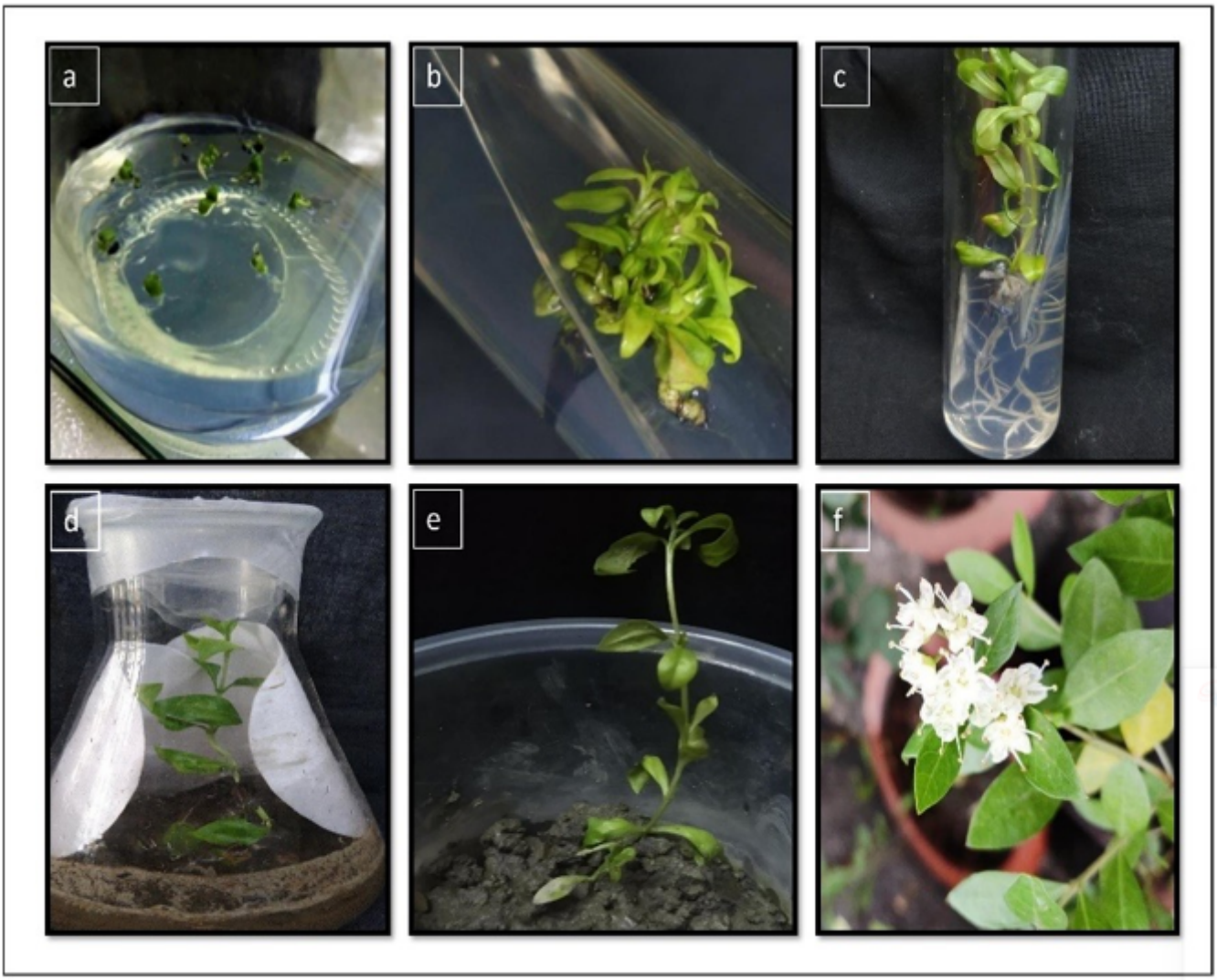

Figure 1

Stages of in vitro regeneration of Lawsonia inermis L. a: in vitro seed germination, b: shoot bud multiplication, c: growth of root, d: acclimatization, e: field transfer, f:In vivo plant of Lawsonia inermis L.

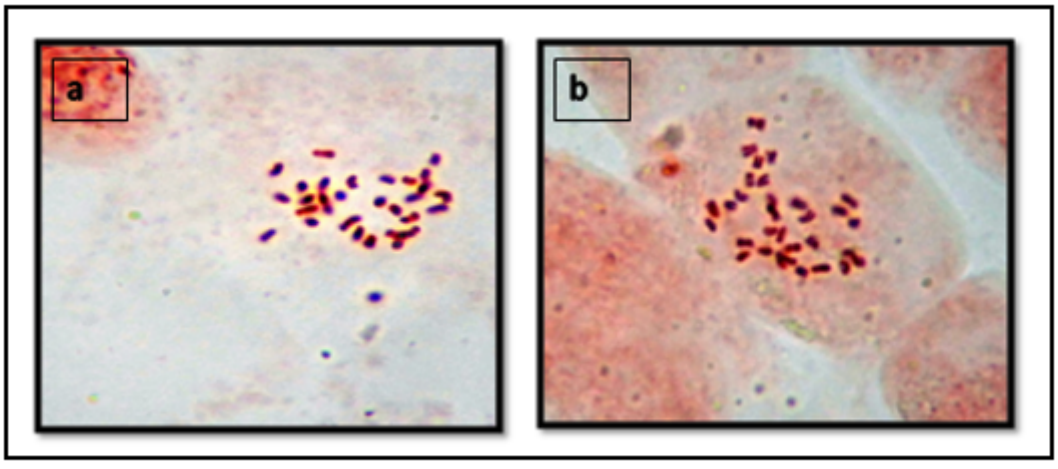

Figure 2

Somatic chromosome analysis in Lawsonia inermis L. a: metaphase plate of in vivo root, b: metaphase plate of in vitro root.

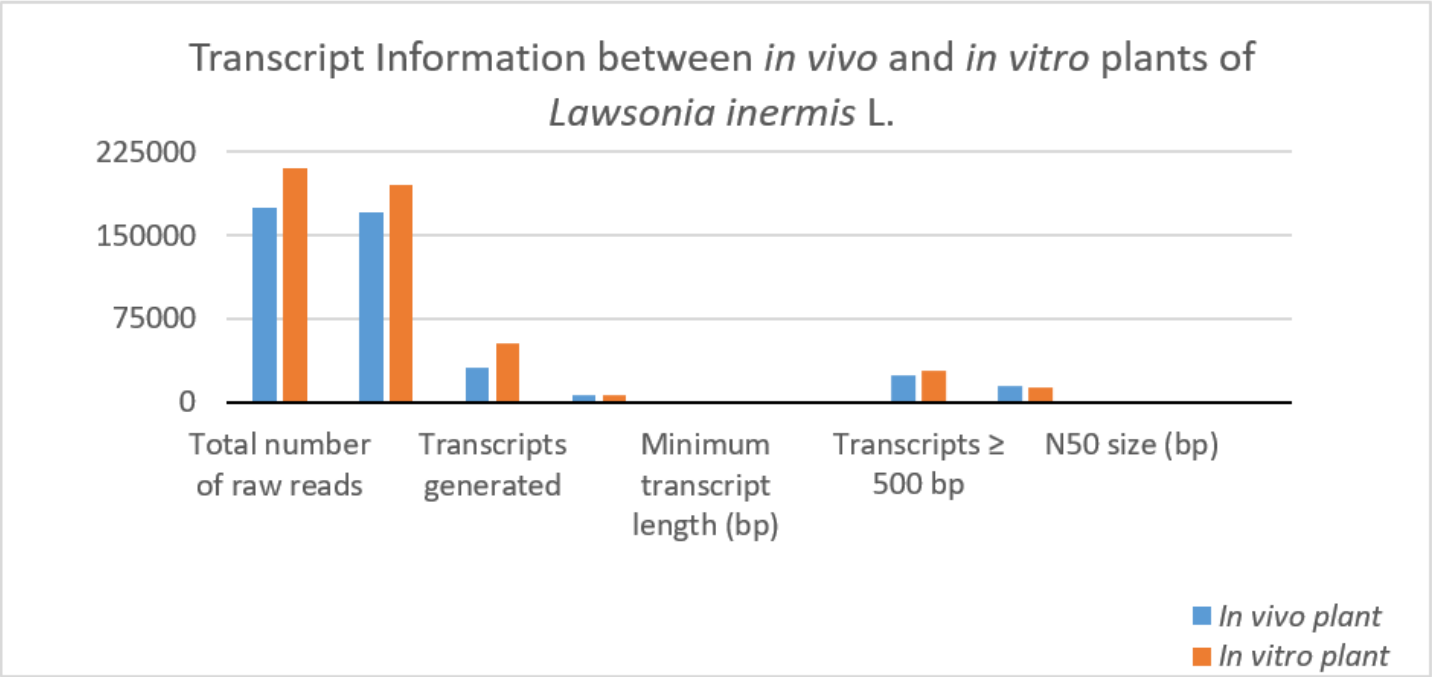

Figure 3

Transcript information between in vivo and in vitro plants of Lawsonia inermis L.

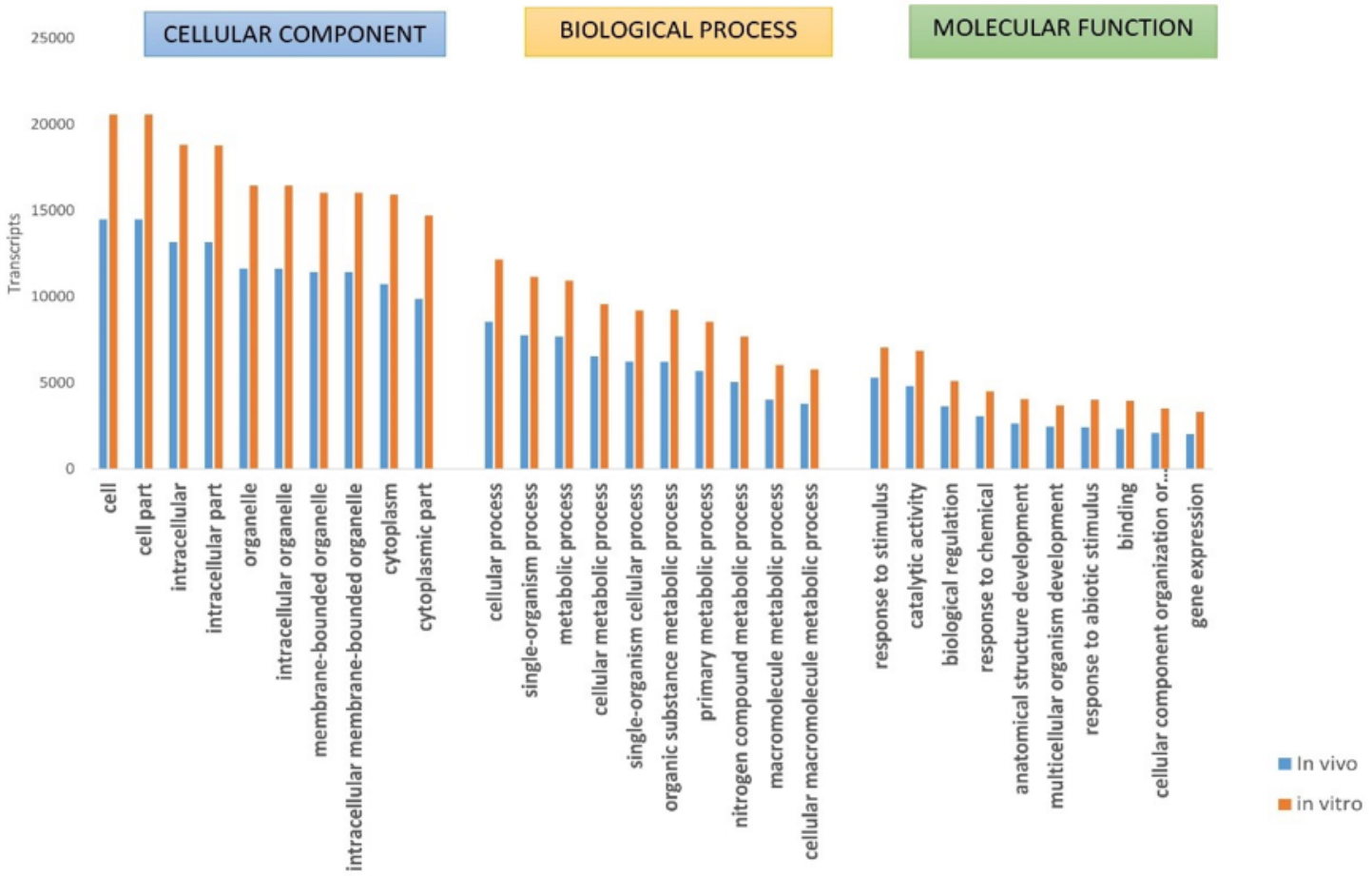

Figure 4

Gene ontology classification of in vivo and in vitro transcripts of Lawsonia inermis L.

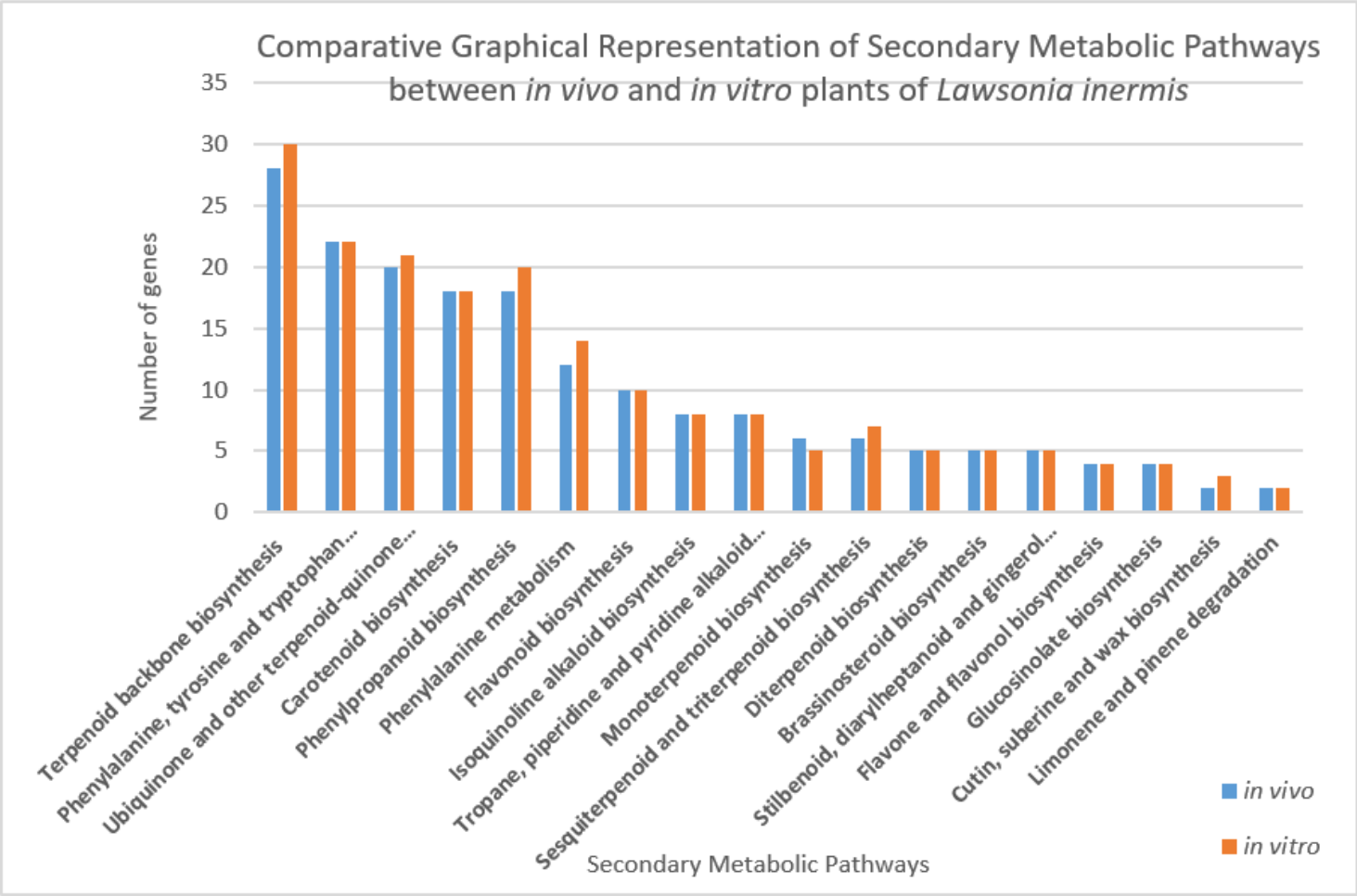

Figure 5

Comparative Graphical Representation of Secondary Metabolic Pathways between in vivo and in vitro plants of Lawsonia inermis L.