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RESEARCH ARTICLE

Callusing and untargeted metabolic profiling in Red sanders var. MTP 1

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Abstract

Red Sanders (*Pterocarpus santalinus* L.) is an economically important forest tree crop that is endangered and endemic to the Indian Sub-Continent. Red Sanders (*Pterocarpus santalinus* L.) is one of the most sought-after timber trees (listed in the IUCN red list) in the international market for its brick / blood red heartwood and is vulnerable to illegal felling and trading. To conserve Red Sanders and prevent its extinction, preserving its germplasm is crucial. Developing a micropropagation protocol is also essential to mass-produce elite genotypes for the timber industry. This study aims to establish a micropropagation method through callus induction using shoot tip explants. Further, the study identified that in tissue culture studies using shoot tip explants, we identified that 5 % $CaOCl_2$ is the best for controlling both epiphytic and endophytic microbes. For callus induction, MS medium modified with different concentrations of TDZ *viz*., 1, 3 and 5 ppm was used. Explants were surface sterilized with 5 % $CaOCl_2$ and inoculated in the MS + TDZ media under complete dark conditions. The calli could be potentially used for indirect organogenesis, somatic embryogenesis as well as suspension cultures. Hence, it is concluded that 1 % TDZ was an effective callus inducer. Comparative GC-MS analyses of *in vivo* grown shoot tips and *in vitro* grown calli identified 290 metabolites in all the samples tested. Detailed metabolomic analyses will shed light on the molecular biology and biochemistry of callus induction pathways in future.

Keywords

callus induction; GC-MS; *Pterocarpus santalinus* L.; Red Sanders; tissue culture

Introduction

Red Sanders (*Pterocarpus santalinus* L.) belonging to the family Fabaceae (1) is an important Forest Tree crop, a source of high-quality timber as well as group of organic compounds namely 'Santalin A / B / Y' and 'Santarubin' (2). Red Sanders is also called Red Sandalwood / Red Chandan / Rakta Chandan / Red Santer Wood / Santali Lignum Rubrum / Rubywood / Sandalwood Padauk / Sappan / Bois de santal Rouge etc. The trees are cross pollinated, but also exhibit lesser amount of self-pollination. Fruit set is high during cross-pollination especially involving

geitonogamy (3, 4). The heartwood of Red Sanders is used for making the musical instruments namely Shamishen and Zither in the South East Asian countries, making furniture, hand carvings etc (5). Also, the trees have the property of alleviating the negative effects of nuclear explosions. Hence, they are widely grown in areas where nuclear work-stations are located (6). The heartwood of Red Sanders with wavy grains fetches premium price in the international timber trade. Because of its high cost, it has attracted the attention of smugglers that lead to illegal felling for timber export. A reduction in the population led to the inclusion of Red Sanders in the IUCN red list of endangered species (7-9).

Tissue culture encompasses techniques that are used for both fundamental as well as applied research. Tissue culture aids in micro-propagation (direct as well as indirect organogenesis) as well as conservation of important plant species, development of suspension cultures for synthesizing metabolites of pharmaceutical significance as well as development of embryoids / somatic embryos for mass multiplication (10). Tissue culture is also used in fundamental research *viz.,* studying the effect of different chemical substances on cellular growth and development, functional genomics studies that involve genetic engineering / genome editing etc. Tissue culture has been successfully employed in commercial (micro) propagation of different crops (*viz.*, fruits: banana (11), pineapple (12), strawberry (13), citrus (14); vegetables: potato (15); ornamentals: orchids (16), gerberas (17), etc., spices: vanilla (18), black pepper (19); forest crops: bamboo (20) etc.). Tissue culture aids in mass multiplication of elite genotypes in all the above said crops and is not limited to the above listed ones.

Since Red Sanders is highly cross pollinated (21), seed-based multiplication will yield progenies that are genetically dissimilar. The only way to obtain true to type plants / genetically identical plants is through tissue culture. Further, the inclusion of Red Sanders in the IUCN red list necessitates conservation of red sanders genotypes (22). In this context, establishment of micro-propagation protocols for mass multiplying Red Sanders genotypes is the need of the hour, both to conserve as well as meet the demands in the international market. So, the work on the development of tissue culture protocols for micro-propagation of Red Sanders var MTP 1 was carried out. Callus can be exploited for development of plants through indirect organogenesis. Besides indirect organogenesis, callus could be used potentially for the development of somatic embryos (for micro-propagation) as well as for the establishment of suspension cultures (for targeting metabolites *viz*., Santalin, Santarubin etc.). Hence experiments were directed towards callus induction as well as metabolomics (for establishment of suspension cultures).

Materials and Methods

Shoot-tip culture method of micro-propagation was attempted in the Red Sanders var. MTP 1. The experiments were carried at both, the Forest College and Research Institute, Mettupalayam and Agricultural College and Research Institute, Coimbatore.

Callus induction

Culture medium: Shoot tip culture was carried out using basal as well as modified Murashige and Skoog medium (pH 5.6 - 5.8). For initial establishment / primary inoculation of the shoot tips, basal MS medium was used initially (first 2-3 rounds of culture initiation), which was then supplemented with a fungicide, Bavistin (100 mg/L). Then, during sub-culturing, for multiple shoot induction, modified MS medium (1, 3 and 5 ppm of thidiazuron) was used. Upon preparation each time, the media bottles were stored in a media storage room for a continuous period of 3-10 days to check for bacterial and fungal growth, before inoculation. The bottles without contamination were used for culture initiation / sub-culturing.

Explant collection: The Forest College and Research Institute, Mettupalayam has a mother bed nursery with a collection of many forestry species, among which Red Sanders var. MTP 1 is one among the tree species maintained (Fig. 1). In the mother bed nursery, about 60 plants are maintained with a spacing of 15 -30 cms between plants in a row and about 1 foot distance between rows. Shoot tips of length 1.5-2.0 cm (with one node) were collected once in a fortnight from the new flushes (Fig. 2A). Upon excision, the shoot tips were placed in a beaker with tap water to prevent dehydration until further steps were carried out.

Fig. 1. Mother bed nursery of Red sanders var. MTP1, FC&RI.

Explant pre-preparation: Upon harvest, the explants were subjected to a series of treatments before being taken to the laminar air flow chamber. In the tissue culture laboratory, collected explants were thoroughly rinsed with tap water to remove adhering particles. Then, the explants were treated with a detergent solution (4-5 drops of Tween 20 per litre of sterile distilled water) for 20 min. After which, they were rinsed thrice in sterile distilled water for 30 - 60 sec per wash. Following this, the explants were soaked in a fungicidal solution containing Bavistin (5 %) for 45 min. with intermittent shaking. Then, the explants were rinsed thrice in sterile distilled water for 30 - 60 sec per wash. Following fungicidal treatment, the explants were soaked in an antibiotic solution (Ampicillin sodium salt @ 250 mg/L) for 45 min. with intermittent shaking. Again, the explants were rinsed thrice in sterile distilled water for 30 - 60 sec per wash. Finally, the explants were subjected to soaking in an anti-oxidant solution (100 mg/L ascorbic acid and 100 mg/L citric acid). This was followed by a final rinsing (three times) in sterile distilled water for 30-60 sec per wash. After this step, the explants were moved to the laminar air flow chamber for further treatments and primary inoculation.

Explant preparation and inoculation: Before primary inoculation, the explants were sterilized using sterilant viz., ethanol and Calcium hypochlorite ($CaOCl_2$). The details of explant treatment are as follows: The explants were initially sterilized with 70 % ethanol for 30 - 45 sec which was followed by rinsing in sterile distilled water (thrice) for 30 - 60 sec per wash. Then, the explants were subjected to treatment with different concentrations of $CaOCl_2$ *viz.*, 1, 3, 5 and 7 % solutions for 20 min. Finally, the explants were washed thrice in sterile distilled water (30 - 60 sec per wash). This was followed by primary inoculation of shoot-tip explants. All the experiments were carried out in three replications with 12 explants per replication and a total of 36 explants per treatment.

Culture conditions: The cultures in basal MS media were placed in a growth chamber maintained at 34.5 °C in dark conditions. Sub-culturing of primary inoculants (for multiple shoot induction) in MS media supplemented with different concentrations of Thidiazuron (1, 3 and 5 ppm) was carried out after a period of 15 - 20 days. The bottles were then moved to the culture room having temperature of 27 °C and placed in complete dark conditions.

Statistical analyses

All statistical analyses reported here were performed using the software JMP Statistics Pro 17, unless otherwise stated.

Standardization of $CaOCl_2$ concentration on reducing bacterial and fungal epiphytes

For the identification of a desired $CaOCl_2$ concentration for surface sterilization of Red Sanders shoot tip explants, Randomized Block Design (RBD) was adopted and the following observations were recorded: *per cent* contamination and survival rate of explants. We define, '*per cent* contamination' as the total number of explants affected by microbial contamination (both bacteria and fungi) and 'survival of explants' as the number of explants that shows positive growth effect. For observing the contamination due to bacteria and fungi, data were recorded 3 DAI and 10 DAI, respectively. The data were subjected to one way ANOVA and Tukey's Honestly Significant Difference (HSD) Test.

Callus induction potential of Thidiazuron

Shoot tip explants were inoculated for callus induction potential under different concentrations of TDZ *viz.,* 1 ppm, 3 ppm and 5 ppm. The observations that were recorded include the initial callus weight (2 weeks after first sub-culture) and final callus weight (4 weeks after second sub culture). Paired T-Test, One-way ANOVA and Tukey's HSD tests for both initial and final callus weight were taken up. The induced calli were then used for GC-MS analysis of untargeted metabolites (to see the potential for use in suspension cultures).

GC-MS analysis for untargeted metabolomics

***Sample preparation and extraction of metabolites*:** About 0.3 g and 0.5 g tissues of *in situ* grown shoot tips (MTP 1) and *in vitro* cultured calli samples (derived from MTP 1 shoot tip cultures), respectively, were taken after grinding using liquid nitrogen in a pre-chilled pestle and mortar. To the powdered sample, 1.4 mL of methanol was added and transferred to microcentrifuge tubes. The samples were vortexed and incubated at 70 °C in a shaking water bath for 15 min. Then, the samples were centrifuged at 12000 rpm for 20 min in a refrigerated centrifuge (Eppendorf Centrifuge 5427 R) maintained at 4 °C. The supernatant was then transferred to a 25 mL falcon tube to which 1.4 mL of water and 750 µL of chloroform were added. The mixture was vortexed and centrifuged at 6000 rpm for 20 min in a refrigerated centrifuge (Thermo Fisher Scientific - Thermo Electron LED GmbH D-37540 Osterode) maintained at 4 °C. Aliquots of supernatant (upper polar phase) were concentrated using a vacuum concentrator (Eppendorf Concentrator plus) at 45 °C for 50 min. Powdered samples derived from *in vivo* grown plants (shoot tips) were used as a control and those derived from calli (TDZ treatments @1, 3 and 5 ppm) were used as test.

Derivatization of polar phase metabolites: Then, 50 µL of methoxamine HCl and pyridine mixture (prepared by mixing 20 mg methoxamine HCl in 1 mL of pyridine) was added to the concentrated polar phasic aliquots. This step enables protection of carbonyl components by methoximation. This was followed by incubating the mixtures at 37 °C for 2 hr. in a shaking water bath. After 2 hr. of incubation, the samples were derivatized [tri-methyl-silation (TMS)] by adding 80 µL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) and again incubated at 37 °C for 30 min in a shaking water bath. Finally, the samples were centrifuged at 10000 rpm for 3 min in a refrigerated centrifuge maintained at 4 °C. The supernatants were pooled and transferred to GC-MS vials for GC-MS analyses. Each sample was analyzed in triplicate.

***Gas Chromatography Mass Spectrometry (GC-MS) analyses*:** The samples were subjected to GC-MS analyses using a Gas Chromatograph (GC) coupled with a Mass Spectrometer (MS) (GC/MS-TQ8040 NX SHIMADZU, Shimadzu Corp., Tokyo, Japan). The GC was equipped with a fused silica capillary column, SH-Rxi -5 Sil MS, measuring 20 m in length and 0.18 mm in diameter, with a film thickness of 0.18 mL. Utilizing a split mode (10:1), the diluted samples were injected, maintaining a constant helium gas flow rate of 1 mL/min. The initial oven temperature of 70 °C was maintained for one minute and then gradually raised to 300 °C at a rate of 5 °C per min, resulting in a total run time of 55 min per sample. The injector port temperature was set to 280 °C, while the ion source temperature was set to 230 °C.

For chromatograph acquisition and peak deconvolution, GC-MS real time analysis software (version Shimadzu) was used. Different biochemical components of the mixture were identified by comparing their linear indices to a series of n-alkanes (C8-C24) under the same chromatographic conditions. Metabolites were identified by similarity searching of each mass spectra obtained against the NIST 10 library (National Institute of Standards and Technology, Gaithersburg, MD, USA), a database (23). The preprocessing of total ion chromatograms viz., alignment, baseline correction and integration (24) was carried out following which the data sets including sample information, retention time m/z and peak intensity were formatted as comma separated values (csv) files and exported as input files for MetaboAnalyst 5.0 data analysis software.

GC-MS data processing and statistical analyses (untargeted metabolite analyses)

The output data (as a csv file) with a list of compounds identified along with different particulars *viz,* retention time, peak area, peak height, peak area *per cent*, peak height *per cent* etc., was used for interpreting the data. For data analysis using the online software MetaboAnalyst 5.0, peak area per cent data was utilized. As a first step, the 290 unique compounds listed out from all the samples were subjected to enrichment analyses which enabled us to classify the metabolites into different classes. Then, The GC-MS output data with top 60 [*in vitro* grown callus samples (MS + TDZ @ 5 ppm)] - 100 [*in vitro* grown callus samples (MS + TDZ @ 1ppm) / *in vivo* grown shoot tip samples] compounds from each sample were converted into a matrix format that represented 290 unique compounds (unique retention times and area) in all the samples tested. This data matrix was subjected to quality check, data filtering, data normalization (sample normalization by median; log10 data transformation and Pareto scaling) before subjecting to One-factor statistical analyses (25). Pareto scaling helps to increase the amplification of low abundance ions without amplification of raw data noise. To visualize the metabolites (significant due to treatment) one factor statistical analyses *viz*., ANOVA, Principal Component Analyses (PCA), Principal Least Square Discriminant Analyses (PLSDA) as well as Heat map analyses (Euclidean distance and Ward's clustering) were carried out. PLSDA is used to analyze large data sets based on variable importance in project (VIP) scores are used. VIP scores rank the overall contribution of each variable using a significance level of $p \leq 0.05$.

Results

Standardization of $CaOCl_2$ concentration on reducing bacterial and fungal epiphytes

The results of experiments on evaluating the effect of different concentrations of $CaOCl_2$ in reducing bacterial and fungal growth upon primary inoculation is given in Table 1-6. Tables 1-3 and tables 4-6 tell us about the effect of different concentrations of $CaOCl_2$ on bacterial and fungal growth, respectively. It is a well-established fact that epiphytic bacterial and fungal growth shall be visible in after 48 and 72 hr. of primary inoculation, respectively. If bacterial and fungal growth is observed beyond this time period, it can be concluded that they are of endophytic origin (26). In both the cases, i.e., for observing bacterial and fungal growth, data was collected, three and 10 days after primary inoculation. The data was subjected to one way ANOVA and Tukey's HSD test. It was observed that, in controlling bacterial growth, all the four different concentrations of $CaOCl_2$ had poor effect in controlling both epiphytic and endophytic bacteria. Beyond three days, bacterial growth was not observed.

On the other hand, fungal growth was observed after a period of seven through 10 DAI revealing the establishment of fungal endophytes. The above observations were recorded in the same culture bottles after a duration of 10 DAI. Upon statistical analysis by ANOVA, significant differences in the

Table 1. Effect of $CaOCl_2$ on sterilization of bacterial contaminants

Treatment	Number	Mean	Std Dev	Std Error
1 % $CaOCl_2$	3	1.33333	2.3094011	0.95743
3 % $CaOCl_2$	3	1.33333	2.3094011	0.95743
5 % $CaOCl_2$	3	0	0	0.95743
7 % $CaOCl_2$	3	0.33333	0.5773503	0.95743

Table 2. Analysis of variance of different $CaOCl_2$ concentrations in controlling bacterial contaminants

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
$CaOCl_2$ concentration	3	4.25	1.41667	0.5152	0.6833
Error	8	22	2.75		
C. Total	11	26.25			

Table 3. Tukey HSD analysis for identifying appropriate $CaOCl_2$ concentrations in controlling bacterial contaminants

Treatment	Level A	Level B	Mean
1 % $CaOCl_2$	A		1.3333333
3 % $CaOCl_2$	A		1.3333333
5 % $CaOCl_2$	A		0
7 % $CaOCl_2$	A		0.3333333

Table 4. Effect of $CaOCl_2$ on sterilization of fungal contaminants

Treatment	Number	Mean	Std Dev	Std Error
1 % $CaOCl_2$	3	7	2.6457513	1.2583
3 % $CaOCl_2$	3	6.66667	3.2145503	1.2583
5 % $CaOCl_2$	3	0.66667	1.1547005	1.2583
7 % $CaOCl_2$	3	6.33333	0.5773503	1.2583

Table 5. Analysis of variance of different $CaOCl_2$ concentrations in controlling fungal contaminants

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
$CaOCl_2$ concentration	3	81.66667	27.2222	5.731	0.0216*
Error	8	38	4.75		
C. Total	11	119.66667			

Table 6. Tukey HSD analysis for identifying appropriate $CaOCl_2$ concentrations in controlling fungal contaminants

Treatment	Level A	Level B	Mean
1 % $CaOCl_2$	A		7
3 % $CaOCl_2$	A		6.6666667
5 % $CaOCl_2$		B	0.6666667
7 % $CaOCl_2$	A	B	6.3333333

effects of different concentrations of $CaOCl_2$ in controlling fungal endophytes was revealed. Tukey's test enabled the identification that a concentration of 5 % $CaOCl_2$ and 7 % $CaOCl_2$ had significant control over fungal endophytes. Among these two concentrations, $CaOCl_2$ at 5 % concentration seemed to provide better fungal control, although minimal endophytic fungal growth was observed. This paved way for using the fungicide Bavistin at the time of media preparation (for primary inoculation of shoot tip explants) to take care of fungal endophytes.

Callus induction potential of Thidiazuron

In case of the experiments on callus induction, MS medium modified with different concentrations of the cytokinin Thidiazuron *viz*., 1 ppm, 3 ppm and 5 ppm were used. The shoot tip explants placed in the initial establishment medium were sub -cultured after 15 days and the culture bottles were placed in dark at 27 °C in the culture room. Follow up experiments to promote growth of callus after two weeks of the first sub-culture. The observation on calli (Fig. 2B-D) development was recorded as calli can be used for inducting somatic embryos or could be used for establishing suspension cultures.

The results of paired T-Test, One Way Anova and Tukey's HSD for evaluating the callus induction potential of different concentrations of Thidiazuron are given in Tables 7-13. Paired T-Test (Table 7) revealed that there existed significant differences in both the initial and final weight of calli from different treatments (both within and among pairs). Among the three different concentrations of TDZ studied for callus induction, TDZ at 1 ppm produced calli of higher weight compared to the other two treatments. Initial weight of calli ranged from 0.35- 0.86 gm across different treatments. Among the three treatments, initial weight of calli produced using 1 ppm TDZ was on the higher side weighing 0.86 gm (Table 8). The final weight of calli ranged from 0.99-3.74 gm across different treatments. Among the three treatments, final weight of calli produced upon treatment with 1 ppm TDA was around 3.74 gm (Table 11).

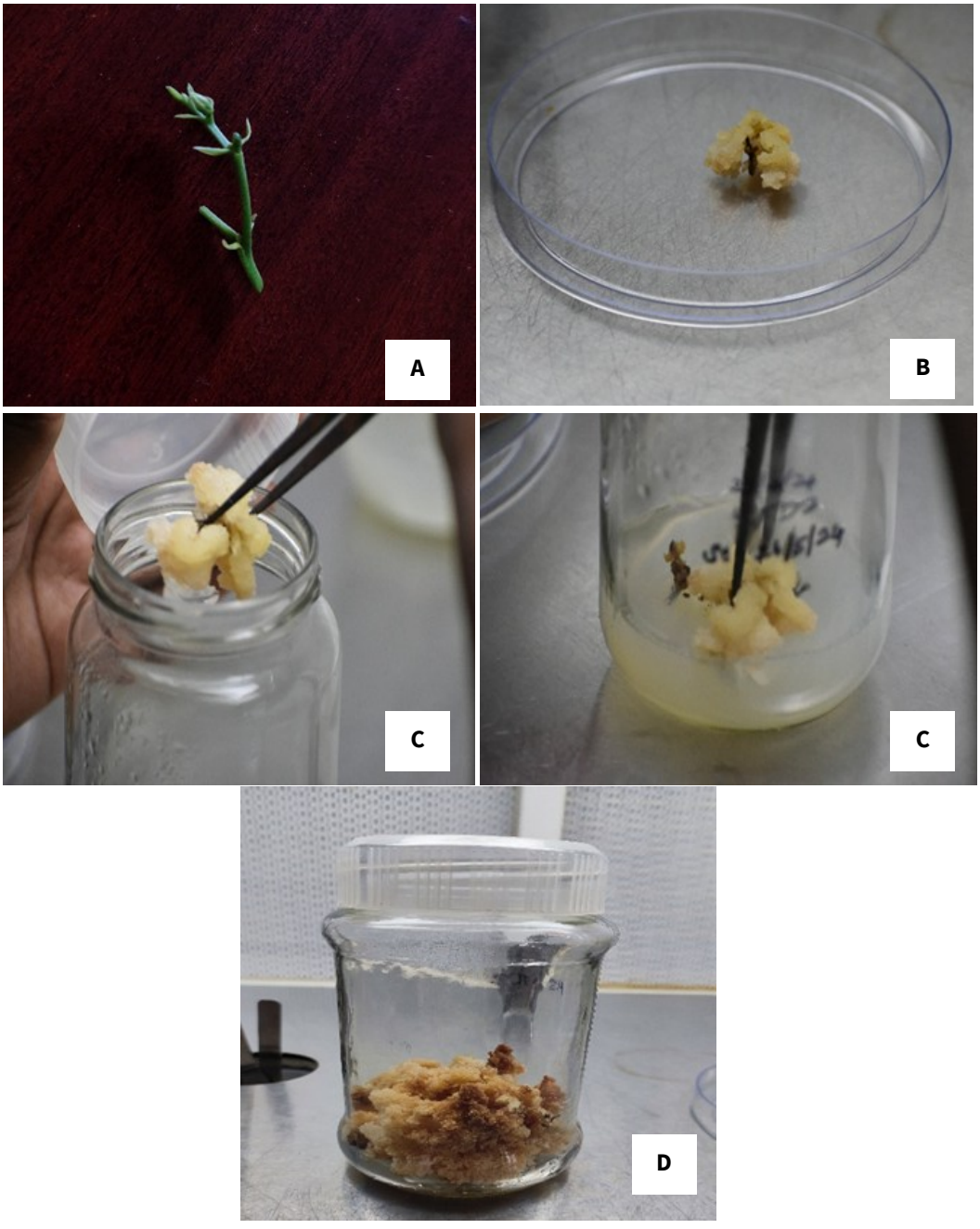

Fig. 2. (A-D): (A) Shoot tip explant; (B) Callus grown after two weeks of first subculture; (C) Subculturing of callus: (D) Callus grown after four weeks of second subculture.

Table 7. Paired T test analysis for initial and final callus weight

TDZ concentration (ppm)	Mean of Difference	Mean of Mean
1	2.8778	2.3028
3	0.675	0.6875
5	0.5917	0.7014
F Ratio	36.2834	26.1073
Prob > F	<.0001*	<.0001*
Test across Groups	Within pairs	Among Pairs

Table 8. Mean and standard deviation of initial callus weight calculation for further statistical analysis

TDZ Concentration (ppm)	Number of explants inoculated	Mean	Std Dev	Std Error
1	36	0.863889	0.649463	0.0803
3	36	0.35	0.343511	0.0803
5	36	0.405556	0.395651	0.0803

Since significant differences were revealed within and among pairs by paired T-Test, for reconfirming the results, one way ANOVA was performed both on the initial and final weights of calli obtained from different treatments of TDZ (Table 9 and Table 12). Also, Tukey's HSD test was carried to identify the best treatment (Table 10 and Table 13). One-way ANOVA and Tukey's HSD test revealed that 1 ppm TDZ was significantly better than 3 and 5 ppm TDZ in inducing callus for subsequent experimental trials.

GC-MS analysis for untargeted metabolomics

The list of unique compounds identified is given in the Supplementary file 1.

Enrichment analysis

The 290 unique compounds identified was subjected to Enrichment analysis option of the software MetaboAnalyst 5.0. A list of compounds was submitted using the interface which was compared to compounds already listed in HMDB, PubChem and KEGG databases. Of the 290 compounds, 17 compounds did not show any matches which could be due to spelling errors or really not listed in the databases. After sorting this, enrichment analysis was carried out by selecting the options Super-class, Main-class and Sub-class available under "Chemical structures".

Table 9. Analysis of variance for different TDZ concentration for initial callus weight

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
TDZ concentration	2	5.726852	2.86343	12.3363	<.0001*
Error	105	24.37194	0.23211		
C. Total	107	30.0988			

Table 10. Tukey HSD analysis for identifying significant TDZ concentrations for initial callus weight

TDZ Concentration (ppm)	Level A	Level B	Mean
1	A		0.863889
5		B	0.35
3		B	0.405556

Table 11. Mean and standard deviation of final callus weight calculation for further statistical analysis

TDZ Concentration (ppm)	Number of explants inoculated	Mean	Std Dev	Std Error
1	36	3.74167	2.711286	0.2877
3	36	1.025	0.903446	0.2877
5	36	0.99722	0.878793	0.2877

Table 12. Analysis of variance for different TDZ concentration for final callus weight

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
TDZ concentration	2	178.9563	89.4781	30.0277	<.0001*
Error	105	312.8847	2.9799		
C. Total	107	491.841			

Table 13. Tukey HSD analysis for identifying significant TDZ concentrations for final callus weight

TDZ concentration (ppm)	Level A	Level B	Mean
1	A		3.741667
3		B	1.025
5		B	0.997222

Classification based on super class

The classification of 290 metabolites under 39 Super Chemical Class metabolites is given in the Fig. 3. Among the 290 metabolites, organic acids and their derivatives predominated the metabolite population followed by organic oxygen compounds. Lipids and lipid like molecules were observed to be present in least amounts.

Classification based on main class

The classification of 290 metabolites under 617 Main Chemical Class is given in the Fig. 4. Among the 290 metabolites, organooxygen compounds predominated the metabolite population followed by carboxylic acids and its derivatives. Indoles, steroids and their derivatives were present in least amounts.

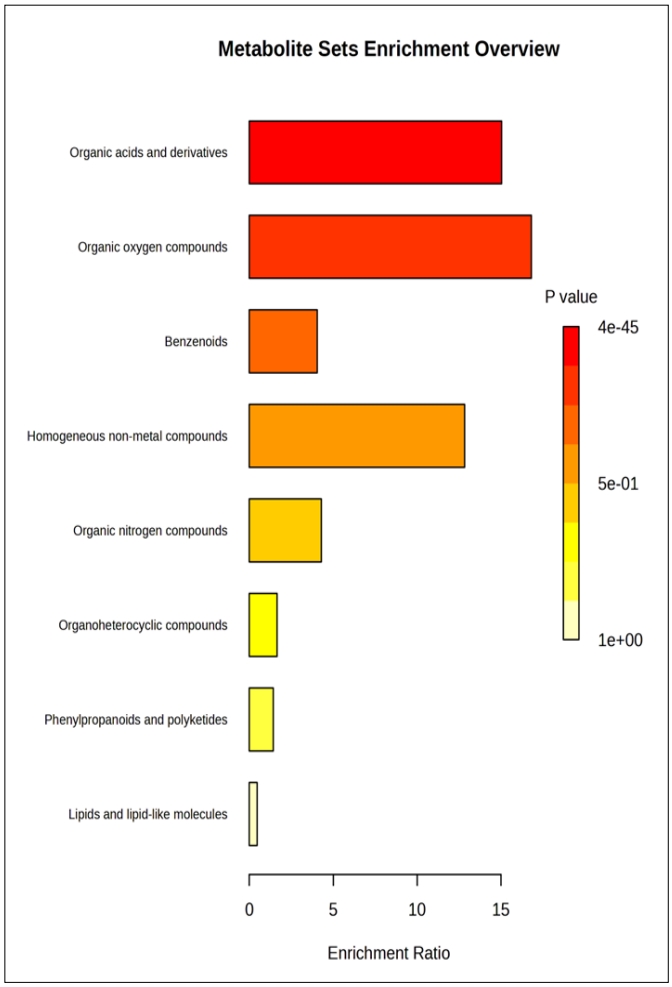

Fig. 3. Enrichment analysis of GC-MS metabolites obtained from *in vivo* shoot tips and *in vitro* developed calli of Red sanders based on super class.

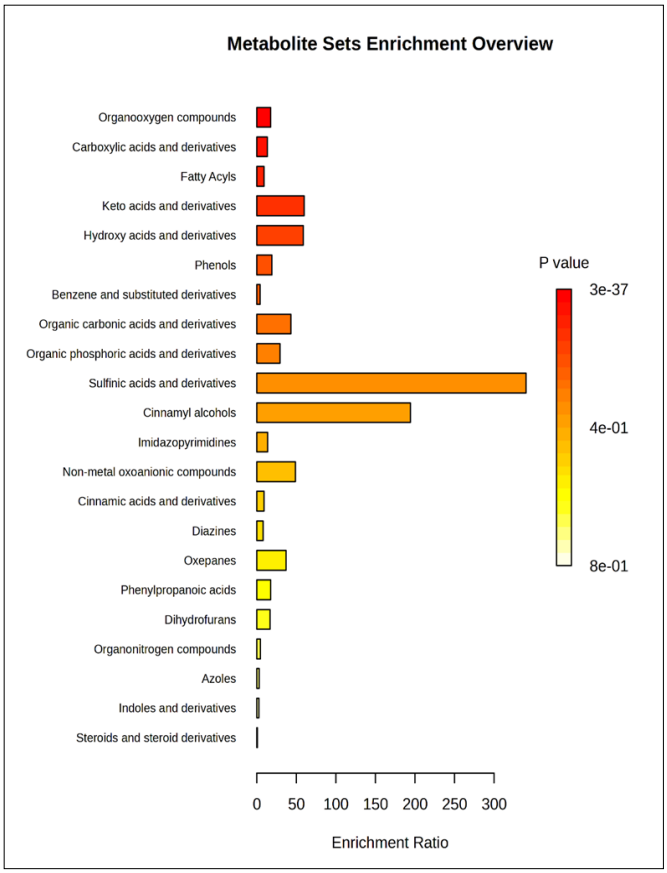

Fig. 4. Enrichment analysis of GC-MS metabolites obtained from *in vivo* shoot tips and *in vitro* developed calli of Red sanders based on main class.

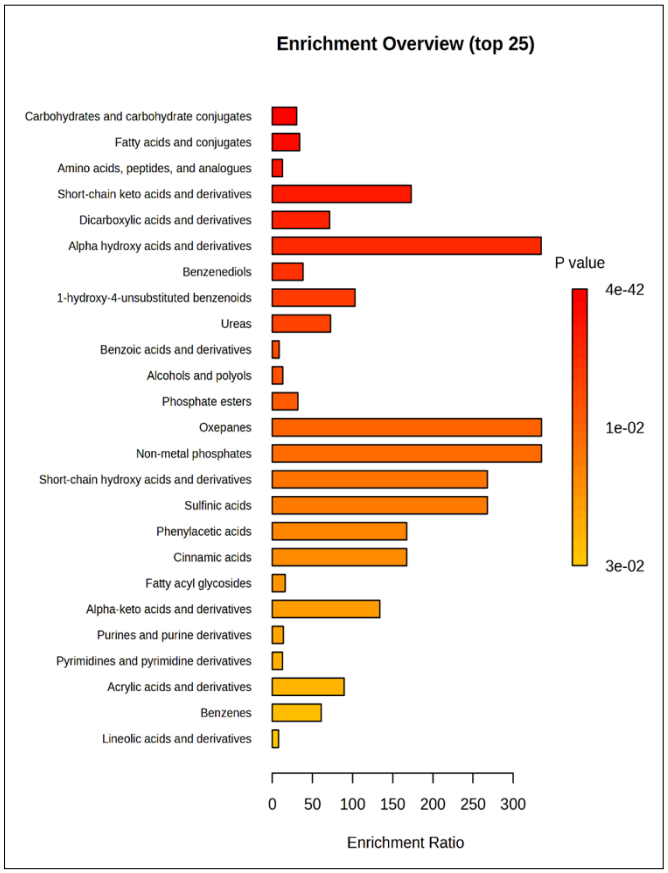

Fig. 5. Enrichment analysis of GC-MS metabolites obtained from *in vivo* shoot tips and *in vitro* developed calli of Red sanders based on sub class.

Classification based on sub-class

The classification of 290 metabolites under 1250 Sub Chemical class is given in the Fig. 5. Among the 290 metabolites, carbohydrates and their conjugates, followed by fatty acids and their conjugates were significantly predominant in the metabolite population. On the other hand, benzenes and linolenic acids and their derivatives were found to be less significant.

One-way ANOVA analysis of metabolites

For carrying out one factor analysis, the per cent area profile of 290 compounds were processed, normalized and subjected to statistical analyses. The results of One-way ANOVA, PCA, PCLSDA and Heatmap analyses are given in Fig. 6-10. One-way ANOVA enabled identification of 60 compounds (out of 290) involved in different biochemical pathways to be significantly present across all the samples *viz*., *in vivo* grown shoot tips as well as *in vitro* grown calli.

PCA of metabolites

PCA based on PERMANOVA analyses revealed the relation between the metabolites from different treatments. It was observed that the metabolite population from the in vivo grown shoot tips and *in vitro* grown calli from the treatments MS + 3 ppm TDZ and MS + 5 ppm TDZ were almost similar in composition. On the other hand, the metabolite population from the *in vitro* grown calli (MS + 1 ppm TDZ) had a significantly different metabolite composition.

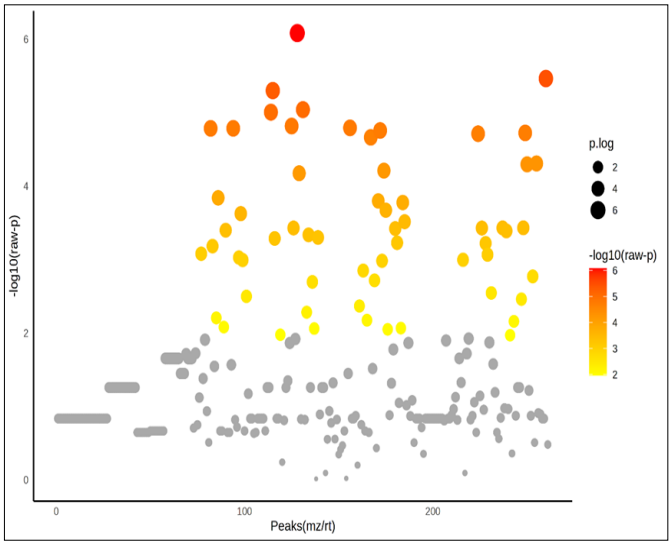

Fig. 6. One-way ANOVA analysis of GC-MS metabolites obtained from *in vivo* shoot tips and *in vitro* developed calli of Red sanders.

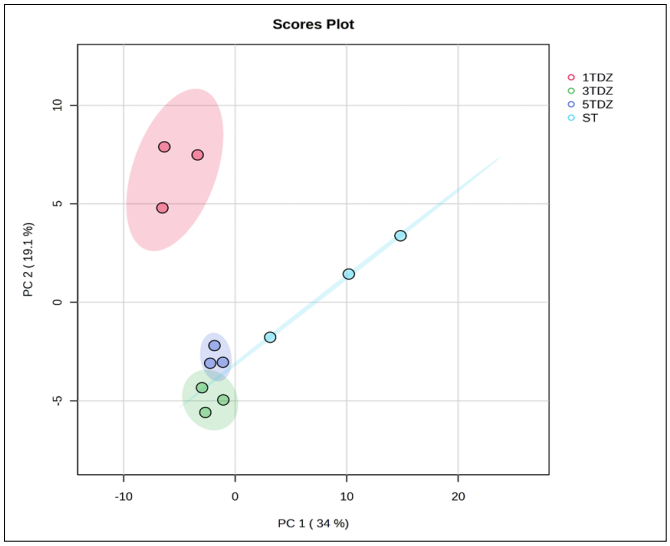

Fig. 7. Principal component analysis of GC-MS metabolites obtained from *in vivo* shoot tips and *in vitro* developed calli of Red sanders.

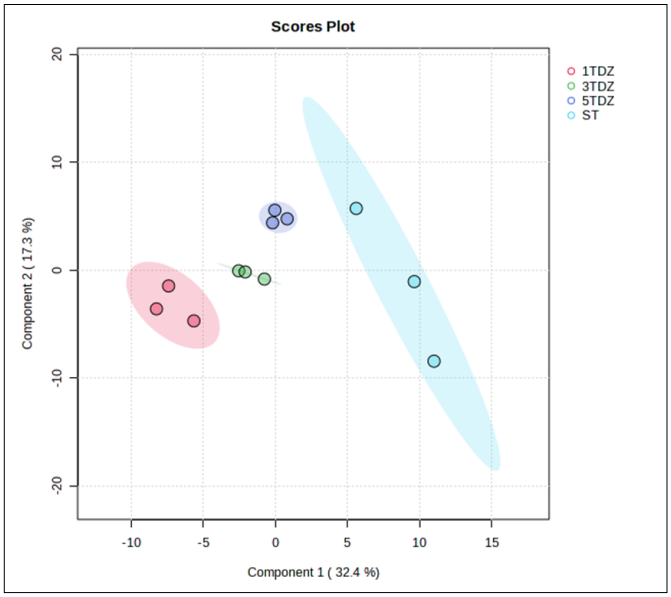

Fig. 8. PLSDA analysis of GC-MS metabolites obtained from *in vivo* shoot tips and *in vitro* developed calli of Red Sanders.

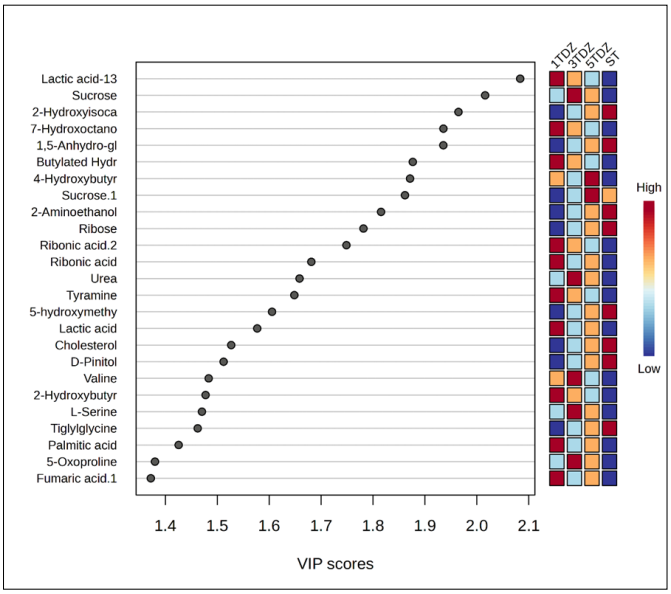

Fig. 9. PLSDA analysis of GC-MS metabolites obtained from *in vivo* shoot tips and *in vitro* developed calli of Red Sanders based on the VIP scores.

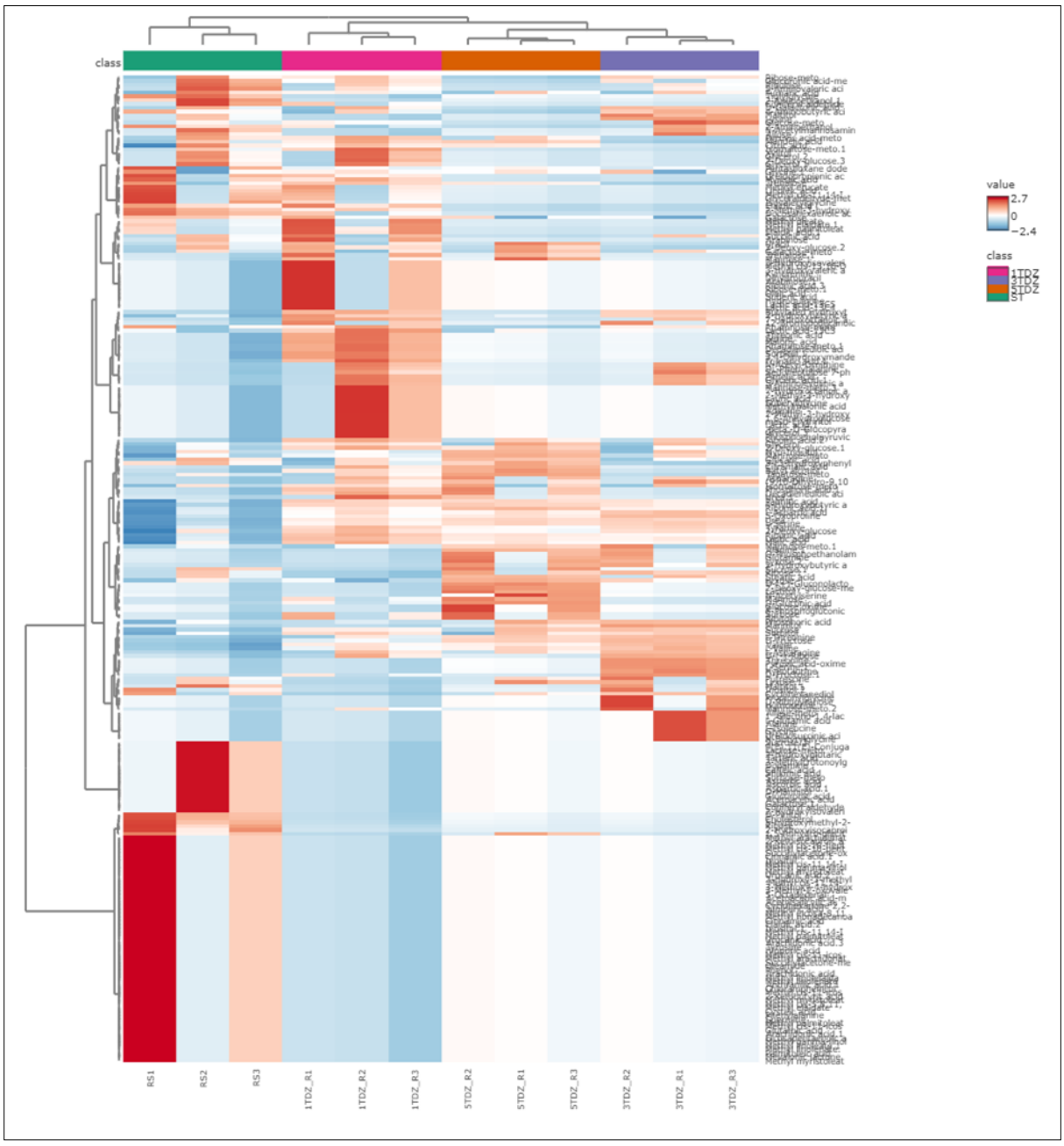

Fig. 10. Heat map analysis of GC-MS metabolites obtained from *in vivo* shoot tips and *in vitro* developed calli of Red Sanders based on the VIP scores.

PLSDA analysis of metabolites

PLSDA analyses provided us with a clear-cut differentiation of metabolites from the four different groups. This is because, grouping of metabolites happen based on the variables involved during experimentation. Based on the variable importance in projection (VIP) scores, it was observed that L-Lactic acid was predominant among the metabolites observed in all the four samples.

Heat map analysis of metabolites

Heat map analysis provides an intuitive visualization of the metabolites from the test samples. Heat map analyses based on Euclidean distance and Ward's clustering provided us with evidence that the metabolites from *in vitro* grown calli (MS + 3 ppm TDZ and MS + 5 ppm TDZ) were almost similar in composition. On the other hand, the metabolite population from the *in vivo* grown shoot tips and *in vitro* grown calli (MS + 1 ppm TDZ) had a significantly different metabolite composition.

Discussion

Standardization of $CaOCl_2$ concentration

Tissue culture demands establishment of explants *in vitro* without any contaminating bacteria and fungi (both epiphytic and endophytic origin). To meet this, the explants are surface sterilized inside the laminar air flow chamber before inoculation in the culture medium. For surface sterilization of explants, sterilant *viz.,* 70 % alcohol, mercuric chloride, sodium hypochlorite (27) or calcium hypochlorite, alone or in combination are used (28). Among the 'chlorine' based sterilant, mercuric chloride and sodium hypochlorite have been widely used. Due to environmental hazards and storage problem, calcium hypochlorite was used as an alternative. Calcium hypochlorite solutions can be prepared afresh and used in surface sterilization and has been found to be effective (29). Therefore, it is essential to identify a proper concentration of calcium hypochlorite that can be recommended for use in tissue culture.

In the present investigation, different concentrations of $CaOCl_2$ were tested on shoot tip explants of red sanders in controlling microbial growth. This suggested that explants harbored endophytic bacteria and fungi. Among the different concentrations of $CaOCl_2$, all of the treatments exhibited identical control of epiphytic bacteria, but only 5 % $CaOCl_2$ was effective in controlling bacterial endophytes. Similarly, observations on fungal growth revealed that all the treatments exhibited identical control of epiphytic fungi, but 5 % and 7 % $CaOCl_2$ exhibited better control of endophytic fungi. Among these two concentrations of $CaOCl_2$, 5 % $CaOCl_2$ was effective in controlling fungal endophytes, but still, growth of endophytic fungi was observed. This paved way for inclusion of Bavistin in the culture medium used for further experimentation.

Callus induction in Red Sanders

Shoot tip culture of red sanders was cultured under complete dark conditions, as this is a common practice in commercial banana tissue culture. The experiments were taken up, which resulted in good callus development (in all of the TDZ concentrations). Callus is a good starting point for different tissue culture techniques *viz.,* indirect organogenesis, induction of somatic embryos, establishment of suspension cultures, starting point for genetic engineering / genome editing in most crops. In our case, the experiments were redirected to establishment of desired TDZ concentration for callus induction as well as metabolomics studies.

Callus induction potential of Thidiazuron

TDZ is a powerful as well as a costly cytokinin based growth regulator that is used in tissue culture, especially in tree crops (30, 31). TDZ has been successfully used to grow coconut plants from zygotic embryos (32). Hence, in this experiment on red sanders, use of TDZ was followed. Observations on callus induction by different TDZ concentrations were taken by comparing the initial and final weight of callus upon sub-culturing. Statistical analyses revealed that TDZ at 1 % concentration was the best for a callus induction as the initial and final weights were higher than in other TDZ concentrations.

GC-MS analysis for untargeted metabolomics

Understanding the metabolite composition in a sample will provide us with a birds-eye view of underlying biochemistry / molecular biology. In this context, untargeted metabolite analysis was carried out using GC-MS from *in situ* grown shoot tip explants as well as *in vitro* grown calli subjected to three different TDZ concentrations *viz.,* 1, 3 and 5 ppm (three replicates for each treatment). This was carried to understand the metabolite composition of *in vivo* grown plant parts and *in vitro* grown calli. Since, red sanders is a medicinally important plant, besides being an economically important timber tree, establishment of suspension cultures with a thorough knowledge on metabolite accumulation will enable devising strategies for mass production of medically important metabolites.

GC-MS analysis revealed the identification of about 290 compounds that were present in all the samples. A close observation of chromatogram peaks of unique metabolites in all the classes revealed that under *in vitro* conditions, the 'number' of metabolites were on the lower side than in the *in vivo* grown shoot tips. On the other hand, metabolites from *in vivo* grown shoot tips had lower peak areas compared to *in vitro* grown calli, indicating the possibility of more metabolite production by calli. Also, comparison of the unique metabolites across different treatments of calli with TDZ, revealed that there is an inverse relation to TDZ concentration and metabolite numbers. At lower concentrations, a greater number of metabolites were observed. As TDZ concentration increased, suppression in metabolite production was observed. This clearly suggests that TDZ, as a plant growth regulator is involved in multiple biochemical pathways that need to be dissected. Further, heat map analysis also revealed that TDZ @ 1ppm produced unique metabolites than at 3 and 5 ppm levels. Correlating the results of callus induction potential by TDZ and heat map analyses of metabolites, it is clearly evident that metabolites accumulated in callus treated with 1 ppm TDZ promoted callus production. Hence, it is concluded that metabolites that are involved in dedifferentiation of shoot tip to callus have accumulated. Careful dissection of the compounds, their sub-class as well as main class will definitely shed light on the metabolic pathway that is responsible for callus induction.

Conclusion

In the present study, we used different concentrations of $CaOCl_2$ of which treatment with 5 % concentration for 20 min proved adequate for controlling the epiphytes and also majority of the endophytes. TDZ @ 1.0 ppm promoted callus growth in red sanders and concluded that for inducing callus using TDZ, culturing shoot tip explants under dark conditions will provide success. To conclude, the study undertaken on tissue culture for callus induction provided us greater insights for future research in Red Sanders tissue culture through indirect organogenesis. A thorough investigation on the unique metabolites and their metabolic pathways shall pave way for future studies on enhancing callus production in other crops species.

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Authors' contributions

MP reviewed the manuscript and was involved in design, evaluation and planning the experiments, SPS performed the experiments and drafted the manuscript. MML assisted in software and data analyses. SKK assisted in data analyses and manuscript preparation. HBC assisted in software and data analyses. MBN involved in critical evaluation of the first author's experiments as research advisory committee member. KC involved in critical evaluation of the first author's experiments as research advisory committee member, PS involved in critical evaluation of the first author's experiments as research advisory committee member. UKS and PKT provided the Red Sander's genotype samples for the study. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors have no conflict of interests to declare.

Ethical issues: None

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