INVESTIGATION ON THE ACCUMULATION OF PLUMBAGIN IN *PLUMBAGO ZEYLANICA*L. HAIRY ROOTS

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ABSTRACT

The study aimed to induce hairy roots in *Plumbago zeylanica* L. And figured out the conditions determining its growth and Plumbagin accumulation capacity. The bacteria *Agrobacterium rhizogens* 11350 was used as a vector for gene transfer. The results showed four clones of successful gene-transferred roots; of which only one clone expressed both *rolB* and *rolC* genes and this clone was used to study the effects of environmental conditions. Using D-Optimal matrix, the optimal conditions were medium with 14.33% coconut water, 100 mg/l Chitosan, 19.40 mg/L salicylic acid and 500 mg/l peptone. Under the model of these investigated conditions, the expected fresh weight, dry weight and Plumbagin concentration were 4.968 g, 0.451 g and 11.135 mg per 60 ml media.

Keywords: Hairy root, D-Optimal matrix, response surface, Plackett-Burman model.

INTRODUCTION

Plumbago zeylanica L., belonging to Plumbaginaceae, was a medicinal plant originated from the North-West Asia (Aditi *et al.*, 1999), and distributed in tropical areas including Australia, Asia and Africa (Vijver and Looter, 1971). *P. zeylanica* L. has been traditionally used as drugs for several diseases (Abebe and Ayehu, 1993). Roots, leaves and stem of *P. zeylanica* L. All contain bioactive compounds useful for medicinal uses. Moreover, the roots consist acrid cryslalline compound which is yellow color and known as Plumbagin. This compound, Plumbagin, was used as a medicinal drug by Indian from 750 B.C. Other compounds in *P. zeylanica* L., roots include phenolic acids, tannins and anthocyanin for their anti-bacteria effects (Rang and Dung, 1996).Some research indicated that Plumbagin extracted from *P. zeylanica* L., has anti-cancer (Ram, 1996), anti-bacteria (Didery *et al.*, 1994; Duraga *et al.*, 1990) and anti-insect (Kubo *et al.*, 1983) effect; inhibits cell division (Bhargava, 1994) and reduce the cholesterol level (Ram,1996).

Plumbago zeylanica L. roots required several years to accumulate active Plumbagin (Kitanow and Pashankov, 1994). Therefore, the collection of Plumbagin was a slow progress and decreased their population in nature. In this study, we studied the capacity of *Plumbago zeylanica* L. hairy roots to accumulate Plumbagin on investigated optimal conditions.

MATERIALS AND METHODS

Induction of *in vitro* roots

Stems with dormant bud were gently washed with soap and water, then washed again with sterilized distilled water. Explants were then washed with 10% Javel solution in 20 minutes and finally washed with ionized water. Explants were cultured in MS media (Murashige Skoog, 1962) supplemented with 2.5 mg/L BA and 0.1 mg/LIBA to induce shoots. Leaves from these shoots were then used to transfer genes.

Leaf explants were soaked and cultured with *Agrobacterium rhizogen*es ATCC 11325 (*rolB*: 610 bp, *rolC*: 530 bp). After three days, cefotaxim (500 µg/ml) was used to washed out *Agrobacterium.* The clean explants were cultured on MS supplemented with cefotaxime in dark condition. The explants with induced hairy roots were then selected to cultured on liquid MS medium. Genetically modified roots were used to extract DNA following Miniprep kit-QIAGEN procedure. PCR reaction was run with primers: *rolB*:5**'**-TCAAGTCGCCGAGGTTTCTT-3'; 3'-AAACGCTCCGCGGTGGT-5'; *rolC*: 5'-TTGACCTATGTGCTCTTT-3'; 3'-CTCCATTCCAAATTTGCATT-5'and reaction components: 2.5 ml 10x buffer PCR, 0.2 mM dNTP's, 0.4 mM primers, 1.0 Unit *Taq* polymerase, 200 ng DNA template, final volume 25 ml. Initial heating of PCR mixture for 2 minutes at 94^{o}C, following 30 cycles at 94^{o}C in 1 minute, 55^{o}C in 1 minute, 72^{o}C in 1 minute, and finally kept at 72^{o}C in 5 minutes. The expression of *rolB and rolC* gene were compared to 10000 bp ladder after electrophoresis.

Determination of total Plumbagin

50 g powder of dry roots was extracted at room temperature with chroloform in Soxhlet machine for 48 hours. Extracted solution was evaporated to eliminate chloroform (acquired dry powder) (Jeyachandran *et al.*, 2009). 10 mg of this dry powder was dissolved in 5 ml acetonitrile and top up to final volume of 10 ml in 10 ml flask (stock solution). Different concentrations were prepared from stock solution and analyzed by HPLC. Samples were filtered by 0.45 µm syringe before loading. HPLC was run using reversed-phase column C-18, (250×4.6), 5 µm. The Solvent was acetonitrile (50 mM potassium dihydrogenphosphate: actonitrile with ratio (45:55)) at pH 3.5, 1.0 ml/minute flow rate. The system was operated at 26^{o}C. Injection volume was 20 µl. Plumbagin and samples were detected at 270 nm.

Using Plackett-Burmanmodel to choose the factors for growth and Plumbagin accumulation capacity in hairy roots

2 g of hairy roots was cultured in 60 ml MS liquid media at 24^{o}C and shook at 80 RPM for 30 days in dark condition.

Seven factors were chosen to conduct 12 experiments: coconut water (10-30%), chitosan (10-100 mg/L), salicylic acid (100-300 mg/L), sugar (10-50 g/L), yeast extract (100-1000 mg/L), casein (100-500 mg/L), peptone (100-500 mg/L) (Table 1). Low level (-1) and high level (+1) of elements established by Plackett-Burman'matrix (Plackett, Burman 1946; Dennis, 1995).

Table 1. Plackett-Burman matrix

Treatment								Fresh weight		Dry weight		Plumbagin concentration	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Experiment	Model	Experiment	Model	Experiment	Model
1	-1	-1	+1	-1	+1	+1	-1	4.82	4.78	0.54	0.56	1.184	1.154
2	+1	-1	-1	-1	+1	-1	+1	4.22	4.02	0.403	0.36	0.899	0.731
3	+1	-1	+1	+1	-1	+1	+1	4.41	4.78	0.415	0.47	0.956	0.591
4	+1	-1	+1	+1	+1	-1	-1	3.37	4.02	0.253	0.36	0.945	1.154
5	+	+	-1	-1	-1	+	-1	4.25	4.02	0.427	0.45	0.008	0.16

	1	1				1							9
6	-1	-1	-1	+1	-1	+1	+1	3.61	4.02	0.439	0.36	0.380	0.591
7	+1	+1	+1	-1	-1	-1	+1	5.14	4.78	0.557	0.45	0.221	0.169
8	-1	+1	-1	+1	+1	-1	+1	5.28	4.78	0.559	0.56	0.860	0.731
9	-1	-1	-1	-1	-1	-1	-1	4.4	4.78	0.423	0.47	0.351	0.731
10	+1	+1	-1	+1	+1	+1	-1	5.32	4.02	0.563	0.45	1.42	1.154
11	-1	+1	+1	-1	+1	+1	+1	4.62	4.78	0.585	0.56	0.353	0.591
12	-1	+1	+1	+1	-1	-1	-1	3.34	4.02	0.346	0.45	0.361	0.169

Four elements chosen based on level effects of them through Plackett-Burman's matrix, twenty five experiments from four elements established dependent on D-Optimal design (Table 2).

Table 2. Experiments, established dependent on D-Optimal design

Treatment	Components				Fresh weight		Dry weight		Plumbagin content	
	Coconut water	Chitosan	SA	Peptone	Experiment	Model	Experiment	Model	Experiment	Model
1	22.55	175	41.93	249.35	2.923	3.004	0.378	0.382	10.592	9.929
2	10	100	100	500	2.95	2.786	0.347	0.341	10.585	10.280
3	30	264.27	100	100	2.944	2.866	0.370	0.373	10.567	11.382
4	20.34	300	42.36	100	2.895	3.012	0.385	0.362	7.612	8.793
5	10	100	42.90	291.88	3.097	3.241	0.356	0.350	10.920	10.293
6	10	278.12	52.77	290.08	4.968	4.893	0.510	0.504	5.0553	5.735
7	10	300	10	500	2.692	2.747	0.428	0.435	6.709	7.456
8	30	100	84.46	100	2.519	2.738	0.336	0.349	7.971	6.913
9	30	100	100	425.96	2.660	2.583	0.375	0.354	14.703	14.558
10	20.34	300	42.36	100	3.097	3.122	0.294	0.313	9.346	8.287
11	30	196.39	42.80	500	2.568	2.531	0.339	0.350	12.456	12.099
12	10	300	100	100	3.06	2.939	0.45	0.431	10.347	10.422

13	19.66	100	10	500	2.985	2.956	0.344	0.393	13.683	11.861
14	30	100	100	425.96	3.32	3.038	0.381	0.367	9.922	10.773
15	30	196.39	42.80	500	2.243	2.247	0.280	0.268	8.327	7.608
16	30	300	100	500	3.662	3.806	0.467	0.472	10.03	11.055
17	17.38	100	11.41	100	3.291	3.311	0.385	0.355	6.863	7.720
18	10	202.60	10	100	2.977	2.966	0.356	0.375	6.777	7.387
19	30	300	10	310.31	2.848	2.939	0.396	0.403	9.185	7.603
20	30	100	10	100	2.921	2.843	0.384	0.385	8.662	10.300
21	30	300	10	310.31	2.828	2.939	0.429	0.431	11.59	10.423
22	10	300	91.56	500	2.580	2.786	0.335	0.341	10.67	10.73
23	10	100	100	500	3.316	3.012	0.34	0.362	8.27	8.19
24	17.79	222.28	100	344.21	2.895	3.038	0.352	0.367	11.14	10.38
25	13.84	100	100	100	3.058	2.956	0.439	0.393	10.60	11.91

Final equation in terms of actual factors: fresh weight Y_1 (g), dry weight Y_2 (g), Plumbagin content Y_3 (mg/g) described:

$$Y_{(n)}=b_o+b_1x_1+b_2x_2+b_3x_3+b_4x_4+b_{12}x_1x_2+b_{13}x_1x_3+b_{14}x_1x_4+b_{23}x_2x_3+b_{24}x_2x_4+b_{34}x_3x_4+b_{11}x_1^2+b_{22}x_2^2+b_{33}x_3^2+b_{44}x_4^2+b_{123}x_1x_2x_3+b_{134}x_1x_3x_4+b_{124}x_1x_2x_4+b_{234}x_2x_3x_4+b_{112}x_1^2x_2+b_{113}x_1^2x_3+b_{114}x_1^2x_4+b_{122}x_1x_2^2+b_{133}x_1x_3^2+b_{144}x_1x_4^2+b_{223}x_2^2x_3+b_{224}x_2^2x_4+b_{233}x_2x_3^2+b_{244}x_2x_4^2+b_{334}x_3^2x_4+b_{344}x_3x_4^2+b_{111}x_1^3+b_{222}x_2^3+b_{333}x_3^3+b_{444}x_4^3$$

In which, b_1, b_2, b_3, b_4; b_{11}, b_{22}, b_{33}, b_{44}; b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, b_{34}, b_{123}, b_{134}, b_{124}, b_{234},b_{112}, b_{113}, b_{114}, b_{122}, b_{133}, b_{144}, b_{223}, b_{224}, b_{233}, b_{244}, b_{334}, b_{344},b_{111}, b_{222}, b_{333},$b_{444}$are system numbers; x_1, x_2, x_3, x_4, x_1x_2, x_1x_3, x_1x_4, x_2x_3, x_2x_4, x_3x_4, x_1^2, x_2^2, x_3^2, x_4^2, $x_1x_2x_3$, $x_1x_3x_4$, $x_1x_2x_4$, $x_2x_3x_4$, $x_1^2x_2$, $x_1^2x_3$, $x_1^2x_4$, $x_1x_2^2$, $x_1x_3^2$, $x_1x_4^2$, $x_2^2x_3$, $x_2^2x_4$, $x_2x_3^2$, $x_2x_4^2$, $x_3^2x_4$, $x_3x_4^2$, x_1^3, x_2^3, x_3^3, $x_4^3$are variables numbers.

Statistics

Results were analysed by Design expert 7.0.0® program of Stat-Ease Inc, USA. From the results, the optimal conditions were selected based on the fresh weight, dry weight and Plumbagin accumulation capacity.

RESULTS AND DISCUSSION

Induction of hairy roots

Based on observation (Figure 1), roots with fast growth rate compared with the control group were proposed to be genetically modified and therefore selected for further experiment (Figure 2). Analyzing DNA of the root samples by PCR reaction with *rolB* and *rolC* primers showed that four samples (P_1, P_2, P_3, P_4) had *rolB* gene expression and one (P_4) with *rolC* gene expression (Figure 3 and 4). The simple P_4 was therefore chosen to investigate on the growth rate and Plumbagin accumulation capacity of its hairy roots.

Figure 1: Hairy roots under microscope

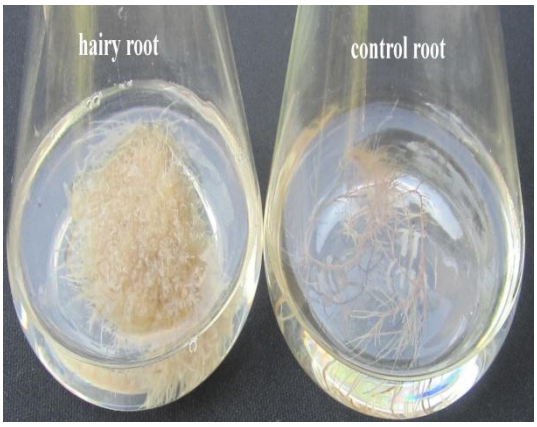

Figure 2: Hairy roots cultured in liquid media after 14 days cultured with bacteriaafter 30 days

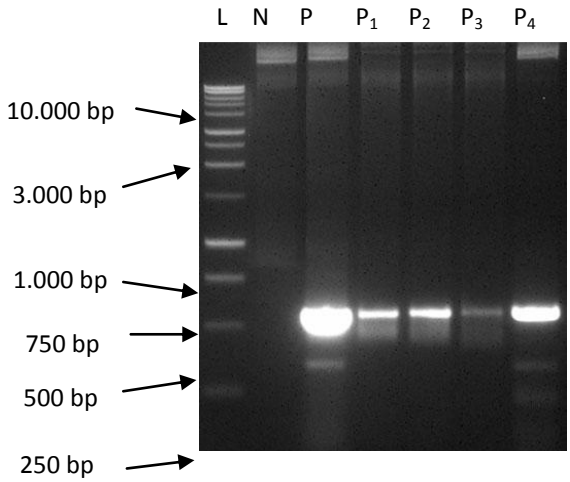

Figure 3: PCR gene *rolB*

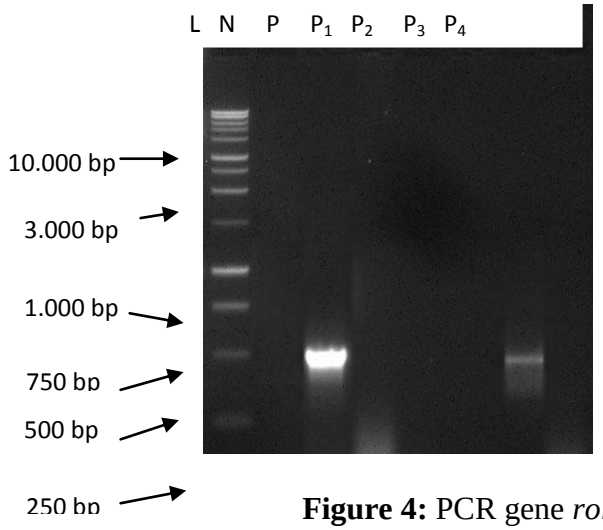

Figure 4: PCR gene *rolC*

Hairy roots cultured in liquid media were collected and quantified accumulated Plumbagin. Based on the standard, Plumbagin was indentified (Figure 5). Following the chromatography, Plumbagin was detected at 7.078 minute. The result was confirmed by Gopinath *et al*. (2009).

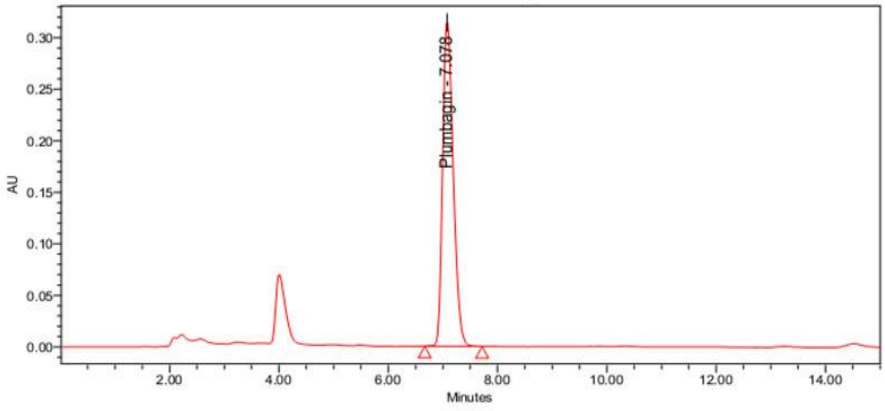

Figure 5: Plumbagin chromatography by HPLC

Selection of important factors on the growth and Plumbagin accumulation capacity of *PlumbagoZeylanica*L. hairy roots

Plackett-Burman matrix indicated 4.02-4.78 g of fresh weight, 0.36-0.56 g of dry weight and 0.169-1.156 mg of accumulated Plumbagin in 60 ml cultured media (Table 1). The Effects of each factor on the fresh weight, dry weight and Plumbagin accumulation capacity were calculated by Design expert® 7.0.0 program (Table 1). Four elements effect to dry weight, fresh weight and Plumbagin content detected by $p < 0.05$: coconut water, Chitosan, salicylic acid and peptone. In which, coconut water has the highest influence of dry weight and fresh weight; peptone and Chitosan effect to Plumbagin accumulation.

Table 3. Effect of coconut water, Chitosan, salicylic acid (SA) and peptone dry weight, fresh weight and Plumbagin content of hairy root.

Factors	values		Level affect		
			Fresh weight	Dry weight	Plumbagin content
Coconut water	10	30	0.76	0.108	-0.01401
peptone	100	500	0.34	-0.00467	0.56296
Yeast extract	100	1000	0.276667	0.020333	0.005491
Chitosan	100	300	-0.08	0.013333	0.422368
Casein	100	500	0.01	0.003333	-0.11887
SA	10	100	0.066667	0.009333	0.106667
Sugar	10	50	-0.41333	-0.08833	-0.07184

Optimizing value factors for the growth and accumulation of Plumbagin capacity of *PlumbagoZeylanica*L. hairy roots

Using Design expert® 7.0.0. Software to establish a response surface method - D Optimal for four elements chosen from seven elements survey. Analysis ANOVA and final equation in terms of actual factors used as the model to detect affect of elements (Table 4).

Table 4. Establish response surface –D Optimal.

Parameter	Fresh weight	Dry weight	Plumbagin content
R^2 (R-Squared)	0.926556	0.864337	0.993905
C.V%	7.851963	6.98845	10.50962
Final equation in terms of actual factors	$Y_1 = 3.278 – 0.352x_1 - 0.536x_2 - 0.144x_3 + 0.426x_1x_2 + 0.439x_1x_3 – 0.983x_1x_4 - 0.526x_2x_3 + 0.240x_3x_4 + 0.386x_1^2 – 1.310x_2^2 - 0.714x_3^2 + 1.265x_4^2 - 0.813x_1x_2x_3 + 0.266x_1x_2x_4 + 1.156x_1x_3x_4 + 0.669x_1^2x_2$	$Y_2 = 0.346 – 0.031x_2 + 0.036x1x_3 - 0.044x1x_4 - 0.038x_2x_3 + 0.025x_3x_4 – 0.068x_3^2 + 0.1x_4^2 - 0.029x_1x_2x_3 + 0.023x_1x_2x_4 + 0.048x_1x_3x_4 + 0.065x_1^2x_2$	$Y_3 = 7.03 + 1.31x_1 - 0.75x_3 - 0.97x_1x_2 + 1.21x_1x_3 + 0.95x_2x_3 – 1.41x_2x_4 – 0.90x_3x_4 + 2.22x_2^2 + 1.48x_3^2 – 2.00x_1x_3x_4 - 0.73x_1^2x_2$
x_1, x_2, x_3, $x_4$for coconut water (10-30%), Chitosan (100-300mg/L), salicylic acid (10-100 mg/L) and peptone (100-500 g/L) respectively			

Response surface shows relation with the elements and detect primary parameters to receive the best response surface (Figure 6). From the actual results, response surface - D Optimal model predicted Optimizing value factors for the growth and accumulation of Plumbagin are coconut water: 14.33%, chitosan 100 mg/l, salicylic acid 19.40 mg/l and peptone 500 g/l, corresponding fresh weight: 4.968 g, dry weight: 0.451 g, and Plumbagin content:11.135 mg

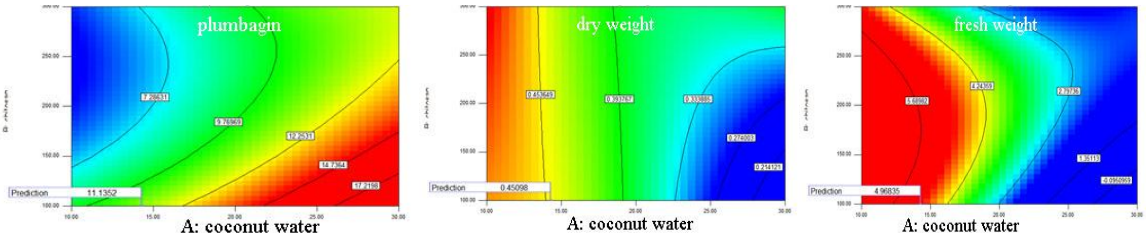

Figure 6.response surface of coconut water and chitosan

Results showed the great effect of factors on the growth and plumbagin accumulation capacity of *Plumbago zeylanica* L. hairy roots. This results were supported by another research of Putalun *et al*. (2010) and chitosan and salicylic acid were used to increase the plumbagin accumulation capacity of *Drosera burmanii* (Naha´lka *et al*., 1998).

CONCLUSION

*PlumbagoZeylanica*L., was used as a medicinal plants for various treatments. There are many studies about *in vitro* propagation and genetically modified hairy roots of *Plumbago zeylanica*L. This study provides the information on some important factors and conditions to achieve optimal growth and Plumbagin accumulation capacity in the hairy roots.

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