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In vitro Secondary Metabolite Production from the Roots of *Decalepis arayalpathra* KMA 05 Clones and its Antimicrobial Potential using *Methylobacterium* sp. VP103 as an Elicitor

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Abstract

Decalepis arayalpathra KMA 05 clones were elicited with a bacterium, *Methylobacterium* sp. VP103 isolated from the leaves of *ex-vitro* established plantlets of *D. arayalpathra* KMA 05 clones, with the aim of increasing the production of root specific secondary metabolites. *D. arayalpathra* KMA 05 shoot tip cultures were triggered for root initiation by keeping the cultures for one month in ½ strength Woody Plant Medium (WPM)+Thidiazuron (TDZ) 0.3 mg/l.

They were then shifted to ½ strength WPM supplemented with auxins [0.5 mg/l indole-3-butyric acid (IBA) + 0.05 mg/l Naphthalene acetic acid (NAA)] along with 40 mg/l activated charcoal with a fixed concentration (0.4 mg/l) of lyophilized bacterium powder (LBP) dissolved in 30% methanol.

The effect of elicitation was studied on three root specific secondary metabolites, i.e. 2-hydroxy 4-methoxy benzaldehyde (MBALD), 2-methoxy-4-vinylphenol (2M4VP) and α-amyrin. The production of α-amyrin was found to be enhanced in all 12, 24 and 36 week old treated root extracts. Antibacterial activity was also found to be enhanced against all the bacteria selected from the 36 week elicited extracts among which *Rhodococcus* sp. UKS7 (34 ± 0.0 mm.) showed maximum inhibition. The findings provide an insight into the use of *Methylobacterium* sp. VP103 as an elicitor in the growth of the roots and enhanced production of α-amyrin.

Keywords: *Decalepis arayalpathra*; Aromatic compound; Secondary metabolites; Antimicrobial; α-amyrin

Introduction

Plant microbe interactions are known since antiquity and many plant growth promoting microbes as well as harmful ones play an important role in the production of secondary metabolites. Now a days these interactions are being exploited for the sake of secondary metabolite production at *in vitro* level. The production and expression of these secondary metabolites are being influenced by the rhizospheric and phyllospheric microflora.

Hence, for a sustainable production of the secondary metabolites at *in vitro* level these microbes are being exploited. Microbes have played an important role in the production / enhancement of secondary metabolites which are of medicinal importance, however very few metabolites are produced at industrial level e.g. Paclitaxel [1] and Shikonin [2]. *Decalepis arayalpathra syn Janakia arayalpathra* [3] is a critically endangered and medicinally important endemic plant.

The moniliform roots of this plant has been used for treating peptic ulcer diseases [4]. Seven root specific secondary metabolites were separated and reported by [5] which are 2-hydroxy-4-methoxybenzaldehyde (MBALD), α-amyrin acetate, 4-methoxy salicylaldehyde, magnificol, 3-hydroxy p-anisaldehyde, naringenin, kaempferol, and aroma dendrin. Antifungal activity of MBALD has been also reported [6]. Micropropagation studies on *D. arayalpathra* were done [7,8] and analyzed MBALD in normal root cultures of this plant. According to the work reported the allied species, *D. hamiltonii* also produce MBALD [9]. *In vitro* secondary metabolite production is enhanced through the application of a variety of elicitors to plant cell or tissue culture systems.

As a step towards conservation, an attempt for clonal propagation in *Decalepis arayalpathra* KMA 05 was done through seeds in CSIR-CIMAP, Lucknow. Acclimatization and field establishment studies using this phyllospheric microbe *Methylobacterium* sp. VP103 were done in the University of Delhi [10]. In the present study, *in vitro* biotic elicitation in the *in vitro* root cultures of *D. arayalpathra* KMA 05 using *Methylobacterium* sp. VP103 and an activity guided fractionation was done in which five microbes were selected for the study.

At different time intervals, the secondary metabolites were harvested and observed for their antimicrobial activity along with their control. The main focus of the study was optimization of elicitation using a treatment consisting of LBP in the root

induction medium [(RIM) 1/2WPM + 0.5mg/l IBA + 0.05mg/l NAA + 40mg/l activated charcoal] which induces the accumulation of α-amyrin in *in-vitro* (IV) root cultures of *D. arayalpathra* KMA 05 clones. One month old thidiazuron (0.25 mg/l, optimized primary inducer dose) treated axillary shoot tip cultures were co-inoculated with sterilized LBP in RIM to assess its potential in production of secondary metabolite in root cultures of *D. arayalpathra* KMA 05 clones.

Materials and Methods

Plant material

In-vitro (IV) cultures of *D. arayalpathra* KMA 05 clones were established from the seeds in the tissue culture facility of CSIR-CIMAP, Lucknow and procured for elicitation experiments. Primary inducer (thidiazuron- 0.25 mg/l) treated axillary shoot tips were used for inducing roots in RIM with 20 g/l sucrose and 8 gm/l agar for solidification. pH of the medium was adjusted to 5.8 prior to autoclaving. Cultures were maintained in 250 ml Erlenmeyer flasks containing 100 ml of solid medium under 18/6 h dark to light conditions at 25°C. The shoot tips were sub-cultured after every 20 days.

A phyllospheric bacterium, *Methylobacterium* sp. VP103 isolated from the leaves of the ex-vitro established plants of *D. arayalpathra* KMA 05 clones were used as an elicitor [10].

Preparation of bacterial elicitor

The bacterial elicitor was prepared by the protocol developed by Srivastava et al. After preparing the stock in 1 mg/ml concentration of lyophilized bacterium powder (LBP), the stock was filter sterilized with 0.22 µm syringe filters (Sterile axiva syringe filters, Cat. no. SFTF25R) and kept in 1 ml aliquot at -20°C for further use in the RIM.

In vitro elicitation, harvesting of roots and extraction of secondary metabolites

For *in vitro* elicitation of the secondary metabolites in *D. arayalpathra* root cultures, the LBP was inoculated in RIM. For elicitation, a modified root induction protocol (Pre-treatment of axillary shoot tips with TDZ and then transferring on RIM) was used in order to trigger the induction of roots. One month old shoot tip cultures kept on ½ strength basal WPM medium supplemented with 0.3 mg/l TDZ were dissected out and transferred on RIM containing 0.4 mg/l LBP [10].

All the IV cultures were kept in the Central Microbial Culture Facility on RIM+LBP at 25 ± 2°C with relative humidity (60-70%) in the Department of Botany, University of Delhi, India. After 12, 24 and 36 weeks of exposure to the biotic stress provided with the *Methylobacterium* sp. VP103, the IV grown roots of the treated plants along with their control were harvested, washed with lukewarm water and blot dried.

The fresh weight of roots was taken and left to dry at room temperature. Completely dried roots were weighed, powdered and extracted to check for the production of secondary metabolites. All the powdered roots taken in equal quantity (5 gm) were extracted with ethyl acetate in 1:6 ratio. The extraction process was repeated thrice by keeping it on a gyratory shaker (Adolf Kuhner AG, CH-4127 Birsfelden/ Switzerland) for 3 h. All the ethyl acetate extracts were pooled and evaporated at 48°C in a rotavapour (Heidolph, Basis Hei-VAP ML). After evaporating, the residue was used as a crude extract. The dried crude extracts were kept at -20°C for GC-MS analysis and to check the antimicrobial activity.

Gas chromatograph-Mass spectrometric analysis

The crude extracts (60 mg), harvested from roots of non-treated and treated IV plants from 12, 24 & 36 weeks were dissolved in 1 ml methanol **(Figure 1)**, filtered with 0.22 µm syringe filter and transferred to the GC-MS vials. Gas chromatographic (GC) analyses of the all samples were done using Shimadzu Gas Chromatograph, 2010 coupled with AOC-20S auto sampler.

The gas chromatograph (GC) was attached to Shimadzu Single Quadruple MS (GCMS-QP2010 Ultra, Serial No.020525174707, Shimadzu Corp.). Fused silica column (Restek chromatography products, Rtx®-5MS (Lx I.D. 30 m×0.25 mm×df 0.25 µm, a low polarity phase; crossbond ®diphenyl dimethyl polysiloxane) was used.

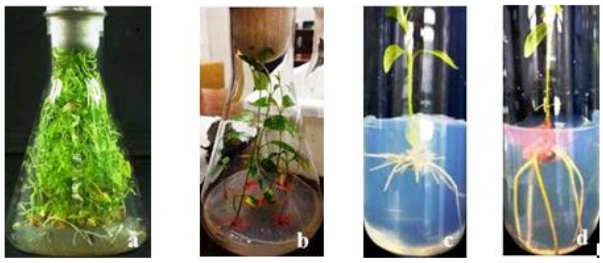

Figure 1. (a) *In vitro* plantlets on RIM (b) *In vitro* plantlets on RIM with 0.4 mg/l LBP (c) Root growth on RIM (d) Root growth on RIM with LBP.

Calibration curve and quantification of α-amyrin

α-amyrin (Sigma-Aldrich) was taken at a conc. of 0.25, 0.5, 0.75 mg/ml in order to quantify the α-amyrin concentration in the different week old root extracts.

Bacterial cultures used

All the bacterial cultures were procured from Applied Microbiology and Biotechnology Laboratory, Department of Botany, University of Delhi, India which were initially isolated from the soil and were maintained on nutrient agar (NA) medium. The nucleotide sequences of all the bacteria have been deposited in the NCBI gene bank **(Table 1)**.

Table 1. List of pure bacterial cultures selected for the present study.

S. No.	Bacterial cultures	Type	Accession no.
1	Myroides indicus	Gram-negative	KC906253
2	Promicromonospora sp. VP111	Gram-positive	KJ024374
3	Rhodococcus sp. UKS7	Gram-negative	KY088272
4	Bacillus sp. SV1	Gram-positive	KJ024372
5	Kocuria sp. UKS5	Gram-positive	KF305935

Anti-bacterial assay

Anti-bacterial activity was checked using agar disc diffusion method. Cultures (48h) were spread on Nutrient agar (NA) plates with the help of a spreader. Sterilized filter paper strips (5 mm) were placed on these NA plates with 20 µl of various extracts along with their controls. The plates were incubated at 37ºC for 24 h and diameter of the inhibition zone (mm) was measured.

Statistical analysis

Results are prepared as mean ± SD of three independent experiments having three replicates each and the level of significant differences between the results was tested by one-way ANOVA followed by Duncan's multiple range test. Comparison between control and means of treatments were done by Duncan's test at significance level P ≤ 0.05.

Results

Shoot tips when inoculated in ½ strength WPM+0.3 mg/l TDZ for one month triggered root induction which were then transferred to RIM+ LBP along with the control and harvested after 12, 24 & 36 weeks **(Figure 1a,1b,1c,1d)**. The crude extracts of the dried and powdered roots when subjected to GC-MS analysis showed peaks consisting of 27 compounds from the 12 week old root extract both in non-treated as well as treated ones with retention times starting from 8.4 to 40.5 **(Figure S2 and S3) (Table S1 and S2)**. Peaks consisting of 35 compounds were observed in the root extract of 24 week old roots of non-treated IV plants with retention times starting from 8.4 to 40.6 **(Figure S4 and Table S3)** and peaks of 22 compounds in the root extract of 24 week old roots of treated IV plants with retention time starting from 8.4 to 40.5 were found **(Figure S5 and Table S4)**. Root extracts of 36 week old non-treated IV plants showed peaks consisting of 78 compounds with retention time starting from 5.6 to 40.9 **(Figure S6 and Table S5)**. Peaks consisting of 73 compounds were seen in the root extract of 36 week old treated plants with retention times starting from 5.6 to 40.8 **(Figure S7 and Table S6)**. It was found that the area percent of MBALD and 2M4VP decreased in the treated root extract of 12 week old plants in comparison to the root extracts of non-treated plants, while it increased in the root extract of the 24 week old plants. Both MBALD and 2M4VP were found to be absent in the root extract of 36 week old plants in both the non-treated as well as treated ones **(Table 2)**. Table 2 clearly shows that the area percent of α-amyrin was significantly enhanced in all the root extracts of the treated plantlets. The concentration of α-amyrin was quantified by plotting the standard calibration curve with 0.25, 0.5 and 0.75 mg/ml concentrations **(Figure S8)**. It was found that α-amyrin concentration was very low in the root extracts of the non-treated plantlets while it was enhanced in the root extracts of the treated plantlets with more than ten folds enhancement in the root extract of 36 week old treated plantlet **(Figure S9, S10, S11, S12, S13, S14)** and **(Table 3)**.

Table 2. Area percent (AP) of 2-hydroxy, 4-methoxy benzaldehyde (MBALD), 2-Methoxy, 4-vinyl phenol (2M4VP) and Alpha-amyrin (AA) in the root extracts of the 12, 24 and 36 week old non-treated and treated plantlets.

No. of weeks	AP of MBALD in root extracts of non-treated plantlets	AP of MBALD in root extracts of treated plantlets	AP of 2M4VP in root extracts non-treated plantlets	AP of 2M4VP in root extracts of treated plantlets	AP of AA in root extracts of non-treated plantlets	AP of AA in root extracts of treated plantlets
12	19.37 ± 0e	14.61 ± 0.006d	1.41 ± 0.01e	0.98 ± 0.006c	1.55 ± 0c	2.14 ± 0.035d
24	10.73 ± 0b	14.45 ± c	0.68 ± 0.006b	1.06 ± 0.023d	1.33 ± 0b	10.78 ± 0.218e
36	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.48 ± 0.015a	12.08 ± 0.046f
Data are mean ± SD of three independent experiments, one way ANOVA was run to check the significance						
(P ≤ 0.05) and different lower case letters indicate significant differences as per Duncan's test						

Table 3. Correlation between area percent and concentration of α-amyrin.

No. of weeks	Area percent of α-amyrin in non-treated root extracts	Area percent of α- amyrin in treated root extracts	Concentration (mg/ml) of α-amyrin in non-treated root extracts	Concentration (mg/ml) of α-amyrin in treated root extracts
12	1.55	2.14	0.005	0.01
24	1.33	10.78	0.004	0.05
36	0.48	12.08	0.014	0.3

The anti-microbial activity of root extracts of non-treated and treated IV plants of *D. arayalpathra* KMA 05 clones were evaluated against some selected bacterial strains isolated from soil. It was observed that most of the root extracts non-treated as well as treated plants were effective in inhibiting the growth of bacterial strains with maximum inhibition (34 ± 0.0) shown by *Rhodococcus* sp. UKS7 by the root extract of the 36 week old treated plantlet **(Figure S15)** and **(Table 4)**. It may be assumed that some other compound in the extract would also be playing an important role in increasing the inhibitory effect present in a lesser amount. Hence, these IV plants could be exploited in future for its anti-bacterial properties against the tested microorganisms. These findings suggest that Methylobacterium sp VP 103 may play an important role in enhancing the production of antibacterial secondary metabolite in this plant.

Table 4. Inhibition zones (IZ) formed by the bacterial strains when treated with root extracts of non-treated (Nt extr) and treated (t extr) plantlets at different time period.

Name of Bacteria	Treatments					
	IZ (mm.) by Nt extr 12 week	IZ (mm.) by t extr 12 week	IZ (mm.) by Nt extr 24 week	IZ (mm.) by t extr 24 week	IZ (mm.) by Nt extr 36 week	IZ (mm.) by t extr 36 week
Myroides indicus	20.67 ± 0.58c	20 ± 1.0c	17.67 ± 0.58b	16 ± 0.0a	17 ± 0.0a	28 ± 1.0d
Promicromonospora sp.VP111	0.0 ± 0.0a	14.67 ± 2.5b	14.33 ± 0.5b	16.67 ± 1.1b	17 ± 0.0b	30 ± 3.0c
Rhodococcus sp. UKS7	20.33 ± 1.1b	17 ± 0.0a	24 ± 1.7c	20.67 ± 2.3b	17 ± 0.0a	34 ± 0.0d
Bacillus sp. SV1	18 ± 3.4ab	18 ± 3.4ab	12.33 ± 1.1c	27.33 ± 2.3b	16 ± 0.0a	18.33 ± 0.5ab
Kocuria sp. UKS5	20.67 ± 2.0b	16.67 ± 1.1a	18 ± 3.4ab	18.33 ± 0.5ab	16.67 ± 0.5a	20.67 ± 0.5b
Data are mean ± SD of three independent experiments; one way ANOVA was run to check the significance						
(P ≤ 0.05) and different lower case letters indicate significant differences as per Duncan's test						

Discussion

The *in vitro* cultures of *D. arayalpathra* KMA 05 clones can serve as a valuable source for antibacterial secondary metabolites. In the present investigation, the biotic elicitation of the secondary metabolites, MBALD, 2M4VP and α-amyrin using *Methylobacterium* sp VP 103 were investigated. 2M4VP, an aromatic and flavoring compound [11] was found to be present as one of the secondary metabolites in the *in vitro* clones of *D. arayalpathra* KMA 05, production of α-amyrin from the bark incision of several species of *Bursera* or *Protium* of the Burseraceae family and has been shown to exhibit various pharmacological activities under *in vitro* and *in vivo* conditions against inflammation, microbial and fungal infections [12]. MBALD, 2M4VP and α-amyrin were expressed in the *in vitro* grown root extracts of the treated and non-treated plants. This indicates that through *in vitro* elicitation methods, these compounds can be obtained without disturbing the natural flora irrespective of climatic constraints as this is an endemic and endangered plant. An ester derivative of α-amyrin i.e α-amyrin-acetate and MBALD were also isolated in the naturally grown tubers of *D. arayalpathra* [1]. MBALD, a specific aromatic compound produced by this plant has been proven to have antioxidant and antimicrobial properties [13]. Biotic elicitation using LBP of *Methylobacterium* sp. VP103 was used in the *in vitro* cultures of *D. arayalpathra* KMA 05 to study the production of the secondary metabolites, the first report so far. Cell-free extracts of *Bacillus subtilis* and *Escherichia coli* were used as biotic elicitors to study the production of secondary metabolite in *Gymnema sylvestre* suspension cultures [14]. GC and HPTLC, the most common techniques to detect and quantitate α-amyrin in plants have been used. GC-MS profiling showed that area percent of MBALD and 2M4VP decreased in the root extract of treated 12 week old plants while it increased in the root extract of 24 week old plants. However, both the metabolites were absent in the root extracts of 36 week old plants **(Figure 2).**

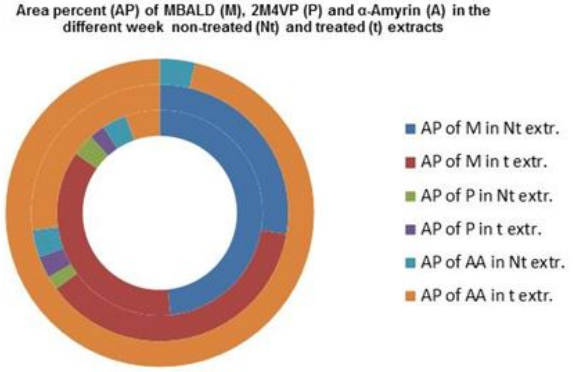

Figure 2. Doughnut chart showing area percent (AP) of 2-hydroxy, 4-methoxy benzaldehyde (MBALD), 2-methoxy, 4-vinyl phenol (2M4VP) and α-Amyrin (AA) in the root extracts of non-treated (Nt) and Treated (t) plantlets.

The area percent of α- amyrin was found to be enhanced in root extracts of all the treated 12, 24, 36 week old plants while the non-treated root extracts showed a decrease. There have been no reports so far on the production of α-amyrin from the *in-vitro* cultures of *D. arayalpathra*. The filter sterilized LBP of *Methylobacterium* sp. VP103 dissolved in 30% methanol at 0.1-0.5 mg/l was effective in root induction and secondary metabolite production, however the maximum biomass

produced was observed at 0.4 mg/l [10], which has been used for elicitation, as secondary metabolite production has been shown to be directly related to the biomass of the roots [15]. Filter sterilized LBP played a significant role in the enhancement of α- amyrin in the root extracts with increase in time period. These root extracts of non-treated and treated *D. arayalpathra* IV plants showed antibacterial activity against all the bacteria selected. However, the 36 week old ethyl acetate root extract of the treated IV plants showed maximum activity (34 ± 0.0 mm) against *Rhodococcus* sp. UKS7 **(Figure 3).**

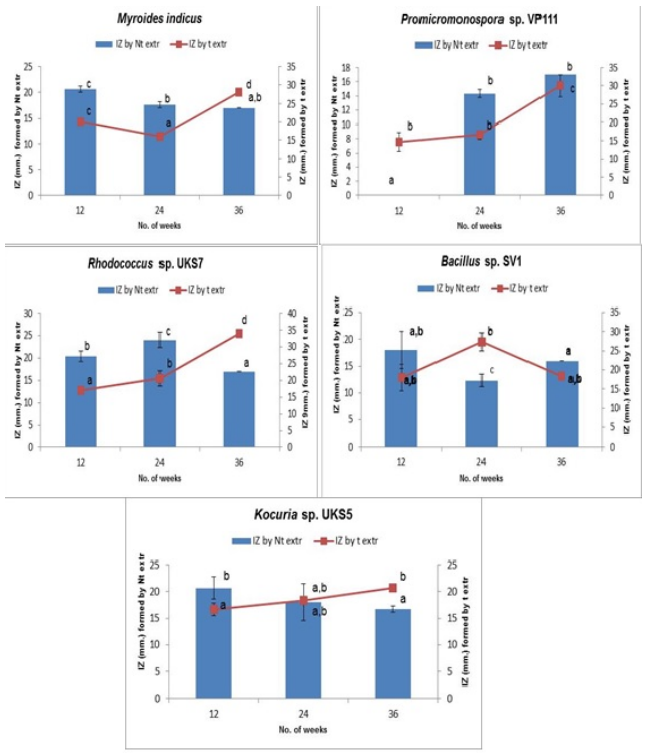

Figure 3. Histogram showing Inhibition zones (IZ) formed by the bacterial strains when treated with root extracts of non-treated (Nt extr) and treated (t extr) plants. Data are mean ± SD of three independent experiments, one way ANOVA was run to check the significance (P ≤ 0.05) and different lower case letters indicate significant difference.

α-amyrin with the presence of other triterpenoids were found to suppress bacteria and *C. albicans* from the methanolic extract of the stem bark of *Klainedoxa gabonensis* Pierre ex Engl. (Irvingiaceae). The antibacterial potential of the root extracts of *Janakia arayalpathra* (Syn: *D. arayalpathra*) against *Pseudomonas aeruginosa*, *Xanthomonas oryzae*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus vulgaris* and *Vibrio cholerae* [16]. *Klebsiella pneumoniae* showed maximum inhibition followed by *Xanthomonas oryzae*. No inhibitory activity was shown by *P. aeruginosa* and *Salmonella typhi*. *Methylobacterium* sp. VP103 can be explored at the molecular level in future to explore the proteins/enzymes responsible for elicitation secondary metabolite production.

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Conflict of Interest

None.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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