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Full Length Research Paper

***In vitro* regeneration of *Neocarya macrophylla* (Sabine) Prance, wild fruit of Niger**

Aboubacar Kolafane* and Sidikou Djermakoye Seyni Ramatou

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Neocarya macrophylla is a spontaneous fruit species that constitutes the dominant population of Dallol Bosso in Southwestern Niger. In order to test the *in vitro* regeneration ability of this species, micro-propagation was applied to micro-cuttings from young almond seedlings. The Murashige and Skoog basal medium was enriched with several concentrations of naphthaleneacetic acid (NAA; 0, 0.5 and 1 mgL⁻¹) and 6-benzylamino purine (BAP; 0, 0.5, 1, 1.5, 2, 2.5 and 3 mgL⁻¹). The first results show that it is possible to obtain *in vitro*-plants by vegetative propagation *in vitro* even at low doses. Thus, in this test, the overall recovery rate was 67.30%, the lag time was 11 days, and the recovery time was spread over 4 days. The statistical analysis at 5% significance threshold showed that there is no significant difference between the different treatments during the emergence of the buds and the neof ormation of the leaves, but at the level of the elongation of the stems, the addition of these growth regulators has significantly impacted the results.

Key words: *In vitro* regeneration, *Neocarya macrophylla*, micro-cuttings, growth regulators, Niger.

INTRODUCTION

Neocarya macrophylla (Sabine) Prance is a wild fruit tree of the Chrysobalanaceae family (Arbonnier, 2009). This woody tree, formerly known as *Parinari macrophylla* of the Rosaceae family, extends in Africa from the Guineo-Congolese region to the Sudano-Zambeian region, especially in the coastal savannahs of Senegal, Liberia, in the woodland of Southern Mali, Southern Niger and North Nigeria. The species grows on Sudanian and Guinean fringes, on forest and lowland areas and on sandy soils (Berhaut, 1967).

In Niger, its range extends the length of the Dallol

Bosso to Fandou Beri and Bonkoukou, on the Dallol Maouri and Foga rivers, on the grounds of Goulbin Maradi, in the Goulbin Kaba to Kanan Bakaché and in the Korama (Dungas, Matameye, Magaria, and East DOUNGOU) (Saadou, 1990).

More abundant in the Dallol Bosso Valley, this species keeps its green leaves especially all year (Sidikou, 1996, 1999). It is used in cooking, traditional pharmacopoeia, pagan religious practices, provides a useful fire wood and offers many services to the populations through the persistent shading it provides.

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It is a tree that produces fruits locally known as Gamsa, consumed and whose trade brings a substantial income to the local population (Balla et al., 2008) and possesses oleaginous properties (Tijani et al., 2010).

The production rate of the fruit estimated at 390.96 kg/tree in 2009 in Niger (Dan Guimbo et al., 2012) remains below the demand.

Although the regeneration of the tree is very important, this species is under increasing pressure, what explains the noticeable decrease of its aging population. This species is threatened as extinction (Saadou, 1990).

Despite its socio-economic importance, very few studies have been conducted on this species, hence the need for assisted regeneration, in order to avoid its very near extinction and perhaps to develop it. This regeneration is only possible with *in vitro* culture. Indeed, today, with plant biotechnology, it is possible to obtain healthy, greenhouse-transferable plants for the industrial production of seedlings of higher health level and high genetic homogeneity.

It is with this in mind that a micropropagation test of this species is proposed to vary the propagation modes and to plan a reintroduction of the same species so as to green the Dallol Bosso with *N. macrophylla* and increase fruit production and local income by producing thousands of seedlings.

The objective of this study, which is an introduction to the *in vitro* culture of *N. macrophylla*, is essentially aimed at testing the ability of the cuttings from young seedlings of this species to an assisted regeneration using micropropagation method.

MATERIALS AND METHODS

Source of transplants

The transplants used in these trials were cuttings of young *N. macrophylla* almond seedlings at seven weeks old, measuring of about 15 cm long and ± 5 leaves (nodes). On each seedling, two thirds of the upper parts were used. The almonds are from the 2016 harvest provided by the National Forest Seed Center (CNSF).

Preparation of the culture medium

Distilled water (100 ml) was poured into a vial with Murashige and Skoog (1962) medium. The powder of the stock solutions was successively added in addition to sucrose (30 g) and the growth regulators to supplement the volume of distilled water at 1 litre and to adjust the pH at 5.7 with HCl or NaOH (0.1 N). Agar (7 g with stirring and heating) was added and the solution dispensed into test tubes (25 ml/tube) and was sterilized.

Aseptic technique

All equipment was disinfected before use:

(1) The culture medium was sterilized in the autoclave at 121°C for

20 min at a pressure of one bar;

(2) The technical material (tongs, scalpels, etc.) was sterilised in an oven at 200°C for minimum of 2 h;

(3) The plant material was sterilised by taking the non-sterile plant material, washing it with water, then with soap and then rinsing it with distilled water. The micro-clippings were then transferred under a hood to be soaked in alcohol (70°) for 30 s and then in bleach (8°) for 10 min and then underwent three rinses with sterile distilled water for 15, 10 and 5 min, respectively.

Implementation

Under the horizontal laminar flow hood and in the vicinity of a ball sterilizer, the disinfected micro-clippings were cut into several fragments on blotting paper and planted in the culture medium using a sterile forceps. About 1.5 cm of explant carrying a single node endowed with a bud was placed in each tube and subsequently closed hermetically and was labelled. The tube was then placed in a culture chamber.

The emergence of the buds and the development of young seedlings took place in the culture chamber set at a temperature of $25 \pm 2^\circ\text{C}$, with a photoperiod of 13/11-h under a light intensity of 3000 Lux.

Experimental apparatus

The experimental device consists of various combinations of concentrations of naphthaleneacetic acid (NAA; 0, 0.5 and 1 mgL^{-1}) and 6-benzylamino purine (BAP; 0, 0.5, 1, 1.5, 2, 2.5 and 3 mgL^{-1}) distributed randomly and conducted in four repetitions. Each repetition was represented by a cutting that carried a bud. For each medium combination, 4 transplants were transplanted.

The MS medium served as a control and, from it, two basal media were defined; in addition to the MS control T0, each medium consisted of six treatments (Table 1):

MS1 = MS supplemented with 0.5 mgL^{-1} of NAA and 0.5, 1, 1.5, 2, 2.5 and 3 mgL^{-1} of BAP.

MS2 = MS supplemented with 1 mgL^{-1} of NAA and 0.5, 1, 1.5, 2, 2.5 and 3 mgL^{-1} of BAP.

Parameters

The selected parameters evaluated are:

(1) The emergence of the bud which was characterized by: (i) the recovery time or latency time, that is, the time that elapses between the sowing of the micro-clipping and the appearance of the first bud; (ii) the duration of recovery or time that elapsed between the appearance of the first bud and that of the last bud; (iii) the recovery rate, which is the ratio between the number of micro-clippings giving buds and the total number of micro-cuttings transplanted;

(2) The elongation of the stem;

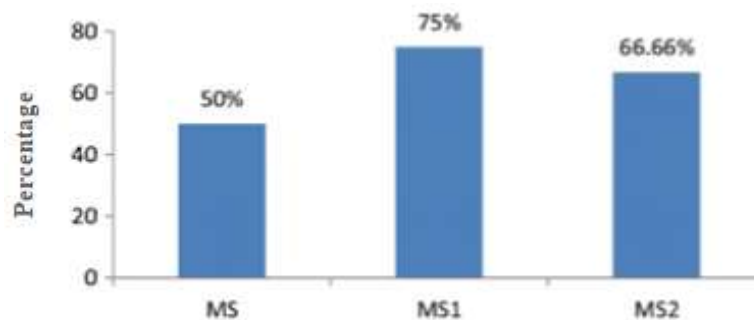
(3) The number of leaves per stem.

Statistical analysis

In order to highlight the effect of hormones on bud recovery and identify the best combination for elongation of stems and leaf formation, statistical analysis of these results was performed using the XL Stat 7.1 software. The test of Newman and Keuls, at the threshold of 5% significance was used to classify the averages.

Table 1. Codification of the different treatments.

Medium	Code	NAA (mgL ⁻¹)	BAP (mgL ⁻¹)
MS	T0	0	0
	T1		0.5
	T2		1
	T3		1.5
	T4		2
	T5		2.5
MS1	T6	0.5	3
	T1'		0.5
	T2'		1
	T3'		1.5
	T4'		2
	T5'		2.5
MS2	T6'	1	3

**Figure 1.** Percentage of the emergence of the bud by culture medium.

RESULTS AND DISCUSSION

At the end of this experiment which was done six weeks at *N. macrophylla*, the following results were recorded.

The emergence of the bud

The recovery time for the emergence of the bud (Figure 1 and Photo 1) took place 11 days after the culture of micro-clippings for all the treatments. This recovery was spread over 4 days. This positive response can be explained by the age of the mother plants used which is young and therefore more reactive.

In a similar study with *Maerua crassifolia*, Diata (2008) noted a lag time of 12 days. Indeed, Gitonga et al. (2008) have shown that the age of transplants has an influence on their performance.

The addition of growth hormones in the culture medium has greatly favoured the emergence of the bud.

**Photo 1.** Budding occurred after 11 days.

The overall recovery rate for micro-clipping is 67.30%. The percentage of success of this regeneration varied

Table 2. Newman-Keuls (SNK)/Analysis of differences between groups with a 95.00% confidence interval.

Medium	Difference	Reduced difference	Critical value	Pr.> Diff	Significant
MS1 ~ MS	0.210	1.176	2.668	0.489	No
MS1 ~ MS2	0.118	0.734	-	-	No
MS2 ~ MS	0.092	0.533	-	-	No

Table 3. Classification and grouping of non-significantly different groups.

Medium	Average	Group
MS1	0.435	A
MS2	0.317	A
MS0	0.225	A

according to the combinations of phytohormones used. Control MS gave 50% response. MS1 gave a more strong response (75%) while MS2 gave a 66.66% response.

These results thus inform us on one hand the quality of the plant material and on the other hand the action of the doses of hormones on the growth of the vitro-plants.

Houédjissin et al. (2015) pointed out that the difference of the recovery and development of the apex of *Pentadesma butyracea* observed between the culture medium used (B5, MS and WPM) would certainly result from the difference in their mineral composition especially the K^+ , NO_3^- and NH_4^{4+} ions.

Also, Zuraida et al. (2015) obtained a rate of 80% over six weeks by adding to the MS medium 3 mgL^{-1} of BAP with micro-clippings of *Plectranthus amboinicus*.

Ndoye et al. (2003) by studying micro-cuttings of *Balanites aegyptiaca* with a combination of 1 mgL^{-1} of BAP and 0.1 mgL^{-1} NAA after 4 weeks recorded 100% recovery.

These results show that one species-friendly medium may not be suitable for another species.

The average elongation of stems

Mean elongation of stems according to auxin dose

Statistical results on the effect of dose of auxin on the elongation of stems are shown in Tables 2 and 3.

These two tables show that the responses obtained are not significantly different; the probability (0.48) being greater than 0.05. The applied dose of auxin therefore does not significantly affect the emergence of the bud. All three media are classified in the same group A with the leading MS1 medium that has an average of 0.435.

Mean elongation of stems according to the combination of auxin and cytokinin

The resumption of vegetative activity characterized by the

elongation of the stem is as shown in Figures 2 and 3.

This height improved significantly in the MS1 medium and the seedlings reached an average size of 0.9 cm in the same time period. The emergence of the stem axis was not observed in all germinated cuttings, in fact, the micro-cuttings of the T5 treatment experienced necrosis just after their emergence.

The behaviour of the transplants was favourable in the presence of the growth regulators as in their absence. However, in the control (T0), the average height of the seedlings did not exceed 0.2 cm after 42 days of growth culture.

From the 37th day, all micro seedlings ceased growing; those of the T4 treatment even experienced a regression. Experimentally, the best result was observed with the T3 treatment where some stems reached up to 2 cm in length. The analysis of curves on MS2 shows that the addition of growth regulators has also had a positive effect on the elongation of the stems. This is relatively higher with the combination T2' which gave an average size of 0.8 cm.

As in the MS1 medium, the MS2 from the 37th day revealed that all growth in the length of the stem remained stationary for all the treatments except for T1' which experienced a slight increase. T3 seedlings suffered necrosis just after emergence of the bud. The maximum length (Photo 2) obtained during this test is 1.8 cm with the combination T4'.

The Newman and Keuls test of the influence of hormonal combinations on the elongation of the stem classifies the treatments into four groups (A, B, BC and C).

Although the elongation of stems was very low in all the tests; the addition of growth regulators had a positive influence, thus there are significant differences in stem growth according to the combination applied and the most significant is the T3 combination. The averages between 0.717 and 0.608 belonging to treatments T3, T4', T4, T2 and T2' are classified in group A with T3 in the lead. Treatments T6', T1 and T6 are classified in group

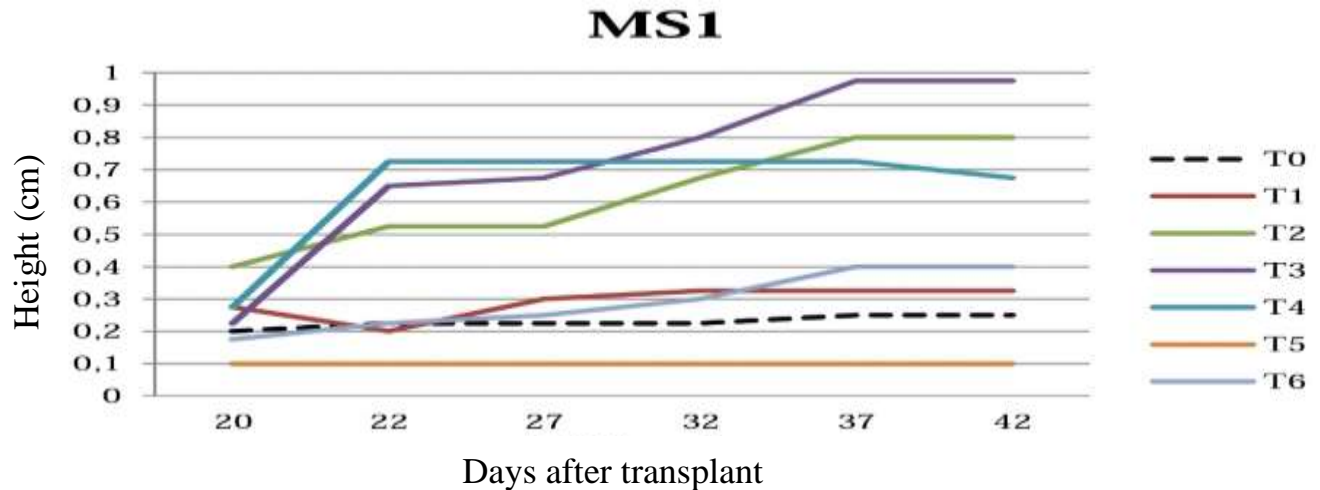


Figure 2. Average elongation of stems on MS1 medium (Effect of doses (0 and 0.5 mg.L⁻¹) of NAA and (0, 0.5, 1, 1.5, 2, 2.5, and 3 mg.L⁻¹) of BAP).

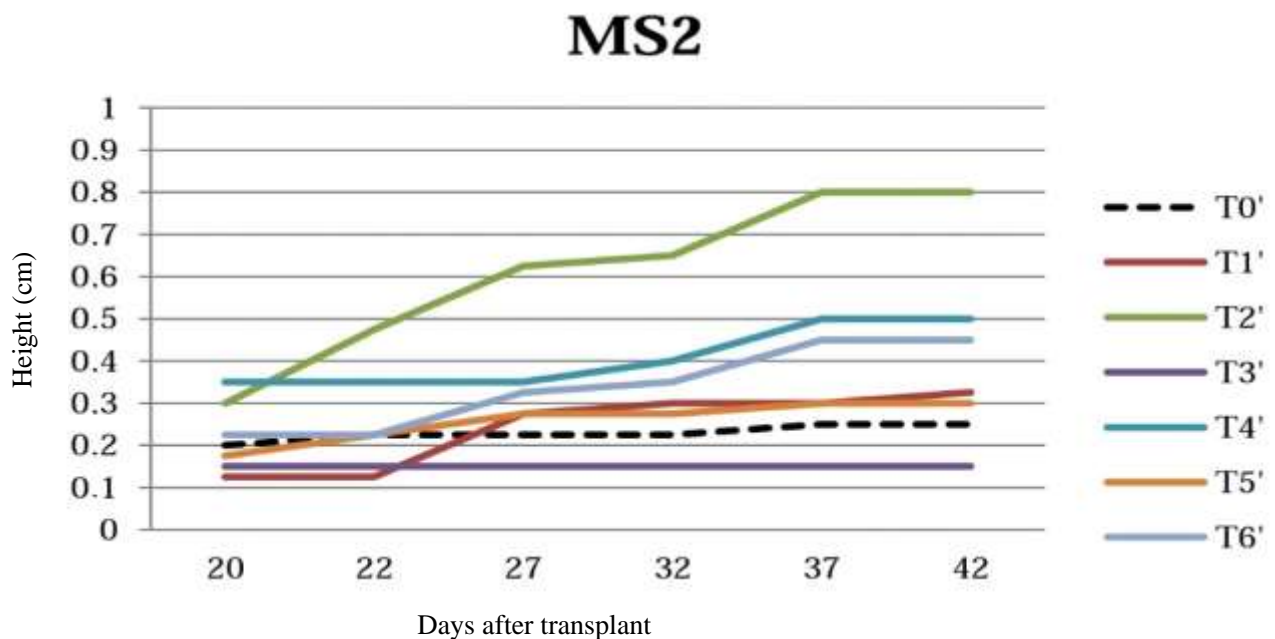


Figure 3. Mean elongation of stems on MS2 medium (Effect doses (0 and 1 mg.L⁻¹) of ANNA and (0, 0.5, 1, 1.5, 2, 2.5 and 3 mg.L⁻¹) of BAP).

B with averages of 0.338 to 0.292. The B and C groups with averages ranging from 0.263 to 0.150 includes the treatments T5', T1', T0 and T3', respectively. The last T5 treatment is placed in the group C.

However, Ndoye et al. (2003) with *B. aegyptiaca* after 4 weeks was found to be 2.29 cm in length with a combination of 2.5 mg.L⁻¹ of BAP and 0.1 mg.L⁻¹ of NAA in MS medium. For Rancillac and Lafargue (1981), benzyladenine was more effective than kinetin in inducing

caulogenesis. A concentration between 2 and 4 mg.L⁻¹ is the most favourable at the sea level. Beyond 30 days, growth in length of shoots stopped for the majority of treatments (except for T4 and T1') which justifies the need to change the medium after one month in culture as nutrients are exhausted.

Le Bellec (2017) attests that the first transplanting which takes place systematically 4 weeks after cultivation makes it possible to renew the culture medium which

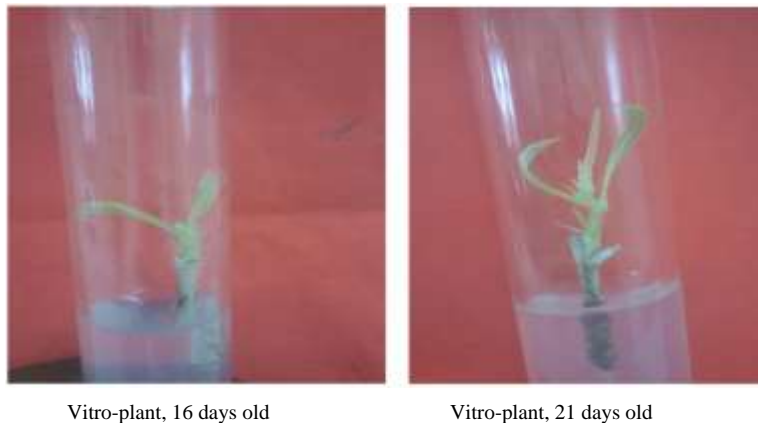


Photo 2. Height of the vitro-plants of *Neocarya macrophylla*.

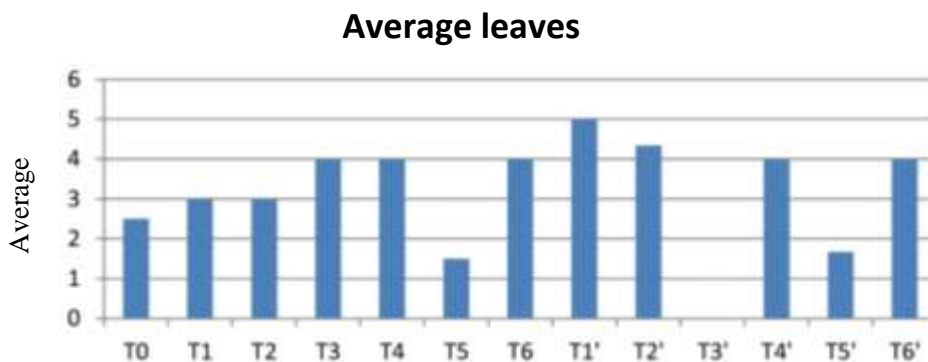


Figure 4. Medium shade of new leaves formed by treatment.

begins to be exhausted and which furthermore sometimes contain inhibiting substances released by the transplant itself during the growth of the bud.

The necrosis observed at T5 level are mainly due to lesions caused by sterilizing agents and transfer stress. The low percentage of T3' could also be the result of this stress.

The middle shade of new leaves formed by stem

After six weeks of culture, the results (Figure 4) show that all combinations are favourable to leaf formation except for one; therefore, the addition of cytokinin (BAP) in the culture medium significantly improved leaf formation by the vitro-plants.

Figure 4 shows that the responses to leaf formation differ according to the hormonal combinations.

The average number of leaves recorded in the control (T0) is 2.5. This number has constantly evolved from 3 to 5 leaves on average in MS1 and MS2 media except for

T5, T3' and T5' treatments (where the minimum number of leaves has been noted). It was higher with T1' treatment (5 leaves on average).

Treatments T3, T4, T6, T2', T4' and T6' all gave almost the same number of leaves (4 on average). This constancy may be related to the purity of the species; micro-plants do not absorb more than their needs for nutrition.

Overall, the MS1 medium was more consistent and more favourable to leaf formation. From 0.5 to 1 mgL⁻¹, the seedlings give 3 leaves on average; but with up to 3 mgL⁻¹ they give up to 4 leaves on average per micro-plant after 42 days.

These results are similar to those found by Adane (2013), with meristems of the Giant Cavendish plantain (*Musa species*) in a combination of 5 mg.L⁻¹ of BAP and 0.5 mg.L⁻¹ of NAA recording a greater mean leaf length of 4.33 after 60 days.

Gbadamosi and Sulaiman (2012) noted for their part up to 6 leaves on average at *Irvingia gabonensis* by combining 0.05 mgL⁻¹ BAP, 0.05 mgL⁻¹ kinetin (KIN), 0.05

Table 4. Analysis of variance.

Source variations	Sum squares	Degree of freedom	Average squares	F	Probability	Value critical for F
Between groups	38.91666667	12	3.24305556	0.756152	0.68335204	2.475312973
Within groups	64.33333333	15	4.28888889	-	-	-
Total	103.25	27	-	-	-	-

**Photo 3.** Development of bud 21-day old leafy plants.

mgL⁻¹ AIB and 10.0% coconut water in ¼ MS. Analysis of variance of the number of sheets is shown in Table 4.

The analysis of variance on the average number of leaves issued by the stems gave a probability of 0.68; this being greater than 0.05, the effect of cytokinin on leaf formation is not significant in this case. Photo 3 shows the T3 treatment leaves.

Overall, the T3 combination appears the best of all and the most economical *in vitro* for regeneration of micro-clippings of this species; a concentration of 0.5 mgL⁻¹ NAA and 1.5 mgL⁻¹ BAP is sufficient to induce the formation of stems with leaves at the buds in *N. macrophylla*. In this trial, callus formation or root formation was not observed.

Conclusion

This study shows that the regeneration of *N. macrophylla* is surely possible using the *in vitro* culture technique, because it has successfully produced *in vitro*-plants from micro-clippings taken from young seedlings. In this work,

challenge was encountered when all the technical advantages of *in vitro* culture for the successful production of plants, which could be used for its propagation and domestication are put at the service of this species. Thus, efforts have been focused on the culture medium to propose the best combinations in terms of hormones.

The basal medium is Murashige and Skoog, the hormones used are NAA (auxin) and BAP (cytokinin). These tests led to the conclusion that:

- (1) Regeneration *in vitro* of *N. macrophylla* can be done with young cuttings;
- (2) The addition of hormones has significantly improved bud recovery capacity (up to 75% for MS1);
- (3) Stem elongation and leaf formation increased considerably with the addition of cytokinin in the medium.

For the remaining of this preliminary work and the success of micro-propagation *in vitro* of *N. macrophylla*, it is necessary to extend research to other combinations, which would favour rhizogenesis, because this phase will depend on the acclimatization and consequently the

success of the propagation of this plant which is heavily exploited in the Dallol Bosso region.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Biomangement of Rose and carnation wastes in flower industries with three epigeic earthworm species: *Eisenia fetida*, *Eisenia andrie* and *Dendrobanae veneta*

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The horticulture sector in Ethiopia is being challenged by multifaceted hitches, among which managing excess wastes produced from the farms and minimizing the cost of inorganic fertilizers are the major ones. The performance of three epigeic earthworm species, *Eisenia fetida*, *Eisenia andrie* and *Dendrobanae veneta*, in managing flower (Rose and Carnation) wastes through vermicomposting was evaluated. The study was done using wastes collected from two flower farms, Dugda Flora (Debre Zeit) and Ethiopian Magical Farm (Legedadi). The wastes were pre-composted by mixing with cow dung and arranged in piles with 80 cm height and 1.5 m width under different treatments. The worms were introduced into the piles when the temperature and moisture of the piles was lowered to a level suitable for the worms. The physico-chemical variables of the vermicompost and size reduction of the wastes were evaluated at the beginning, middle and conclusion of the experiment. It took 3 months for all Rose wastes to be converted into vermicompost and 6 months for Carnation wastes. There could be multifaceted reason for the delay of Carnation, but the recalcitrant nature of the plant seems to be the main reason. Size reduction ranged from 49.6 to 87.5%, Total Nitrogen (TN) (1.43 to 2.5%), Available Phosphorus (P_2O_5) (1879 to 2600 ppm), Available Potassium (AV.K) (73.3 to 105.5 c mol(+)/kg), Carbon to Nitrogen ratio (C:N) (12:1 to 28:1) for Rose while TN ranged from 1.6 to 2.3%, P_2O_5 from 1867 to 2112 ppm, AV.K from 73.3 to 103 c mol(+)/kg, and C:N from 14.4:1 to 25:1 for carnation during the same study period. There was no significant variation in terms of the quality of vermicompost produced by the worms and between the waste types. The overall results showed that all the three worms can be employed to manage both types of wastes.

Key words: Earthworms, *Eisenia fetida*, *Eisenia andrie*, *Dendrobaena veneta*.

INTRODUCTION

Ethiopia ranked second in Africa in exporting cut flowers (Getu, 2009). The horticulture sector is growing rapidly in the country in the last two decades and hugely contributing to the Gross Domestic Production (GDP) of the country. It is also providing job opportunity to more than hundred thousands of Ethiopians, mainly females,

throughout the country. However, the sector is currently being challenged by multifaceted hitches problems; among which is the management of excess wastes produced from the farms and minimizing the cost of inorganic fertilizers which are the major ones. Thus, managing these wastes at low capital, in eco-friendly,

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and environmentally harmonious ways has become the main issue of these farms recently (Manaf et al., 2009). There are over 100 flower growers on 1700 ha (Bogale, 2017) in the country and are producing, with rough estimation, between 500 to 3000 kg dry flower waste per day. Furthermore, they are expending at least 1USD per m² to buy inorganic fertilizers (Unpublished data). Such figures show how much the sector is being challenged in the country. Thus, managing these wastes at low capital, in eco-friendly, and environmentally harmonious ways along with producing biofertilizers have become the main issue of these farms recently (Manaf et al., 2009).

Nowadays, vermicomposting is being considered as an important means for managing solid wastes that have been applied in various parts of the world. This method is highly advantageous in decreasing the need for landfill space alongside successfully diminishing the volume of wastes. Concurrently, this technology produces excellent biofertilizer (vermicompost) which has an important role in sustainable agriculture (Sharma et al., 2005).

The main actors in vermicomposting process are earthworms and microorganisms. Earthworms consume biomass and excrete it in digested form called worm casts which are rich in nutrients, growth promoting substances, and having properties of inhibiting pathogenic microbes (Maheswari and Ilakkiya, 2015). Earthworms have physical and biochemical role in the vermicomposting process (Aalok et al., 2009); they grind the waste, aerate the substrate and the biochemical process produce vermicompost (Frederickson et al., 1998).

Hitherto, earthworms have been employed and were able to successfully manage wastes such as garden waste (Dickerson, 2001), sludge and fibers (Garg et al., 2006), agricultural and domestic wastes (Handreck, 1986). These worms were also able to completely degrade vegetable waste, coffee husk and 'Khat' (Degefe et al., 2016). However, the performance of these worms in managing wastes of cut flowers has never been evaluated locally as well as internationally. In this study, the appropriateness and efficiency of three earthworm species; *Eisenia fetida*, *Eisenia andrei* and *Dendrobaena veneta* was investigated in managing wastes of Rose and Carnation flowers. These worms were preferred for vermicompost because they are resilient earthworms that can be readily handled and tolerate wider moisture and temperature ranges (Dominguez and Edwards, 2011). It was hypothesized that these worms can effectively manage cut flower wastes and produce good quality of soil amendments.

MATERIALS AND METHODS

Experimental design

Wastes were collected from the farms; Dugda flora (Debre Zeit) and Ethiopian Magical Flora (Legedadi). The wastes were chopped,

mixed with cow dung in 3:1 ratio and 24 piles with eight treatments (in triplicate) and were established separately for each type of wastes. Each pile had 1.5 m width and 0.8 m height (approximately 40 kg). The piles were pre-composted so as to stabilize the substrate in terms of pH, temperature and moisture following the procedures of Azizi et al. (2014) and watered considering the optimum moisture level (70%) at frequent interval until the day of worm introduction. The three worms; *E. fetida*, *E. andrei* and *D. veneta* were introduced to the piles, except in the controls, when the temperature lowered (< 27°C). Introduction of worms considered optimum feeding rate of 0.75 kg feed/kg worm/day (Ndegwa et al., 2000). The synergetic effect of efficient microorganisms (EM) with the worms in managing these wastes was also assessed by inoculating them in the piles and measuring the changes in physico-chemical parameters. After the introduction of worms, the treatments were labelled as T1 (*E. andrei*), T2 (*D. veneta*), T3 (*E. fetida*), T4 (Control), T5 (Control + EM), T6 (*E. andrei* + EM), T7 (*D. veneta* + EM) and T8 (*E. fetida* + EM). The experimental setup was similar for both types of waste.

Physico-chemical analysis

The height of each pile was measured at the end of the experiment (at Dugda Farm) and compared with the initial so as to observe the efficiency of the worms in reducing the height of waste piles. Initial samples were taken from each treatment piles before introduction of the worm and at the end of the experiment sent to laboratory for physico-chemical analysis. Moisture level was determined using AOAC Official Method 923.10 while pH was determined using FAO potentiometric–water extract procedure. Total nitrogen was determined using Kjeldahl method (Bremner and Mulvaney, 1982), available phosphorous determined using Olson (1963) and available potassium determined using ammonium acetate extract (Garg et al., 2005). TOC was measured after igniting the sample in a Muffle furnace at 550°C for 50 min by the method of Nelson and Sommers (1982).

Statistical analysis

Data were analyzed using SPSS software 15 version. Analysis of variance was used to analyze the significance in variation in the physico-chemical parameters between the treatments in each site.

RESULTS AND DISCUSSION

The worms in both (Rose and Carnation) treatment piles, were able to survive and successfully multiply; however, the rate of vermicomposting process was not uniform. The size of the piles was more reduced at Rose wastes (Tables 1 and 2) than at Carnation. The overall vermicomposting process took three months at Rose waste while it took nearly six month for Carnation waste. The change in height of the piles at the end of the experiment is presented in Tables 1 and 2. The reduction ranged from 78.75 to 87.5% in the piles where worms were introduced, whereas it was 49.25% for the control and 51.25% for control with EM. The highest reduction was observed in the pile with *D. veneta* worm with EM, although there was no significant variation statistically ($P>0.05$), and the lowest in the control. The overall result

Table 1. Height reduction of the piles during the vermicomposting process of Rose waste.

Treatment	Initial Height (cm)	Final height T1 (cm)	Final height T2 (cm)	Final height T3 (cm)	Average height (cm)	Reduction (%)
<i>E. andrie</i>	80	15	16	16	15.6	80.5
<i>D. veneta</i>	80	10	11	10	10.3	87.1
<i>E. fetida</i>	80	16	16	17	16.3	79.5
Control	80	38	41	43	40.6	49.25
Control + EM	80	41	40	36	39	51.25
<i>E. andrie</i> + EM	80	19	17	18	17	78.75
<i>D. veneta</i> + EM	80	10	10	10	10	87.5
<i>E. fetida</i> + EM	80	15	17	14	15.3	80.3

Table 2. Height reduction of the piles during the vermicomposting process of Carnation waste.

Treatment	Initial Height (cm)	Final height T1 (cm)	Final height T2 (cm)	Final height T3 (cm)	Average height (cm)	Reduction (%)
<i>E. andrie</i>	80	25	26	22	24.3	69.2
<i>D. veneta</i>	80	28	26	27	26	67.5
<i>E. fetida</i>	80	21	23	22	22	72.5
Control	80	45	47	44	45.3	43.4
Control + EM	80	44	48	40	44	45
<i>E. andrie</i> + EM	80	23	20	27	23.3	70.8
<i>D. veneta</i> + EM	80	29	29	26	28	65
<i>E. fetida</i> + EM	80	26	25	24	25	68.7

showed that the worms were able to reduce the size of the pile highly in Rose waste. Unlike in Rose waste, the height reduction of waste was lower for Carnation during the vermicomposting process. The reduction ranged from 65 to 72% for the piles with the worms. The variation in reduction was not statistically significant ($P>0.05$) among the treatments.

Change in physico-chemical parameters during the vermicomposting process for the Rose and Carnation waste

The physico-chemical analysis showed that there was significant increment of TN, P_2O_5 and AV.K while C:N ratio reduced in all the treatments. Nitrogen level was slightly higher in T3 and T6 which implied that *E. fetida* can contribute more N than other worms particularly when synergistically used with EM (Figure 1); however, the variation was not statistically significant ($P>0.05$). Phosphorous content was also increased during the vermicomposting process in all the treatments (Figure 1). The increment rate was not uniform among the treatments, although it was not significant. More increment was observed in *D. veneta* and *E. fetida* piles with EM. As in the Rose waste, increment in TN, P_2O_5 and AV.K concentration and reduction of C:N ratio was

observed in all the treatments for the Carnation waste. Generally, total nitrogen and available phosphorus was observed to be relatively lower in Carnation waste compared to Rose waste, although the difference was not statistically significant. Similarly, the rate of reduction of C:N ratio was more pronounced in Rose waste than Carnation. The final concentrations of total nitrogen, available phosphorous and potassium concentration were slightly higher in *E. andrie* and *E. andrie* + EM piles than other treatments, though it was not statistically significant (Figure 2). The reduction of C:N ratio in vermicompost was also relatively higher in these piles.

The higher reduction of volume of waste for both types of waste implied that all the three worms can be employed in managing the waste. However, the vermicompost process seems to be rapid for Rose waste than Carnation. The increase in nitrogen, phosphorus and potassium concentration was a common phenomenon for other types of wastes such as vegetables during vermicomposting process and similar results were observed by earlier researchers. For example, Azizi et al. (2014) observed an increasing trend in nitrogen and potassium concentration for vegetable and paddy straw wastes. Similarly Chauhan et al. (2010) reported similar trends of nutrient concentration for N, P and K in vegetable wastes. The higher concentration of TN in the produced vermicompost from both types of

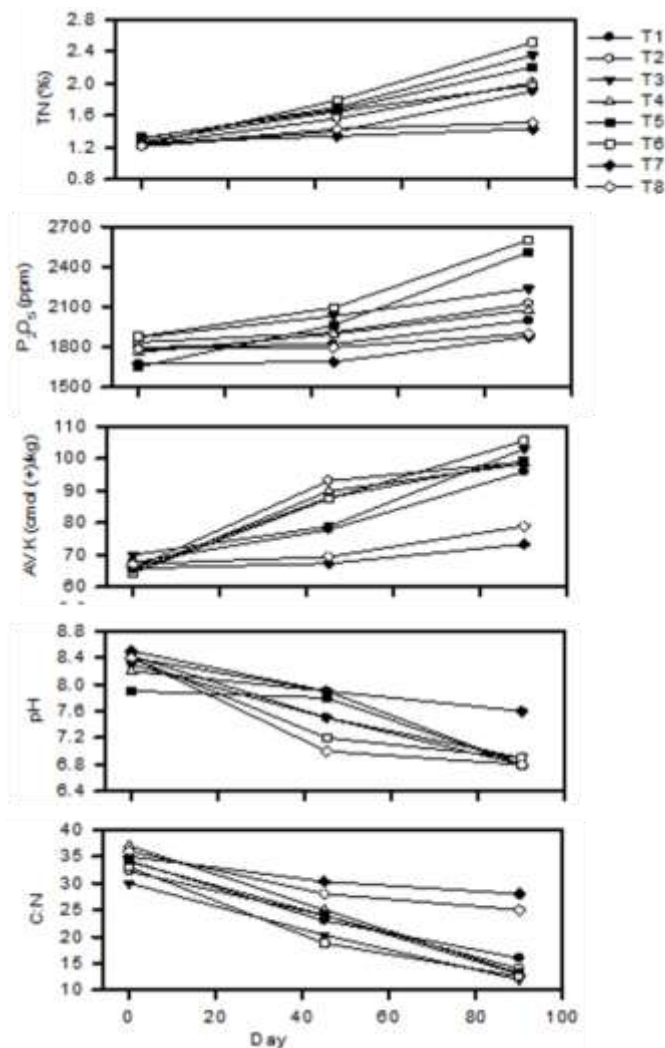


Figure 1. Changes in chemical variables during vermicomposting process in the rose waste. TN: Total Nitrogen, P₂O₅: Available Potassium, AV.K: Available Phosphorus, C:N: Carbon to Nitrogen ratio.

wastes can be attributed to mineralization of carbon rich materials, which could be facilitated by microbes, and the role of nitrogen fixing bacteria (Plaza et al., 2008). High level of nitrogen can also have been contributed by earthworms through the excretion of ammonia (Ansari and Rajpersaud, 2012). The phosphorus increase during vermicompost process can be attributed to mobilization and mineralization of phosphorus due to bacterial and fecal phosphatase activity of the worms (Asnari and Ismail, 2008).

Reduction trend in C:N during the process of vermicomposting is also a common phenomenon and observed by many researchers (Sharma et al., 2005; Ansari and Rajpersaud, 2012). The overall quality of the vermicompost produced from both waste was with high-quality in terms of nutrient content and C:N ratio and it is

within the standard level for vermicompost (MoFARA, 2016). However, the rate of waste conversion by the worm was slower in Carnation wastes. While there could be multifaceted reason for the delay, the recalcitrant nature of the plant seems to be the main reason as the environmental condition was maintained at similar level for both experiments at both flower wastes. Therefore, further research should be conducted to confirm or refute this hypothesis and the cellulose and lignin content of the plants should be determined.

Conclusions

All the three worms used in this study were able to manage both types of wastes; however, their performance

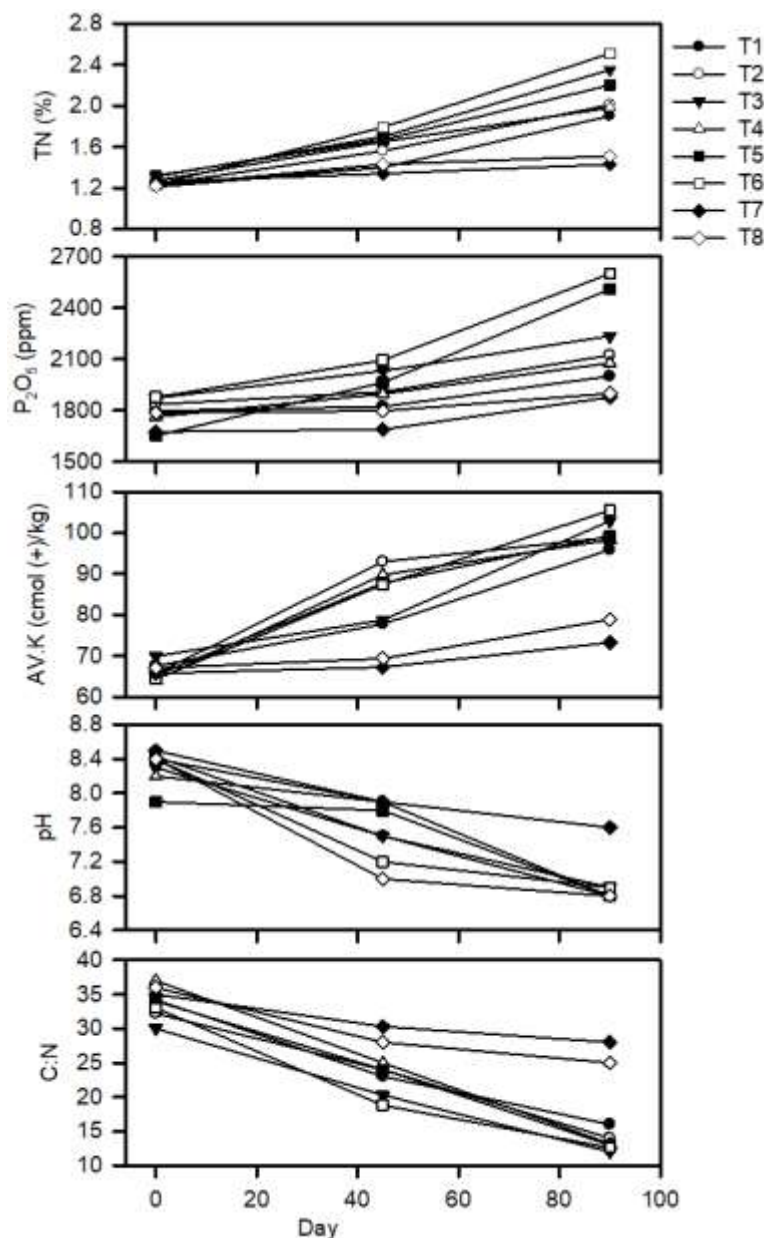


Figure 2. Changes in physico-chemical variables during vermicomposting process in the Carnation waste. TN: Total Nitrogen, P₂O₅: Available Potassium, AV.K: Available Phosphorus, C:N: Carbon to Nitrogen ratio.

and time taken in managing the wastes varied depending on the nature of the waste material. The rate of waste conversion by the worms was more brisk on Rose wastes than on Carnation. However, there was no significant variation among the quality of vermicompost produced by the worms in terms of nutrient content and C:N ratio. Therefore, it can be concluded from this study that all the three worms can be employed to manage both Rose and Carnation; however, managing the Carnation waste need relatively longer period. Also, the application of Efficient Microorganisms (EM) hastened the vermicomposting

process.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of sample extraction, preparation methods on HPLC quantification of plumbagin in *in vivo* and *in vitro* plant parts of *Plumbago zeylanica* L.

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Plumbagin is an important therapeutic compound of *Plumbago zeylanica* L., the commercial demand of this compound warrants optimizing a suitable method to isolate plumbagin in large scale. The present study was undertaken to obtain the maximum recovery of plumbagin content by employing different extraction methods viz., ultra-assisted extraction (UAE), maceration extraction (ME), soxhlet extraction (SE), serial soxhlet extraction (SSE), and serial maceration extraction (SME) from plant parts of *P. zeylanica*. Plumbagin content of two different sources such as field grown and hardened *in vitro* regenerated plants were quantified using reversed-phase high-performance liquid chromatography (RP-HPLC). For *in vitro* cultures, 6-benzylaminopurine (BAP) and Kinetin (KIN) were used for multiple shoot induction from nodal segments of *P. zeylanica*. Maximum percentage of shoot induction was obtained on MS medium fortified with BAP (6.66 μM) from nodal segments exposed for 6 weeks. Further multiple shoot proliferation and elongation was achieved in MS medium with a combination of BAP (6.66 μM) and KIN (4.44 μM), with the maximum number of shoots (47.3 ± 0.06) and shoot length (2.0 ± 0.06 cm) per explant after 6 weeks of culture. The optimum root induction was observed on MS medium supplemented with 1.23- μM indole-3 butyric acid (IBA) which produced 10.02 ± 0.2 mean roots with 6.2 ± 0.8 cm root length. Among the extraction methods, the SME method yielded maximum recovery (99.5%) of plumbagin as compared to others. *In vitro* leaf extract yielded high content of plumbagin (152.02 mg/g^{-1} DW) as compared to other plant parts (root 115.41 mg/g^{-1} dry weight; stem 98.02 mg/g^{-1} dry weight) whereas *in vivo* leaf, stem and root samples yielded 96.7, 38.59, and 86.35 mg/g DW of plumbagin, respectively. The present observation suggests that the SME was more efficient for obtaining the maximum recovery of plumbagin and it was confirmed with HPLC quantification. Among the field grown and *in vitro* regenerated plants, the *in vitro* culture shows more accumulation of plumbagin and is found suitable for commercial extraction.

Key words: Micropropagation, cytokinin, serial maceration extraction (SME), optimization, methanol, quantification.

INTRODUCTION

Plumbago zeylanica L. is an important medicinal shrub commonly known as 'Chitramoolam' in Tamil, and

'Chitrak' in Sanskrit belonging to the family Plumbaginaceae (Nisha and Purshotam 2014). It is a

native of South Asia; the species are distributed throughout most of the tropics and subtropics; growing in deciduous woodland, savannas and scrublands from sea level up to 2000 m altitude (Jain et al., 2014). Plumbagin is a natural naphthoquinone compound (5-hydroxy-2-methyl-1, 4-naphthoquinone) found in three genera (*Plumbago*, *Plumbagella*, *Ceratostigma*) of the tribe Plumbagineae (Van der Vijver, 1972) and also from the insectivorous genera such as *Drosera*, *Dionaea*, and *Nepenthes* (Widhalm and Rhodes, 2016). This compound is reported for pharmacological activities like antifertility, antimalarial, antiviral, antimicrobial, anticancer and leishmanicidal (De Paiva et al., 2003; Premakumari et al., 1977; Thaweesak et al., 2011; Aziz et al., 2008). Most importantly, the radio sensitizing effect of plumbagin was demonstrated in *in vitro* cell cultures and in *in vivo* mouse tumour models (Matamoros et al., 2012). Plumbagin being a potential anticancer compound (Cao et al., 2018), the commercial demand for this compound is increasing due to its pharmacological activity. As plumbagin is being isolated from the wild plant population, continuous exploitation may lead to extinction of this plant in future. Therefore, there is a need for developing alternative sources towards plumbagin production. *In vitro* culture (Hu and Wang, 1983) system offers an alternative source of secondary metabolite production in variety of medicinal plants. Various extraction and quantification methods of plumbagin from different plant species have been reported earlier which includes maceration, dynamic maceration, assistance of ultrasonic waves, Soxhlet apparatus, cold maceration and homogenization (De Paiva et al., 2004; Hajnos et al., 2007; Gangopadhyay et al., 2008; Putalun et al., 2010; Chellampillai et al., 2011; Thaweesak et al., 2011) with limited percent of plumbagin recovery. Solvents based on polarity were employed for the extraction of this valuable compound. Hsieh et al. (2005) developed a maceration method using ethanol and water for extraction of plumbagin from the roots of *P. zeylanica*. Chellampillai et al. (2012) developed a novel solvent-free extraction method for plumbagin using hydrophilic liquid Gelucire 44/14. Qualitative and quantitative analyses of plumbagin were carried out from roots of *P. scandens* (De Paiva et al., 2004), leaf and roots of *P. europea* (Muhammad et al., 2009). Gas chromatography-mass spectrometry (GC-MS) based analysis of 1, 4-naphthoquinones in different species of Droseraceae (Bonnet et al., 1984) and normal phase liquid chromatography coupled with UV-detector method (Marston and Hostettmann, 1984) has been studied. Reversed-phase high-performance liquid chromatography (RP-HPLC) method was also used for the analysis of plumbagin (Stensen and Jensen, 1994; Unnikrishnan et al., 2008). Sakomoto et al. (2008) developed enzyme-

linked immunosorbent assay (ELISA) method for detection of plumbagin using monoclonal antibody. Nevertheless, a significant sample preparation step is required for the aforementioned quantification methods. Recovery of the compound is augmented by the methods and selection of solvents used. Although plumbagin was extracted from *P. zeylanica* using various methods, to the best of our knowledge there is no report on the standardized extraction method to maximize the plumbagin content. Hence, in the present study, to find out a suitable extraction method, five different extraction methods were evaluated for maximum recovery of plumbagin from plant parts of *P. zeylanica*. Plumbagin content of *in vivo* and hardened *in vitro* regenerated plant parts of *P. zeylanica* using the extraction method with maximum recovery of plumbagin was standardised and the same has been validated using RP-HPLC.

MATERIALS AND METHODS

Plant

The stem cuttings of the medicinal plant *P. zeylanica* L., was procured from the Centre for Indian Medicinal Heritage, Kanjikode, Kerala, and grown in the Garden of Department of Biotechnology, Bharathiar University, Coimbatore. The Botanical Survey of India, Coimbatore, authenticated the identification; voucher specimen was deposited at the Herbarium, Department of Botany, Bharathiar University, Coimbatore.

In vitro culture

The sterilised nodal explants of *P. zeylanica* were placed on MS media (Murashige and Skoog) 1962 fortified with various concentrations (2.21 to 11.09 μM) of 6-benzylaminopurine (BAP) individually and in combination of BAP (6.66 μM) with Kinetin (2.32 to 11.61 μM). The cultures were maintained at a photoperiod of 16 h light/day at $25 \pm 2^\circ\text{C}$ for multiple shoot regeneration. The regenerated 40-days-old shoots were transferred to MS medium with different concentrations (1.2 to 4.9 μM) of indole-3-butyric acid (IBA) for root induction. The *in vitro* regenerated plantlets were transferred into mixture of sand: soil: vermiculite (1:1:1). The six-month-old *in vitro* hardened plantlets were used for the analysis of plumbagin content.

Chemicals and reagents

Plumbagin (>99% purity) purchased from Sigma Aldrich, USA (Cat. No. P7262) was used as standard. Solvents (HPLC grade) like methanol, hexane, chloroform, ethyl acetate, water and trifluoroacetic acid were obtained from Merck (Mumbai). All other chemicals with the highest purity used for plant tissue culture were purchased from Himedia (Mumbai).

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Sample preparation and extraction

Leaf, stem and root parts of *P. zeylanica* from six-month-old field grown and hardened *in vitro* regenerated plants of the same age were selected for sample preparation after several trails. The plant materials were shade-dried, powdered and sieved (20 to 40 μ mesh). One gram of each plant material was used for extraction of plumbagin.

Extraction methods

For the optimization of extraction and efficient recovery of plumbagin, following extraction techniques viz., ultrasonication, maceration, Soxhlet, serial Soxhlet, and serial maceration, with five different solvents with varying polarity (hexane, chloroform, methanol, ethyl acetate and water) were chosen.

Ultrasonication assisted extraction (UAE) method

UAE was performed according to the method described by Ying et al. (2011). UAE method was performed using ultrasonic apparatus (First source Laboratory solution, LLP). Plant material was soaked in 100 ml of different solvents separately in an Erlenmeyer flask. The flask was placed in an ultrasonic bath for 60 min at 30°C. The samples were centrifuged at 5000 rpm for 10 min and the supernatant was taken for the analysis.

Maceration extraction (ME) method

Extraction of plumbagin was carried out using a method described by Jin et al. (2011) with minor modifications. The dried plant material was extracted with 100 ml of different solvents on a shaker with 150 rpm at 30°C for overnight. The extract was filtered using Whatman No. 1 filter paper.

Soxhlet extraction (SE) method

Okoduwa et al. (2016) method was performed using exhaustive Soxhlet extraction of plumbagin with classical extraction apparatus (Soxhlet apparatus, Borosil) with minor modifications. The plant materials were continuously extracted with cooled, condensed solvents individually for 5 h. After extraction, the methanol solvent was evaporated by concentrating under vacuum with rotary evaporator (Cyber Lab) at 40°C under reduced pressure. The solvent free extract was thereafter evaluated.

Serial Soxhlet extraction (SSE) method

For the SSE method, the plant material was packed in thimbles and was continuously extracted with cooled, condensed solvents from non-polar to polar for 5 h using Soxhlet apparatus (Borosil). After extraction, the methanol solvent was evaporated by concentrating under vacuum with rotary evaporator (Cyber Lab.) at 40°C under reduced pressure. The solvent free methanol extract was thereafter evaluated.

Serial maceration extraction (SME) method

For the extraction of plumbagin, the SME was carried out following the protocol of Balasubramanian et al. (2018). The plant material was extracted with 100 ml of different solvent serially based on the polarity. The samples were then macerated on a shaker with 150

rpm at 30°C overnight. The extract was then filtered through Whatman No. 1 filter paper. For quantification, the extracts obtained using the above different methods were dried using rotary vacuum evaporator (Cyber Lab). 1.0 mg of dried solvent extract powder was dissolved in 1 ml of HPLC grade methanol (99.9%) and filtered using 0.45- μ m polyvinylidene difluoride (PVDF) syringe filter. The concentrated filtrate was further used for the quantification of plumbagin using HPLC. All the samples were preserved at -20°C until analysis.

Quantification of plumbagin

Quantification of plumbagin was performed using Waters2998Liquid Chromatography equipped with the Photodiode Array Detector. The data was processed with Empower2 software. The separation was achieved on Symmetry® C18column (4.6 mm \times 250 mm, 5 μ m). The mobile phase used was methanol: water with 0.1% Trifluoroacetic acid at a ratio of 20:80 v/v. With the flow rate of 1.0 ml/min at 30°C, temperature at 254 nm, injection volume was set at 20 μ l. Standard stock solution of plumbagin was prepared with HPLC grade methanol (concentration of 1 mg/ml). The presence of plumbagin in the sample was analysed by comparing it with retention time of the standard plumbagin. The standard and sample solutions were injected in triplicate. The amount of plumbagin present in each sample was calculated by comparing the standard area with sample area as:

$$\text{Plumbagin (mg/g)} = \frac{\text{Peak area of the sample}}{\text{Peak area of the standard}} \times 1000$$

Validation of analytical method

Analytical methods used for plumbagin quantification was validated for specificity, linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ). The specificity of the HPLC method was validated by injecting 20 μ l of standard plumbagin (1.0 mg/ml) and methanol (100%) as control individually. Five different concentrations (10 to 50 μ g/ml) of the standards were analysed in triplicate and the respective calibration curve was generated.

The linearity between peak areas and the concentration of the plumbagin was calculated using linear regression analysis. Accuracy of the analytical condition was determined by spiking known concentration of the standard to the various extracts obtained using different extraction methods and solvents and calculating the percentage of recovery. The recovery of plumbagin was calculated by subtracting the mass concentration of non-spiked extract using external standard linear regression. The experiment was repeated three times and the replica were evaluated, the values were represented as relative standard deviation (RSD%) and standard error (SE). Recovery (%) and RSD (%) were calculated using the following formula:

$$\text{Recovery (\%)} = \text{RC/IC} \times 100$$

where RC is the recovered concentration and IC is the injected concentration.

$$\text{RSD (\%)} = (\text{SD/M}) \times 100$$

where SD is the standard deviation and M is the mean.

The LOD and LOQ were calculated based on the standard deviation of the y-intercept (σ) and slope of the calibration curve (S) obtained from linear regression. LOD was calculated using the expression $3.3 \sigma/S$ and LOQ was calculated using $10 \sigma/S$.

Table 1. Effect of different concentration of 6-Benzylaminopurine (BAP) and Kinetin (KIN) combination with BAP (6.66 μM) exposed cultures on multiple shoot induction using nodal explants of *P. zeylanica* L.

BAP (μM)	6 Weeks		
	Response (%)	Mean shoot number	Mean shoot length (cm)
0.0	0.0 \pm 0.00 ^e	0.0 \pm 0.00 ^e	0.0 \pm 0.00 ^e
2.21	30.0 \pm 1.3 ^d	12.2 \pm 0.5 ^c	0.6 \pm 0.02 ^c
4.44	50.0 \pm 1.1 ^a	15.6 \pm 0.4 ^b	1.2 \pm 0.06 ^a
6.66	52.3 \pm 1.7 ^a	18.4 \pm 0.4 ^a	1.8 \pm 0.01 ^a
8.88	44.4 \pm 0.2 ^b	16.5 \pm 0.2 ^b	0.8 \pm 0.03 ^b
11.10	39.2 \pm 0.4 ^c	8.6 \pm 0.8 ^d	0.3 \pm 0.01 ^d
BAP+KIN (μM)			
0.0	0.0 \pm 0.00 ^f	0.0 \pm 0.00 ^f	0.0 \pm 0.00 ^e
6.66+2.21	60.12 \pm 1.6 ^d	25.1 \pm 0.32 ^b	0.6 \pm 0.08 ^d
6.66+4.44	92.06 \pm 1.8 ^a	47.3 \pm 0.06 ^a	2.0 \pm 0.06 ^a
6.66+6.66	79.06 \pm 0.8 ^b	20.4 \pm 0.08 ^c	1.0 \pm 0.01 ^b
6.66+8.88	72.04 \pm 1.0 ^c	14.8 \pm 0.2 ^d	0.8 \pm 0.01 ^c
6.66+11.10	58.06 \pm 1.0 ^e	8.2 \pm 0.02 ^e	0.2 \pm 0.04 ^e

Values represent mean \pm standard error of 20 replicates per treatment in three repeated experiments. Values within column followed by the same letter are not significantly different. Mean values of three independent experiments (\pm) with standard errors. Values with the different letters within columns are significantly different according to Duncan's multiple range test (DMRT) at a 5 % level.

Statistical analysis

The analysis was put up according to a thoroughly randomized design. All the experiments were repeated three times. The data (percent of regeneration, shoot number and shoot length, root number and root length) were statistically analysed using one-way analysis of variance and pair wise means compared using Duncan's multiple range test ($p = 0.05$).

RESULTS AND DISCUSSION

Plant parts of six-month-old field grown and six-month-old *in vitro* hardened plant parts of *P. zeylanica* was found suitable for extraction of plumbagin. For *in vitro* propagation, the shoot tip and nodal explants are valuable culture technique for the large-scale production of secondary metabolites (Sen and Sharma, 1999). In the present study, after 10 days of inoculation, shoot initiation in nodal explants was observed. The regenerated shoots produced abundant adventitious shoots with subsequent sub-culture. Efficient regeneration response was observed in medium supplemented with 6.66 μM of BAP with the highest mean number (18.4 \pm 0.4) of shoots per explants at the end of 6 weeks (Table 1). The shoot multiplication rate was decreased at high concentration of BAP, which corroborate with Ashok et al. (2011) where BAP above 6.66 μM was not effective for shoot regeneration in *Plumbago* species. The primary effect of BAP in inducing multiple shoot has been previously reported (Chen et al., 2001; Huang et al., 2000). In the present study, among the five different concentration (2.2 to 11.10 μM) of KIN along with BAP (6.66 μM) tested,

combination of 6.66 μM BAP along with 4.44 μM KIN was found to be effective in enhancing shoot induction. The highest mean number of shoots obtained was 47.3 \pm 0.06 (Figure 1a, b and c) with an average shoot length of 2.0 \pm 0.06 cm per explants at the end of 6 weeks. Root initiation was observed within 5 to 7 days after inoculation on MS medium enriched with different concentrations (1.23 to 4.92 μM) of IBA. Maximum number of roots (10.02 \pm 0.2) was produced when media was supplemented with 1.23 μM of IBA with an average of 6.2 \pm 0.8 cm root length per explants at the end of 4 weeks (Table 2 and Figure 1d). Similar results were also reported in *Plumbago* spp. (Ashok et al., 2011).

The first prime step in secondary metabolite extraction is to determine the solvent efficiency. For the present study, five different solvents were used based on their increasing polarity *viz.* hexane, chloroform, methanol, ethyl acetate and water for plumbagin extraction. Previously, solvents like chloroform, toluene, water, ethanol, methanol and n-hexane were used for the extraction of plumbagin from *Plumbago* spp. (De Paiva et al., 2003; Hseih et al., 2005; Gupta et al., 1993; Pawar et al., 2010; Komaraiah et al., 2004). In the present study, a significant variation in the area of the chromatogram was obtained with respect to the solvent used for the isolation. The maximum recovery of plumbagin from the plant parts of *P. zeylanica* was achieved using methanol as solvent in the extraction method, which might be due to the lower boiling point of methanol. Water, a highly polar than all solvents used was least effective in the extraction of plumbagin, while chloroform was found to be suitable solvent for maximum recovery of plumbagin from stem

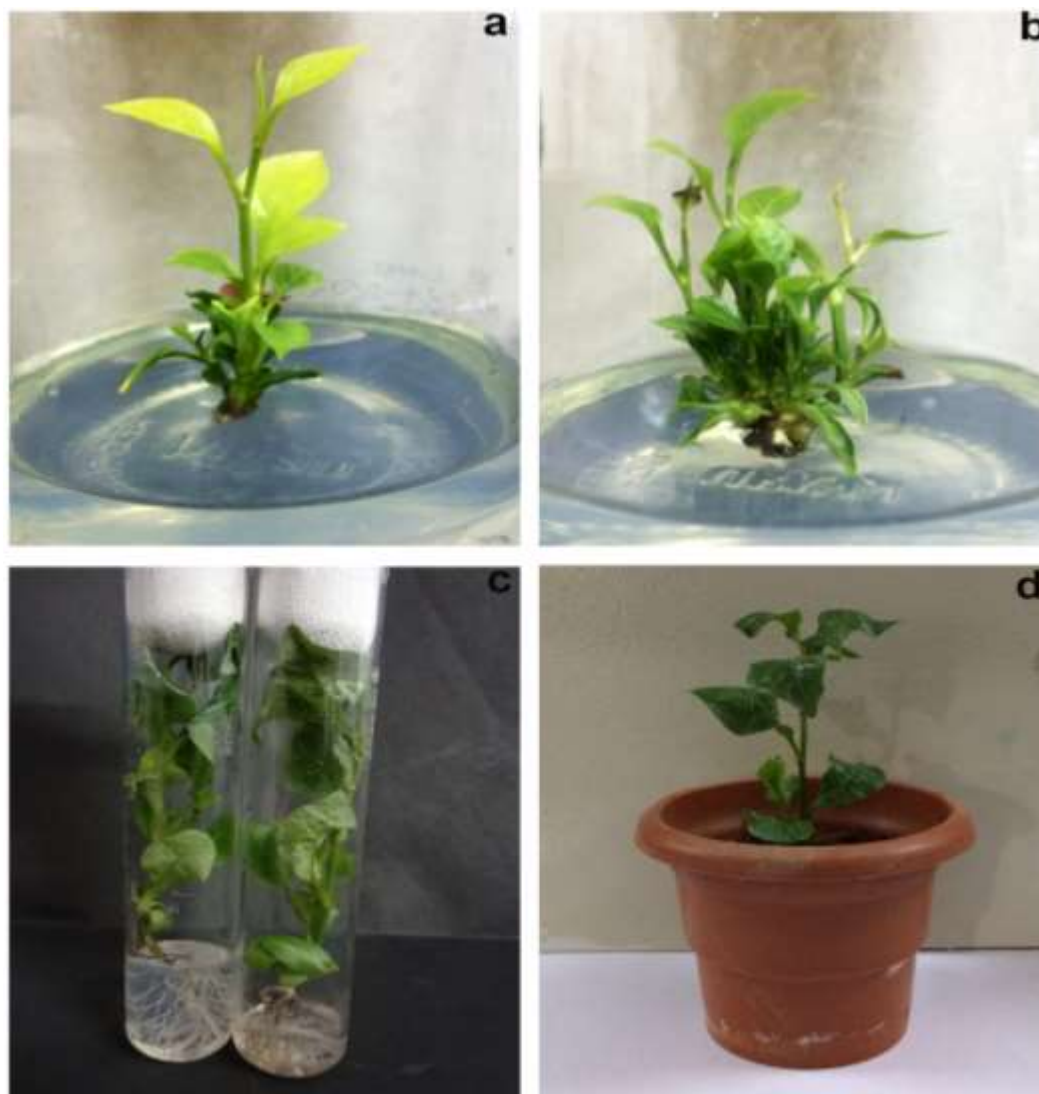


Figure 1. (a) Bud breaking on BAP combination Kinetin (6.66+4.44 μM) from nodal segments after 30 days. (b) Shoot multiplication in BAP combination Kinetin (6.66+4.44 μM) exposed cultures growing on MS basal medium after 6 weeks of transfer. (c) Shoot multiplication in BAP combination Kinetin (6.66+4.44 μM) exposed cultures growing on MS medium after 6 weeks of incubation. (d) Rooting in the regenerated microshoots on MS media containing 1.23 μM IBA after 4 weeks of incubation.

Table 2. Effect of IBA augmented with MS media on root induction from *in vitro* raised *P. zeylanica* L. after 6 weeks.

IBA (μM)	4 Weeks	
	Mean of roots number	Mean of roots number (cm)
0.0	0.00 \pm 0.00 ^d	0.00 \pm 0.00 ^d
1.23	10.02 \pm 0.2 ^a	6.2 \pm 0.8 ^a
2.46	8.6 \pm 0.1 ^b	3.0 \pm 0.0 ^b
3.69	2.4 \pm 0.0 ^c	1.8 \pm 0.6 ^c
4.92	2.3 \pm 0.1 ^c	1.6 \pm 0.0 ^d

Values represent mean \pm standard error of 20 replicates per treatment in three repeated experiments. Values within column followed by the same letter are not significantly different. Mean values of three independent experiments (\pm) with standard errors. Values with the different letters within columns are significantly different according to Duncan's multiple range test (DMRT) at a 5 % level.

Table 3. Accuracy of recovery with standard plumbagin spiked to the leaf, stem and root different extraction methods in field grown *P. zeylanica* L.

Extraction method	Solvent	Recovery (%)			RSD (%)		
		Leaf	Stem	Root	Leaf	Stem	Root
UAE	Hexane	73.0±2.6	43.8±1.3	74.9±0.6	3.62	2.99	0.86
	Chloroform	68.3±2.8	76.0±1.0	64.3±1.1	4.22	1.32	1.71
	Methanol	93.6±1.5	51.4±1.2	95.3±0.9	1.63	2.47	1.01
	Ethyl acetate	69.6±0.6	24.4±0.4	84.3±1.2	0.88	2.01	1.42
	Water	10.6±0.5	9.6±0.5	12.1±0.3	4.94	5.97	2.51
ME	Hexane	58.3±2.8	75.1±0.9	87.9±0.7	4.94	1.27	0.79
	Chloroform	83.3±2.8	23.8±1.3	54.0±1.6	3.46	5.81	3.07
	Methanol	92.3±0.5	63.1±2.7	63.9±0.6	0.62	4.29	1.02
	Ethyl acetate	83.0±2.6	9.6±0.5	74.2±1.0	3.18	5.51	1.34
	Water	51.6±2.8	5.4±0.4	26.6±0.6	5.58	7.40	2.49
SE	Hexane	91.0±1.0	73.6±1.5	45.6±1.0	1.09	2.16	2.24
	Chloroform	72.3±2.5	23.7±1.5	65.8±0.8	3.47	6.63	1.32
	Methanol	83.0±2.6	76.0±1.6	89.4±0.9	3.18	2.11	1.02
	Ethyl acetate	61.6±2.8	15.1±0.3	75.5±1.2	4.68	2.01	1.65
	Water	11.6±0.5	8.8±0.6	16.0±0.8	4.94	7.87	5.0
SSE	Hexane	14.3±0.5	87.1±1.4	54.9±0.5	4.02	1.63	1.06
	Chloroform	9.6±0.5	64.2±1.1	33.7±1.6	5.97	1.72	4.97
	Methanol	24.1±0.9	23.7±1.5	74.3±1.2	3.73	6.63	1.62
	Ethyl acetate	9.6±0.5	14.9±0.7	26.0±0.8	5.97	4.70	3.07
	Water	5.3±0.5	7.6±0.5	14.0±0.2	10.82	0.06	1.42
SME	Hexane	64.1±0.9	72.5±0.6	76.0±0.4	1.48	0.84	0.52
	Chloroform	76.6±1.4	54.7±0.7	70.7±0.6	1.82	1.42	0.94
	Methanol	98.9±0.8	90.8±0.3	99.5±0.2	0.81	0.38	0.20
	Ethyl acetate	9.6±0.5	14.6±0.4	24.8±0.2	5.97	3.37	0.00
	Water	4.6±0.5	5.06±0.6	10.3±0.4	0.12	2.99	4.02

The data were tabulated recovery with standard plumbagin content spiked to the six month old *in vivo* of *P.zeylanica* L plant. Mean values of three independent experiments (\pm) with standard errors.

part of *P. zeylanica*. These results corroborate with the previous studies, where methanol was found to be pertinent for the extraction of quercetin (Balasubramanian et al., 2018). Methanol was found to be ideal for the separation of phenolic substance from field-grown plants and *in vitro* cultures of *Hypericum* species (*Hypericum perforatum* and *Hypericum androsaemum*) (Dias et al., 1999).

Among the extraction methods such as UAE, ME, SME, SE and SSE evaluated for their efficiency to extract plumbagin from different parts of *P. zeylanica*, the recovery of plumbagin using SME method was efficient as compared to ME, SE, UAE and SSE (Table 3). The extraction conditions such as time duration and temperature for each method were resolved based on the previous report (De Paiva et al., 2004). The relative recovery of plumbagin varied among the extraction

method tested. The highest percent of plumbagin recovery from leaf extract was obtained using SME (98.9%) method, followed by UAE (93.6%), ME (92.3%), SE (83.0%), and SSE (24%). The recovery in stem part was the highest in SME (90.8%) followed by SSE (87%), UAE and SE (76%), and ME (75.1%). Similarly, in root extract, the highest recovery was obtained in SME (90.8%), followed by SSE (87.1%), UAE and SE (76%), and ME (75.1%) (Table 3). Plumbagin extraction efficiency was found to be in the order of SME > ME > SE > UAE > SSE. The poor recovery of plumbagin using SSE method could be due to long solvent-reflex (5h). Likewise, the setback of SE method include time consumption, high quantity solvent requirement and economically non-feasible. Further, plumbagin could be degraded due to long extraction process (De Paiva et al., 2004; Matamoros et al., 2012). UAE method was

Table 4. Serial maceration method (SME) using extraction of different solvent system on Plumbagin content in *in vivo* and *in vitro* of *P. zeylanica* L. plant.

Extraction Method	Solvent	<i>In vivo</i> of <i>P. zeylanica</i> L. (mg g ⁻¹) ^a			<i>In vitro</i> of <i>P. zeylanica</i> L. (mg g ⁻¹) ^b		
		Leaf	Stem	Root	Leaf	Stem	Root
SME	Hexane	0.69	0.99	33.45	2.42	41.0	80.49
	Chloroform	2.4	0.5	26.14	8.26	18.9	45.32
	Methanol	96.7	38.59	86.35	152.02	98.02	115.41
	Ethyl acetate	0.0592	0.005	0.002	5.78	6.33	75.42
	Water	0.0512	0.04	0.04	0.02	0.01	0.13

All the extracts were dried and about 1 mg extract powder was dissolved in 1 ml of HPLC grade methanol (99.9%). About 20 μ l of extracts (1 mg ml⁻¹) and standard (1 mg ml⁻¹ plumbagin) were taken as injection volume. mg^a plumbagin equivalent/g⁻¹ DW of *in vivo* of *P. zeylanica* L. extract. mg^b plumbagin equivalent /g⁻¹ DW of *in vitro* of *P. zeylanica* L. extract.

effective for the extraction of plumbagin from stem part; this might be due to the penetration efficacy of the ultrasound. UAE reportedly improved the extraction rate and yielded isoflavone from the stem part of *Pueraria lobata* (Willd.) Ohwi (Huaneng et al., 2007). The SME method was found suitable for maximum plumbagin recovery for *in vitro* culture plants and same was validated using RP-HPLC (Table 4). The conditions for HPLC such as mobile phase composition, temperature and flow rate were optimized to accomplish a good resolution and symmetrically shaped peak for plumbagin in less run-time. Similar separation of plumbagin was reported by Pereira et al. (2015). Although, flow rates of 1.0 ml/min and 0.75 ml/min were reported earlier for the isolation of plumbagin from the plant extract (Stensen and Jensen, 1994; Gangopadhyay et al., 2008; Gupta et al., 1993; Muhammad et al., 2009), in the present study, the resolution of the chromatogram was satisfactory at the flow rate of 1.0 ml/min and can be claimed as significant condition. Similarly, in the present study, methanol: water with 0.1% TFA was efficient with good peak resolution (Figure 2a) of plumbagin when compared with the mobile phase composition, like acetonitrile: water, n-hexane: chloroform-2-propanol, methanol: water reported earlier (Stensen and Jensen, 1994; Gangopadhyay et al., 2008; Gupta et al., 1993; Muhammad et al., 2009). The chromatography peak obtained under the optimized condition applied was efficient and reproducible similar to the earlier findings of Hsieh et al. (2005).

The validation parameters including specificity, linearity, accuracy, precision, limit of Detection (LOD) and limit of quantification (LOQ) were examined according to the ICH Guidelines (2005). Different methods have been reported earlier for the analysis of plumbagin in *Plumbago* spp. (Unnikrishnan et al., 2008; Hsieh et al., 2005; Gupta et al., 1993). However, a full validation report of RP-HPLC method for the comparative analysis of plumbagin from the field-grown and hardened *in vitro* regenerated plant material is reported here for the first time. The least square linear regression data of

plumbagin were used to determine the calibration parameter. Excellent linearity of the standard plumbagin (linear regression of R² = 0.9994) was obtained. The LOD and LOQ values were found to be 2.2 and 2.4 μ g/L, respectively, which indicate that the developed method is suitable and sensitive for the determination of plumbagin. The percentage of recovery was good with relative standard deviation of \leq 2%, which signifies the method is accurate.

The samples prepared from leaf, stem and root parts of *in vivo* and *in vitro* samples of *P. zeylanica* by serial maceration method (SME) using different solvents (hexane, chloroform, methanol, ethyl acetate and water) were used to quantify plumbagin content by HPLC. Methanolic extract of field-grown root samples of *P. zeylanica* showed plumbagin content of 86.35 mg/g dry weight, whereas the content was higher (115.41 mg/g dry weight) *in vitro* roots. This is the first report on the quantification of plumbagin from *in vitro* hardened plant parts of *P. zeylanica*. Dorni et al. (2007) reported the total plumbagin content in the methanolic extract of roots of *Piriformospora indica* L. and *P. zeylanica* L., were 0.569 and 0.247% w/w, respectively. There are reports in other plants (Bhardwaj et al., 2018) which suggest growth hormones applied during *in vitro* propagation may be one of the factor and the other factor hypothesized as formation of chemicals like phenyl amides and accumulation of polyamines during stress conditions during the hardening process of *in vitro* plants (Ramakrishna, 2011). Plumbagin also being a secondary metabolite, the results of the present study could be related to earlier reports. Bonnet et al. (1984) reported an increase in naphthoquinone level up to five times in *in vitro* root and shoot samples of *Drosera intermedia* and *Drosera rotundifolia*. The increase in naphthoquinone level of the plant parts was attributed to *in vitro* culture conditions. HPLC quantification of plumbagin content in methanolic extract of *in vivo* leaf samples of *P. zeylanica* was 96.7 mg/g dry weight, which is lower than the *in vitro* leaf sample (152.02 mg/g dry weight) (Table 4 and Figure 2b, c, d). The results corroborate with the earlier reports

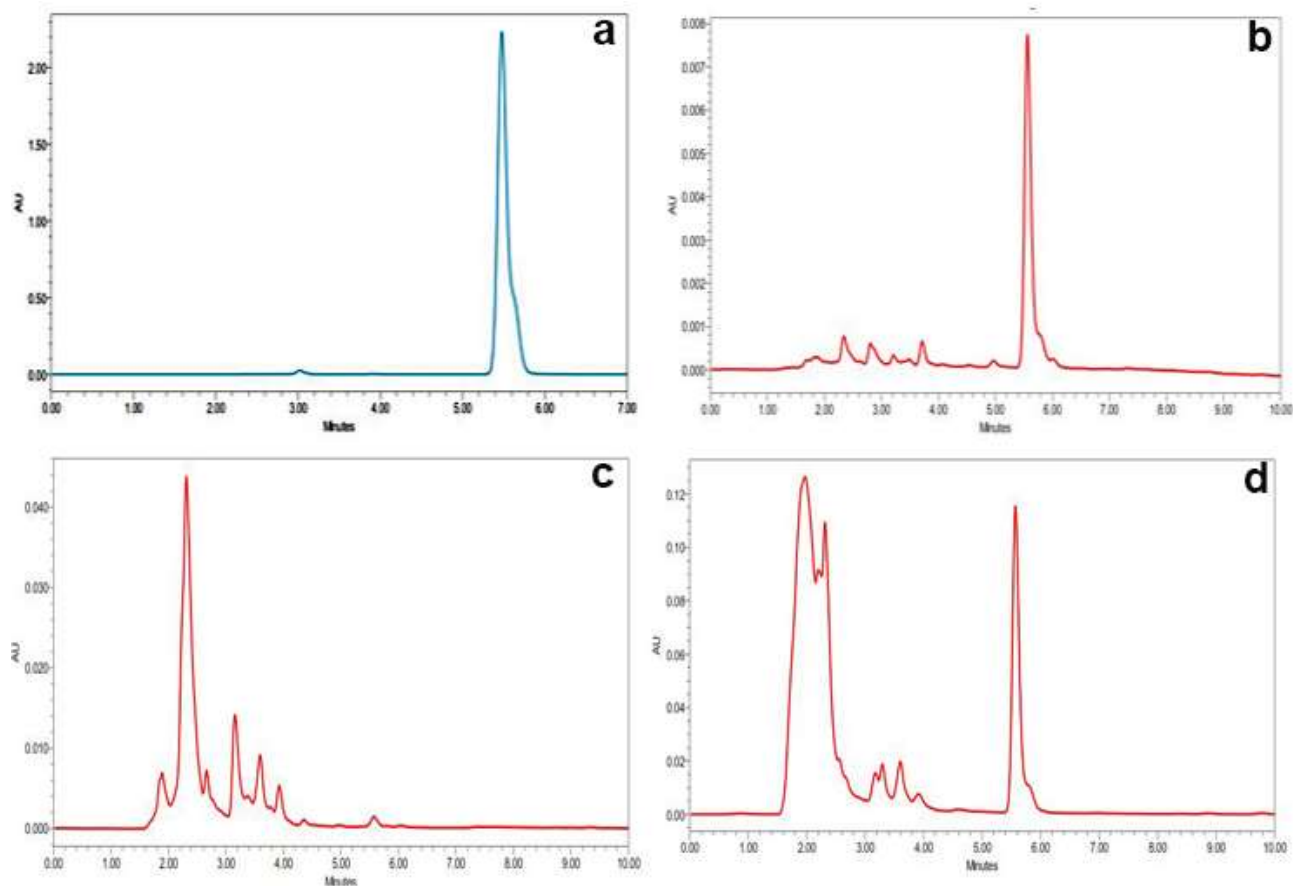


Figure 2. HPLC chromatograms of plumbagin in the *in vitro* *P. zeylanica* L. plant of leaf stem and root methanolic extract of Serial Maceration Method (SME): (a) Standard of plumbagin, (b) methanolic extract of leaf, (c) methanolic extract of stem, (d) methanolic extract of root.

in other plants (Bhardwaj et al., 2018) and Karuppusamy (2009) that the hormones supplied exogenously during tissue culture not only influence shoot proliferation but also *in vitro* bioactive secondary metabolites. Abiotic stress signals creating stress on plants during hardening process was another factor attributed for increased secondary metabolite secretion. Bryant et al. (1983) hypothesized that when plants are stressed, an exchange occurs between carbon to biomass production or formation of defensive secondary compounds. A stress response is induced when plants recognize stress at the cellular level. Secondary metabolites are involved in protective functions in response to both biotic and abiotic stress conditions. The *in vitro* leaf, stem and root of *P. zeylanica* showed 63, 40 and 74%-fold increase in plumbagin content as compared to the field grown parts. The yield of plumbagin from *in vitro* roots of *P. zeylanica* was higher (115.41 mg/g dry weight) than *in vivo* root extract. The *in vitro* leaf samples of *P. zeylanica* showed the highest plumbagin content (152.02 mg/g dry weight) compared to *in vitro* roots (115.41 mg/g dry weight). This finding has implications in plant biodiversity conservation

as *P. zeylanica* L. is also reported to be a threatened taxon and the *in vitro* leaves can be better source of plumbagin rather than *in vivo* and *in vitro* roots, thereby conserving the wild population. *In vitro* propagation and hardening of propagated plantlets of *P. zeylanica* (L.) in greenhouse, is commercially feasible, considering the cost of plumbagin and its pharmacological significance.

Conclusions

The extraction methods optimized in the present study to quantify plumbagin were simple, less time-consuming and reproducible for industrial production. The HPLC method validated for linearity, repeatability and reproducibility was satisfactory. The results from the present study clearly indicate that the SME method-using methanol as solvent is efficient in maximum recovery of plumbagin from *P. zeylanica*. The leaf samples of *in vitro* hardened plants were found to be suitable source for plumbagin isolation than the field grown plants of *P. zeylanica*. Hence, this can be recommended for

production of plumbagin.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Co-expressing house fly cytochrome P450 6A1 and human cytochrome P450 reductase in *Escherichia coli* to bioconvert insecticide aldrin

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Aldrin used in agricultural as well as industrial or home pest control is known to be a persistent organic pollutant, having chronic adverse effects on wildlife and humans. The degradation of aldrin has drawn a lot of attentions. In this study, house fly cytochrome P450 6A1 (CYP 6A1) or both house fly cytochrome P450 6A1 and human NADPH-P450 reductase (CPR) together was expressed in *Escherichia coli* cells to study their activity on aldrin conversion. It was found out that the membrane fractions prepared from the recombinant *E. coli* cells expressing human CPR with CYP 6A1 have catalytic activity on aldrin using enzyme analysis. The catalytic activity of the recombinant *E. coli* cells was also investigated on aldrin. After adding aldrin to the co-expression of human CPR and CYP 6A1 recombinant *E. coli* cells culture with 1 $\mu\text{mol L}^{-1}$ final concentration, 163.36 nmol L^{-1} of dieldrin was detected after 8 h, the product of aldrin epoxidation. These results indicate that co-expressing house fly CYP 6A1 and human CPR can convert aldrin to dieldrin.

Key words: Aldrin, bioconversion, cytochrome P450 6A1, cytochrome P450 reductase, dieldrin.

INTRODUCTION

Aldrin is an organochlorine insecticide which has been used in some developing countries to kill rootworms, beetles, and termites because of its low cost and ability in controlling insects (Costa, 2015). The difficulty to degrade aldrin leads to its extremely accumulation in environment and living organisms (Jhamtania et al., 2018). Thus, aldrin pollution still remains a serious problem in environment and it is required to find some efficient methods for its remediation. Degrading aldrin by microorganisms including bacteria and soil fungi has been reported (Erick et al., 2006; Ferguson et al., 1977;

Xiao et al., 2011). The possibility of using *Pseudomonas fluorescens* cell cultures to biodegrade aldrin in water, obtaining 94.8% degradation of aldrin in 120 h was demonstrated (Erick et al. 2006). Ferguson et al. (1977) reported that members of the order Actinomycetales can epoxidize aldrin to exo-dieldrin with their bacterial enzyme system (Ferguson et al. 1977). Xiao et al. (2011) investigated *Phlebia acanthocystis*, *Phlebia brevispora*, and *Phlebia aurea* to value their degradation capacities on aldrin and the subsequent metabolic products. Over 90% of aldrin was degraded after 28 days and several

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new metabolites including 9-hydroxyaldrin and two carboxylic acid products were also detected in the fungal cultures (Xiao et al., 2011).

Although using natural microorganisms to degrade aldrin in polluted soil is promising, there has been no report of the use of engineered microorganisms to degrade aldrin till now. The construction of an aldrin-metabolizing enzyme and its over-expression in microorganisms are useful for bioremediation. Cytochrome P450s from insects are involved in the metabolism of synthetic insecticides (Goff et al., 2003), secondary plant chemicals (Wiseman and Lewis, 2007), and insect hormones (Liu et al., 2015). The characteristics of insect P450s indicate that they can be used for the study of the metabolism and biodegradation of insecticides. It has been reported that phenobarbital treatment increases cytochrome P450 6A1 gene levels in house fly and increases the epoxidation of cyclodiene insecticides in particular (Cariño et al., 1994). This suggests that cytochrome P450 6A1 are related with aldrin metabolism. A homogeneous recombinant cytochrome P450 6A1 from house fly was obtained by expressing it in *Escherichia coli* and was exploited to monitor aldrin pollution in the environment (Wu et al., 2011; Zhang et al., 2010). However, microsomal P450s depend on their redox partner, NADPH-cytochrome P450 reductase (CPR), to deliver electrons from nicotinamide adenine dinucleotide phosphate (NADPH). In eukaryotic cells, the endogenous electron transport system is enough to support the P450's activity (Zhang et al., 2017); however, the reduction rate of heme iron of cytochrome P450s is very low because of the insufficient endogenous electron transport system in *E. coli*. To enhance the *in vivo* catalytic activity of P450 in *E. coli*, addition of CPR is required. It was reported that the co-expression of cytochrome P450 and cytochrome-450 reductase in the same *E. coli* cells can eliminate the need to add reductase (Grinkova et al., 2010). Like cytochrome P450s, cytochrome-450 reductase can be easily expressed in *E. coli* with certain activity (Kimura and Iyanagi 2003). Considering the availability of human cytochrome P450 reductase gene in our laboratory, choice was made to express house fly cytochrome P450 6A1 and human cytochrome P450 reductase in *E. coli* in the present study. It was found out that the genetically engineered *E. coli* strains have metabolic activity of converting aldrin to dieldrin by gas chromatography-mass spectrometry (GC-MS) analysis.

MATERIALS AND METHODS

The expression vector pCW and human *cpr*/pCW were given by Dr. F. Peter Guengerich (Medical Center of Vanderbilt University). The gene *cyp6a1* and *E. coli* DH5 α cell were preserved in our laboratory. Aldrin, lysozyme, dithiothreitol (DTT) and Triton N-101 were bought from Sigma, America. δ -Aminolevulinic acid (δ -ALA) was obtained from Aldrich, Merck. The restriction enzymes, *Nde*I and *Hind*III, and Isopropyl- β -D-thiogalactopyranoside (IPTG) were

from Takara, Japan. Terrific broth (TB) and Luria-Bertani (LB) media were from Invitrogen, America. All other chemicals were of reagent grade and were used directly without other treatment.

Construction of co-expression plasmids for house fly CYP6A1 and human CPR

The *cyp6a1*/pCW was constructed and preserved in our laboratory. Full length of *cyp6a1* was cloned from house fly, digested with *Nde*I/*Sal*I and then ligated into *Nde*I/*Sal*I-cut the vector pCW (Zhang et al., 2010). In this study, *Nde*I and *Hind*III sites were created for *cyp6a1* by PCR. The forward 5'-AGCGACATATGGCTTTTGGTTCATTTCT-3' (the underlined sequence is the *Nde*I site) and reverse 5'-GCCAAGCTTTTATTTAATTTCTTTCTT -3' (the underlined sequence is the *Hind*III site) were used as the primers. A full-length human *cpr* was obtained by digesting with *Hind*III enzyme from *cpr*/pCW vector. To link *cyp6a1* with *cpr*, the 16 bp sequence TAACTTTAAGAAGGAGAT, which contained an optimized Shine-Dalgarno ribosomal binding site (RBS), was designed to enhance its production in *E. coli*.

Expression of house fly CYP6A1 and human CPR

A single ampicillin-resistant colony of *E. coli* BL 21 cells transformed with plasmid DNA was grown overnight at 37°C in Luria-Bertani medium supplemented with 100 μ g mL⁻¹ ampicillin. A 1 mL aliquot was used to inoculate each 1.0 L of the TB containing 0.02% bacto-peptone (w/v). The TB medium was supplemented with ampicillin (50 μ g mL⁻¹), 1.0 mM thiamine, 0.5 mM δ -ALA and trace elements [27 g FeCl₃·6H₂O, 2.0 g CoCl₂·6H₂O, 2.0 g ZnCl₂·4H₂O, 2.0 g Na₂MoO₄, 1.0 g CaCl₂·2H₂O, 1.0 g CuCl₂, 0.5 g H₃BO₃, and 100 mL concentrated HCl in 1 L ultrapure water]. Induction of the tac promoters was done by adding 1.0 mM IPTG when the OD₆₀₀ of the expression culture reached 0.6. Then it was shaken at 37°C and 180 rpm and the culture was incubated for another 36 h at 28°C and 160 rpm in a shaker.

Cell harvesting and lysis

The pelleted cells were resuspended in 2×TSE (100 mM Tris-acetate, pH 7.6, containing 500 mM sucrose, and 0.5 mM EDTA) according to 5 mL of 2×TSE versus 100 mL of culture. Following the addition of lysozyme (0.25 mg/mL), the cells were gently shaken for 30 min after the addition of an equal volume of H₂O. The lysed cells were centrifuged by 2800 *g*, 4°C for 15 min. The resulting spheroplasts (4 mL/100 mL of culture, in 100 mM pH 7.4 potassium phosphate buffer), which contains 6 mM magnesium acetate, 20% glycerol (v/v) and 0.01 mM DTT, were frozen at -70°C until further use. During the thawing phase, the protease inhibitors of PMSF were added at final concentration of 2 μ M. Cells were sonicated for 20 min with 5 s bursts (at 50% full power) while they were kept in an ice-salt bath. The resulting lysate was centrifuged at 12000 rpm for 12 min, and the resulting supernatant was then centrifuged at 180000 *g* for 65 min; the pellet (that is, membranes), which contains 0.25 mM EDTA and 0.25 M sucrose, was resuspended in 20 mM Tris-acetate buffer (pH 7.4) and then was stored at -70°C.

Characterization of CYP6A1 and CPR expression

CYP 6A1 content was calculated by the general Omura and Sato method using an extinction coefficient of 91 mM⁻¹ cm⁻¹ (Omura, 1964). Total protein concentration was determined by the Lowry assay (Lowry et al., 1951). Activities of CPR were determined by

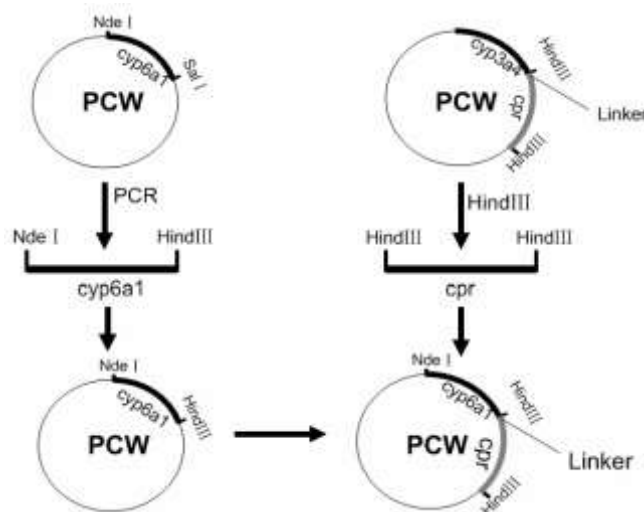


Figure 1. Construction of expression vector pCW/cyp6a1-cpr.

the reduction of cytochrome *c* (20 μM) with addition of 100 μM NADPH. All assays and incubations were carried out in a 100 mM pH 7.4 potassium phosphate buffer. The reduction rate of CPR was monitored by the increased absorbance at 550 nm (Guengerich et al., 2009). For calculating the reduction rate of CPR, a specific molar absorption coefficient (21.1 $\text{mM}^{-1} \text{cm}^{-1}$) of equine heart cytochrome *c* was used.

Measurement of catalytic activity of CYP6A1 and CPR towards aldrin

The reaction mixture contained 2 to 200 μM aldrin (dissolved in acetone) and 50 nM of CYP6A1 and 7 nM of CPR in a final volume of 1.0 mL 50 mM pH 7.4 potassium phosphate buffer. The reaction was initiated after adding NADPH with 0.5 mM final concentration. Incubations were carried out at 37°C for 30 min and terminated by vortexing with 4 mL of ethyl acetate, and 5 μM heptachlor was added to the mixture as an internal standard for GC-MS analysis. Aliquots of the reaction mixture were collected at varying time intervals up to 30 min and the reaction product was extracted with four volumes of petroleum ether. The organic phase was recovered and dried up by a rotary evaporator. The resulting residue was resolubilized with 50 μL of petroleum ether. A 1 μL aliquot was injected into GC-MS equipped with a DB-5MS capillary column and an electron capture detector. Helium was used as the carrier gas with a flow of 36.8 $\text{cm}^3 \text{s}^{-1}$. Samples were injected automatically in splitless mode by HP 7673 auto sampler with 1.00 μL each time. The injection port temperature was kept at 260°C. The column temperature was maintained at 80°C for 1 min, and then programmed at 25°C min^{-1} to 200°C, which was held for 1 min, then at 5°C min^{-1} to 250°C, and then increase at 20°C min^{-1} to 290°C, which was held for 5 min. The mass-spectrometer acquisition parameters were as follows: electron energy 70 eV, solvent delay 10 min, electron multiplier voltage 2000 V. Metabolism product was detected with selected ion monitoring (SIM).

Determination of catalytic activity of the recombinant *E. coli* cells towards aldrin

The substrate aldrin was dissolved in acetone. To study the metabolism of aldrin by the recombinant *E. coli* cells, the final

concentration was adjusted to 1 μM . The recombinant *E. coli* cells were cultivated at 30°C after adding aldrin. At varying time intervals, up to 8 h aliquots of the cell culture were sampled and extracted with four volumes of diethyl ether-petroleum ether (1:1). The organic phase was collected then dried up. The resulting residue was resolubilized with petroleum ether and applied to the aforementioned GC-MS analysis. Samples were analyzed three times in the same assay and in three different runs. The mean data was obtained to analyze the catalytic activity of the recombinant *E. coli* cells towards aldrin.

RESULTS

Construction and expression of CYP6A1 and CPR in *E. coli*

Figure 1 shows the schemes to construct the expression plasmid. *cyp6a1* and *cpr* gene fragment was cut from the recombinant plasmid pCW with *Nde*I/*Hind*III and *Hind*III, respectively and subcloned into the expression vector pCW. A 16 bp linker containing a Shine-Dalgarno ribosomal binding site was employed to link *cyp6a1* and *cpr*. After ligation and transformation, the positive recombinants were subsequently screened out by using ampicillin selective media. Figure 2A shows the nucleic acid electrophoresis of pCW, pCW/CYP6A1 and pCW/CYP6A1-CPR, respectively. The CYP6A1 recombinants were identified by PCR with the forward and reverse primers (Figure 2B). The CYP6A1 and CPR recombinants were proven to contain the 1.5 and 2.0 kb fragments by enzyme cutting with *Nde*I and *Hind*III, respectively (Figure 2C).

The temperature and time for *E. coli* growth were optimized and the highest expression level was obtained at 28°C and 38 h. Compared with no insertion, the expression of CYP 6A1 was obvious (Figure 3). The molecular weight of P450 6A1 is about 58 kDa, which is

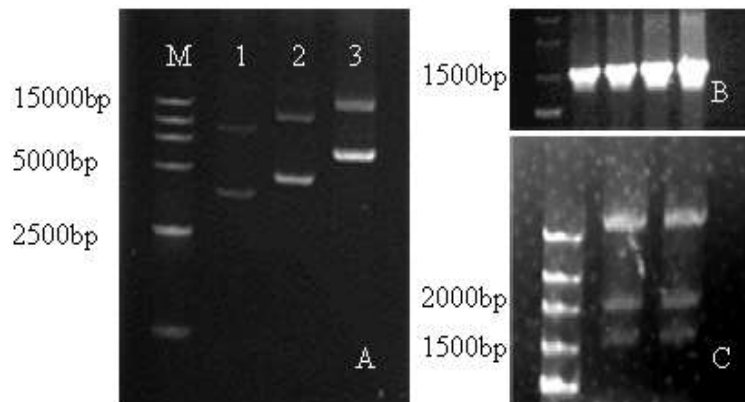


Figure 2. Nucleic acid electrophoresis of pCW (lane 1), pCW/cyp6a1 (lane 2) and pCW/cyp6a1-cpr (lane 3), respectively (A). Identification of the recombinant expression plasmid of pCW/ cyp6a1 by polymerase chain reaction (B) and identification of the recombinant expression plasmid of pCW/cyp6a1-cpr with *NdeI/HindIII* digestion(C).

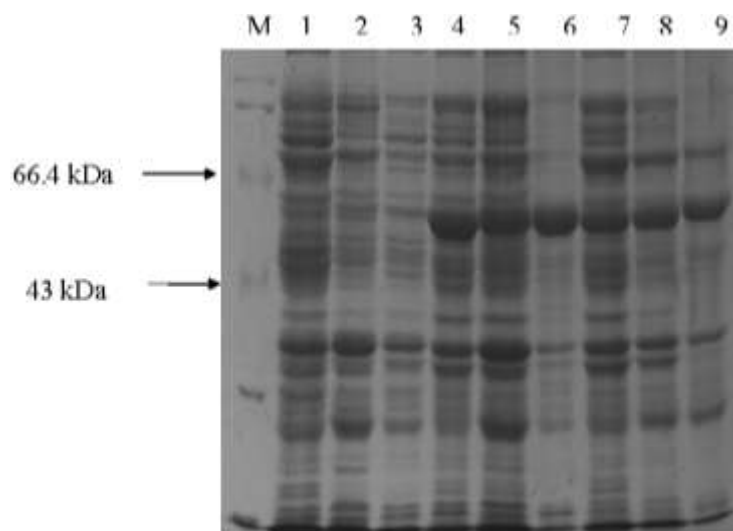


Figure 3. Characterization the expression of recombinant P450 6A1 at the optimized condition of 28°C and 38 h by SDS-PAGE Lane M: Molecular size markers; Lane 1-3: the whole cell lysate, the supernatant and the deposit of *E. coli* with the pCW vector with a non-expressed insert, respectively; Lane 4-6: the whole cell lysate, the supernatant and the deposit of *E. coli* with the pCW vector containing the P450 6A1 modified cDNA induced by the IPTG, respectively; Lane 7-9: the whole cell lysate, the supernatant and the deposit of *E. coli* with the pCW vector containing the P450 6A1 and human CPR modified cDNA induced by the IPTG, respectively

consistent with the gene product. However, the expression of CPR is too low to be detected by SDS-PAGE.

The determination of the CYP6A1 content was based on the method of Omura and Sato (1964) and the result is shown in Table 1. As shown in Figure 4A, the membrane fraction of CYP6A1 showed a maximum

absorption peak at 447 nm with a small peak at 420 nm.

The results indicate the recombinant protein was produced in good quality. On the basis of reduced CO difference spectrum, the expression level in the membrane fraction of CYP6A1 in *cyp6a1/pCW* construct or in *cyp6a1-cpr/pCW* construct was estimated to be 350 nmol L⁻¹ or 158 nmol /L⁻¹ culture, respectively. The

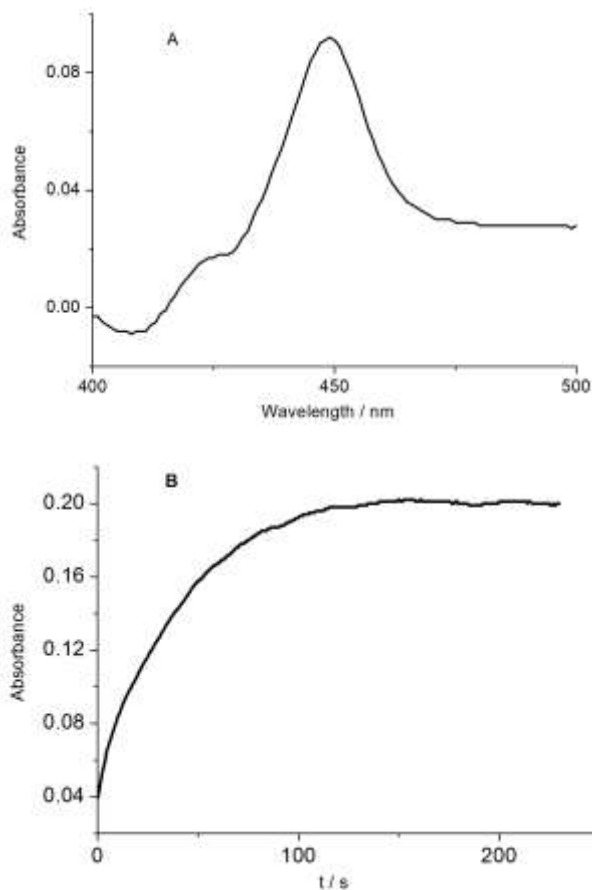


Figure 4. Determination the expression level of enzyme house fly cytochrome P450 6A1 (A) and human cytochrome P450 reductase (B) in recombinant *E. coli* cells.

expression level of P450 in CYP6A1-CPR co-expression is almost half of that in the expression of P450 alone. The limit of the capacity of protein synthesis in *E. coli* for the additional two proteins synthesis may cause the decrease in the expression level of P450. Compared with the previous reported data about the expression level of cytochrome P450s (Hiroshi et al., 1998), the highest expression level of CYP2C8 with 381 nmol L⁻¹ of culture and the lowest expression level of CYP2A6 with 66 nmol L⁻¹ of culture, the expression level of CYP6A1 in this report is medium.

Cytochrome c reductase activity was used to characterize the expression level of CPR in the recombinant *E. coli* cells (Figure 4B). From the results in Table 1, it can be concluded that the cytochrome c reductase activity in the recombinant *E. coli* cells expressing both CYP6A1 and CPR was 137-fold higher than that expressing CYP6A1 only. In the previous report, the expression level of human CPR, co-expressed with cytochrome P450, ranged from 68 to 312 nmol L⁻¹ of culture (Hiroshi et al., 1998). The expression level of CPR in this report is very low. The low yield of CPR in this

report may be caused by the case of co-expression of proteins.

Metabolism of aldrin by CYP6A1 and CYP6A1/CPR

CYP6A1 or CYP6A1-CPR in membranes collected from the recombinant *E. coli* cells were examined on the metabolism of aldrin. GC-MS method was applied to analyze the metabolites as reported previously (Xiao et al., 2011). The membrane fraction prepared from the control pCW and *cyp6a1*/pCW showed no activity on aldrin in this study. Figure 5 shows GC-MS profiles of the metabolites by the reconstituted CYP6A1/CPR. At 30 min of incubation, 25% of aldrin was converted into its metabolite. Mass spectrum of aldrin (retention time 8.4 min) and its metabolite (retention time 10.6 min) is as shown in Figure 6. Aldrin with molecular weight 365 has a mass spectrum showing the following fragment ion results: m/z 329[M-Cl]⁺, m/z 293 [329-HCl]⁺, and 263 [293-CH₂CH₃]⁺. Its metabolite had fragment ions at m/z 345 [M-HCl]⁺, m/z 263 [345-OCH₂CH₃Cl]⁺, m/z 237

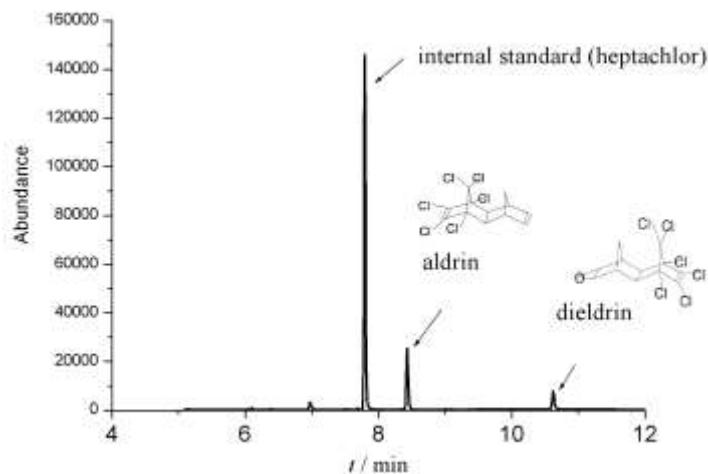


Figure 5. GC-MS profiles of aldrin and its metabolites by the membrane fractions prepared from the recombinant CYP6A1-CPR *E. coli* cells.

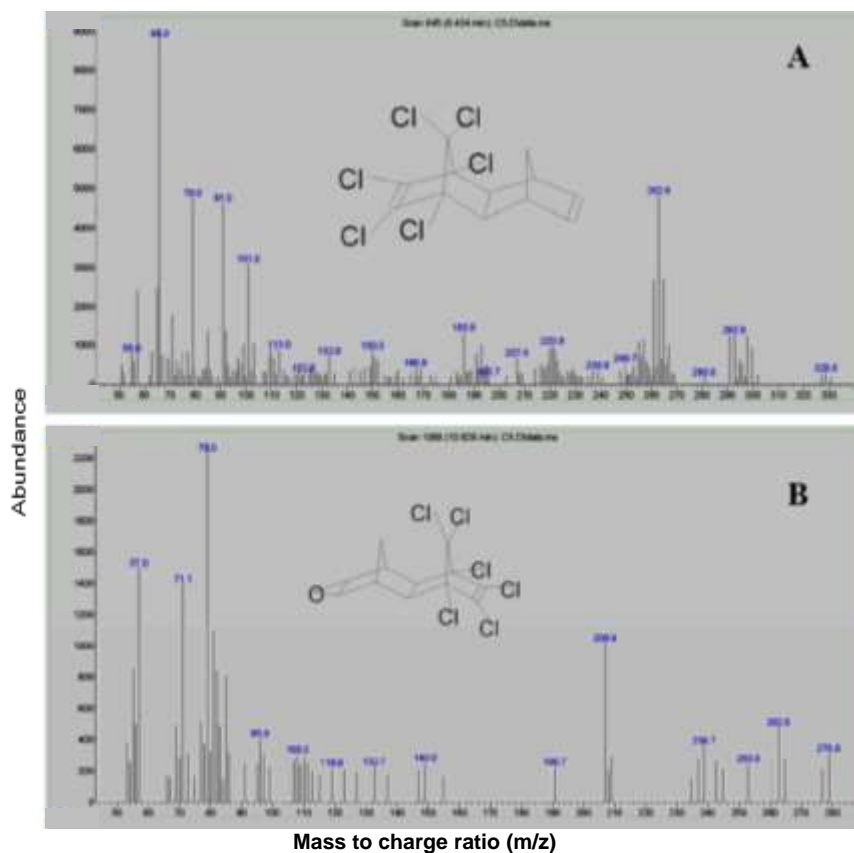


Figure 6. Mass spectra of aldrin (A) and its metabolite dieldrin (B).

$[C_5Cl_5]^+$, and 79. Based on comparison with published data (Xiao et al., 2011), it was concluded that metabolite was dieldrin, the epoxidized product of aldrin.

According to the product dieldrin turnover and the

double reciprocal method, the kinetic parameters, K_m and V_{max} , for the CYP6A1/CPR dependent epoxidation activity towards aldrin were estimated to be 46.1 μM and 27.3 nmol/nmol P450/min, respectively. The kinetic study

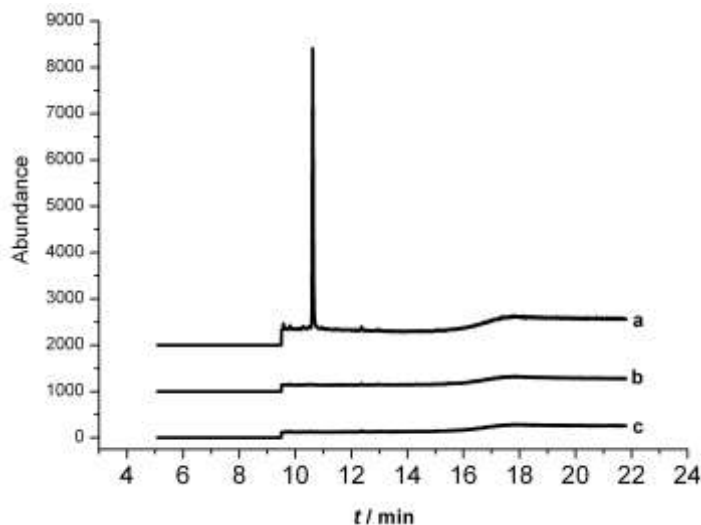


Figure 7. Detection of metabolism product dieldrin by the membrane fractions prepared from the recombinant CYP6A1-CPR *E. coli* cells (a); the recombinant CYP6A1 *E. coli* cells (b) and the control DH5 α /pCW cells (c) by selected ion monitor (SIM) method.

using membrane fractions demonstrated that the enzymatic affinity of cytochrome P450 6A1 was higher than that of CYP6A1 immobilized in dioctadecyl dimethyl ammonium bromide (DDAB) film (Wu, 2011). However, aldrin epoxidase from rat liver was also examined on the activity of epoxidized aldrin with a V_{max} of 5.11 nmol/nmol P450/min and a K_m of 1.64 μ M (Norie et al., 1984). The results indicate that the affinity of aldrin epoxidase with aldrin from rat liver microsomes is much higher than that of CYP6A1.

The study of the metabolism of aldrin by membrane fractions prepared from the recombinant *E. coli* cells indicated that CYP 6A1 alone has no activity to aldrin, but expression of human CPR with CYP 6A1 in the presence of NADPH has enzymatic activity to aldrin (Figure 7). The results indicate that human CPR played an important role in the CYP 6A1 enzyme activity.

Metabolism of aldrin by the recombinant *E. coli* cells

The control DH5 α /pCW cells and the recombinant *E. coli* cells expressing CYP6A1 or CYP6A1/CPR were examined for the metabolism of aldrin. 1 μ M aldrin was added to the cells culture after the cells were in logarithmic growth period. The addition of aldrin caused no growth inhibition in both recombinant cells and the control BL21/pCW cells. Cell culture (2 mL) was sampled every 2 h, then it was processed for GC-MS analysis described earlier. As shown in Figure 8, remarkable metabolism was observed towards aldrin in the recombinant CYP6A1/CPR *E. coli* cells, and lower activity was observed in the recombinant CYP6A1 *E. coli* cells,

while no activity was observed in the control BL21/pCW cells. When aldrin was added into the recombinant CYP6A1/CPR *E. coli* cells, the epoxidation production of aldrin, dieldrin can be detected after 2 h. It indicated that the hydrophobic substrate and product can diffuse into and out of the membranes of *E. coli* cells. Expression of CYP6A1 alone in *E. coli* showed low catalytic activities in the oxidation of aldrin, indicating that some similar factors like reductase existed in *E. coli* cells with the ability to deliver electrons to P450. However, the co-expression of CPR and CYP 6A1 in *E. coli* cells led to a big increase in the catalytic activity of P450. It indicates that *E. coli* strains co-expressing house fly CYP 6A1 and human CPR can efficiently catalyze aldrin without addition of CPR in culture. From Figure 8, it was observed that approximately 15% of aldrin was degraded within 8 h. The results suggest that the human CPR can efficiently give electron to house fly CYP 6A1 in *E. coli* inner membranes to maintain the highest activity of house fly CYP 6A1.

DISCUSSION

In this study, cytochrome P450 6A1 was expressed from house fly or both house fly cytochrome P450 6A1 and human cytochromes reductase in *E. coli* cells. It was demonstrated that the recombinant *E. coli* membrane fractions containing CYP6A1 have no catalytic towards aldrin, but the recombinant *E. coli* membrane fractions containing CYP6A1-CPR can metabolize aldrin to dieldrin. These results indicate that CPR plays an important role in the catalytic reaction of CYP6A1. The

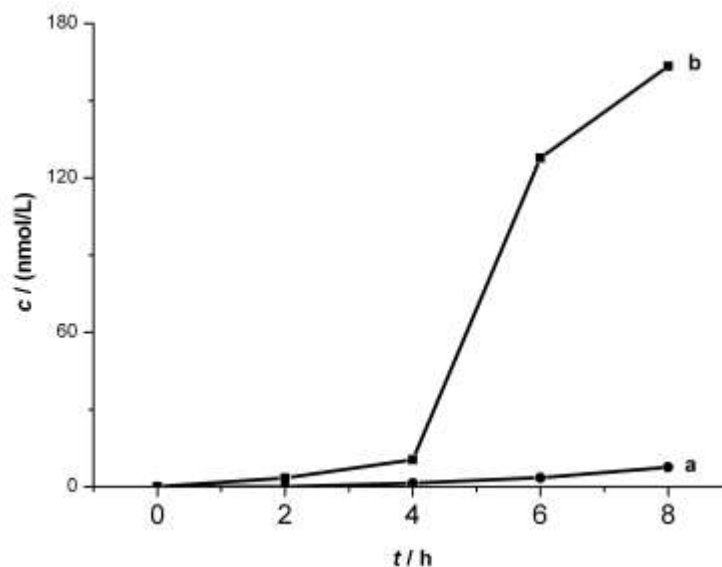


Figure 8. Time course of aldrin metabolism by the recombinant CYP6A1 *E. coli* cells (a) and the recombinant CYP6A1-CPR *E. coli* cells (b). The substrate aldrin was added to the cell culture at a final concentration of 1 μ M. Conversion concentration of aldrin into the metabolite dieldrin was calculated based on GC-MS analysis.

control DH5 α /pCW cells and the recombinant *E. coli* cells expressing CYP6A1 or CYP6A1/CPR on the metabolism of aldrin have also been examined. The results show the recombinant CYP6A1/CPR *E. coli* cells can metabolism towards aldrin remarkably and the recombinant CYP6A1 *E. coli* cells show lower activity, while the control BL21/pCW cells show no activity. These results in this study show the promise of using engineered microorganisms to degrade aldrin by expressing both CYP6A1 and CPR.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Isolation and identification of bacterial endophytes from *Crinum macowanii* Baker

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The widespread distribution of *Crinum macowanii* across the African continent has entrenched the plant's medicinal usage in treating diverse diseases. While its phytochemistry is well established, its microbial symbionts and their utility have not been described. As such, five bacterial endophytes, viz. *Staphylococcus* species C2, *Staphylococcus* species C3, *Bacillus* species C4, *Acinetobacter* species C5 and *Staphylococcus* species C6 were isolated from fresh *C. macowanii* bulb and their phenotypic and genotypic profiles verified by Gram staining and 16S rRNA gene sequencing; respectively. The latter was used to construct a phylogenetic tree that showed similarities (higher than 50 bootstrap values) among the endophytic bacterial isolates. Chemical analysis of bacterial endophytes was done by extracting the crude extracts of each endophyte. Antibacterial activity of each endophyte was performed against a few selected bacterial pathogenic strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus cereus*) using the disk diffusion method with Streptomycin used as a positive control. The crude extracts of all the endophytes showed no bioactivity against *K. pneumoniae*, though the inhibition was observed against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. cereus*. These results suggest that crude extracts of endophytic bacteria from *C. macowanii* have the potential to be used as antimicrobial agents.

Key words: Antibacterial activity, *Crinum macowanii*, endophytes, phylogenetic analysis.

INTRODUCTION

Endophytes, fungi and bacteria are microbial symbionts that occupy internal tissues of plants such as leaves, stems, roots and flowers without causing diseases to their plant hosts (Alvin et al., 2014; Nisa et al., 2015; Wu et al., 2016). The plant-endophyte interaction is a mutual

relationship (Pimentel et al., 2011; Ginting et al., 2013), with plants offering residence, nutrients and protection to the endophytes; whilst endophytes provide several benefits to improve growth and health of their plant hosts (Eljounaidi et al., 2016; Pereira et al., 2016). These

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microorganisms unlike pathogens serve as protective agent of plants by synthesizing secondary metabolites that protect their hosts against pathogens and insect attack (Ellouze et al., 2015). Another mechanism utilised by endophytes to prevent pathogenesis is by inhibition of pathogenic microorganisms (Wu et al., 2016). Endophytes also enhance plant growth by improving nutrient absorption, production of plant hormones and nitrogen fixation (Jin et al., 2014; Santoyo et al., 2016). This defence mechanism against pathogens and insects show potential of endophytes as bio-control agents in agricultural applications (Ryan et al., 2008). Secondary metabolites produced by endophytes have shown various biological activities such as anti-microbial, anti-oxidant, anti-cancer and anti-diabetic (Nair and Padmavathy, 2014). In this study, bacterial endophytes were isolated from medicinal plant, *Crinum macowanii* and tested for anti-bacterial activity against human pathogens.

C. macowanii Baker is a highly valued medicinal plant distributed in tropical areas of eastern and southern regions of Africa. It is found among the 130 species of genus *Crinum*, in a family of Amaryllidaceae. Plant species in this family are known to produce alkaloids as a largest group of secondary metabolites (Elgorashi et al., 2003). Amaryllidaceae alkaloids clinically approved are lycorine and galathamine, with anti-tumor and acetylcholine esterase inhibitory activities, respectively (Acosta et al., 2014).

C. macowanii is mainly used as a healing agent for treatment of sexually transmitted diseases, backaches and is used to stimulate lactation in women and cows (Nair et al., 2000). Other medicinal applications of *C. macowanii* include treatment of kidney and bladder infections, tuberculosis, swelling of the body, scrofula, rheumatic fever, itchy rashes, sores, boils and acne, backache, and venereal disease (Maroyi, 2016). Due to the extensive medicinal application, following its slow reproduction system, this plant is gradually being an endangered species (Nair et al., 2000). With *C. macowanii* endangered, there is a need to explore the species endophytes and their biological activities. Thus, the aim of this study was to isolate, characterize and identify bacterial endophytes from *C. macowanii* and test their secondary metabolites on pathogenic bacterial species.

MATERIALS AND METHODS

Collection and identification of plant material

C. macowanii plant materials were collected in Makonde village with geographical co-ordinates 22°48'18.4"S 30°35'41.4"E, Thohoyandou, Limpopo province, South Africa. Disease free plant materials (leaves, bulbs) were collected and placed in sterile polyethylene bags and transported to the laboratory at 4°C. The plant material was identified at the herbarium of University of Johannesburg (JRAU). The identification of the plant was based on the plant parts collected. The plant specimen was deposited in the herbarium and assigned voucher number Morare-Serepa-Dlamini 1.

Isolation of bacterial endophytes

For isolation of bacterial endophytes, the bulb was the only part utilised by following a protocol by Jasim et al. (2014), which was slightly adjusted to achieve *C. macowanii* bulb surface sterilization. The first outer layer of the bulb (covered with heavy soil) was peeled off and the bulb washed several times with tap water to remove soil on the second layer. The bulb was treated with sufficient volume covering the whole bulb, of Tween 80 with vigorous shaking for 10 min. This was followed by several washes with sterile distilled water, after which the bulb was immersed in 70% ethanol for 1 min with shaking. The ethanol was rinsed off with sterile distilled water and the bulb further sterilized with 1% sodium hypochlorite (NaOCl) for 10 min. The sample was finally rinsed with sterilized distilled water 3 times. The last distilled water rinse was plated on nutrient agar plates as control.

The sterilized bulb outer surface was trimmed off (using sterile blades); the sample was cut into pieces, which were further macerated in phosphate buffered saline (PBS). Serial dilutions of up to 10^{-3} were done using the liquid from the macerated material. From the dilution, a volume of 0.1 mL was spread plated on nutrient agar plates. The control and experiment plates were incubated at 30°C for 2 days and observed daily for bacterial growth. Each bacterial isolate was transferred to sterile nutrient agar plates for purposes of obtaining pure cultures. Glycerol stock cultures for each bacterial isolate were prepared and stored at -80°C for future use.

Morphological identification of bacterial endophytes

Macroscopic methods were used for morphologically identifying endophytic bacteria, the colony shape, size and colour were the first characteristics used for identification. Endophytic bacterial isolates were further identified by traditional Gram stain reaction (Cruikshank et al., 1975) and viewed using a compound bright-field microscope (OLYMPUS CH20BIMF200) with 100x magnification.

Scanning electron microscopy (SEM)

SEM was used to confirm and study the putative bacterial endophyte isolates. This was done following the method reported by Golding et al. (2016). Bacterial endophytes were grown overnight in Luria-Bertani (LB) broth at 30°C in a shaking incubator at 150 rpm. The bacterial suspension of each isolate was centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the pellet cell were rinsed with sterile distilled water and chemically fixed by 1% formaldehyde and 2% of glutaraldehyde (1:1 volume) for 24 h at room temperature (25°C). The samples were serially dehydrated with different concentrations of ethanol (30, 50, 70, 90, 95 and 100%) at 10 min intervals for each concentration. The samples were left to dry overnight in Eppendorf tubes. Dried samples were sputter coated with gold using emscope SC 500 (Goulding et al., 2016), and viewed using the TESCAN VEGA SEM (VG9731276ZA) connected to a monitor.

Molecular identification of bacterial endophytes

Extraction of genomic DNA

Genomic DNA was extracted from obtained pure colonies of each bacterial endophyte isolate using the ZR fungal/bacterial DNA MiniPrep kit (Zymo Research, catalog No R2014) following manufacturer's protocol. The concentration of the extracted DNA was determined using a NanoDrop ND-2000 UV-Vis spectrophotometer (Thermo Fisher scientific, USA).

Table 1. Phytochemical tests for bacterial endophytes extracts.

Phytochemical test	Methods	Observations
Tannins	Add 2-3 drops of 10% FeCl ₃ to 1 ml of endophytes extract	Blackish-blue or blackish-green colour
Alkaloids	Add few drops of Dragendorff's reagent to 1 ml of extract.	Turbidity or precipitation formation
Flavonoids	1 ml of extract + few drops of NaOH	Yellow formation
Saponins	Add few drops of olive oil to 5 ml of extract. shake vigorously	Froth formation
Steroids	1 ml extract + 1 ml of CHCl ₃ . add few drops of conc. H ₂ SO ₄	Reddish brown ring

Polymerase chain reaction (PCR) of the 16S rRNA gene and sequencing experiments

The 16S rRNA gene of each bacterial endophyte was amplified following protocol described by Tsuchida et al. (2002). Briefly, the 16S rRNA gene was amplified using the primers (16S-27F: 5'-AGAGTTTGTATCMTGGCTCAG-3' and 16S-1492R: 5'-CGGTTACCTTGTTACGACTT-3') with 2x PCR master mix with standard buffer. The PCR products and primers were sent for sequencing at Inqaba Biotechnical Company (Pty) Ltd, Pretoria, South Africa.

Phylogenetic analysis

Subsequent to sequencing, the obtained 16S rRNA gene sequences (base pairs) were screened for chimeras using DECIPHER (Wright et al., 2012). The 16S rRNA gene sequences were subjected to BLAST (v.2.6.0) at NCBI to obtain closely related bacterial species. Highly similar sequences with a 96 to 100% identity were aligned with bacterial endophyte sequences isolate using MUSCLE (Edgar, 2004); phylogenetic trees were drawn using the Neighbour-Joining method based on Tamura-Nei model (Tamura and Nei, 1993). Bootstrap values of 1000 replicates were used to determine the tree strength (Pattengale et al., 2009). All evolutionary analysis was performed on MEGA 7 software (Kumar et al., 2016). All obtained sequences of the bacterial endophytes were deposited into GenBank and assigned accession numbers: MF085046 *Staphylococcus* species C2, MF085048 *Staphylococcus* species C3, MF509594 *Bacillus* species C4, MF509593 *Acinetobacter* species C5 and MF509595 *Staphylococcus* species C6.

Production of secondary metabolites by bacterial endophytes

Bacterial endophytes were cultured in 10 Erlenmeyer flasks each containing 500 mL of sterile nutrient broth media, which was shaken at 200 rpm at 30°C for 7 days. An amount of 20 g of XAD-7-HP resin (SIGMA, South Africa, BCBP6698V) was added to the culture after 7 days. The resin was filtered through a cheesecloth, which was washed with deionized water and eluted with 100 ml acetone. The acetone-soluble fraction was dried using a rotary evaporator to yield a crude extract (Hu et al., 2012).

Qualitative analysis of phytochemicals of *C. macowanii* and endophytes crude

Phytochemical screening of *C. macowanii* was adopted from (Trease and Evans, 1983; Harbourne, 1983). Same methods were followed for phytochemical screening of endophytes crude extracts (Trease and Evans, 1983; Harbourne, 1983) with some modifications shown in Table 1.

Antimicrobial activity of the crude extracts from bacterial endophytes

The disc diffusion method as described by Hoelzer et al. (2011) and Zhang et al. (2012) was carried out to evaluate the anti-bacterial nature of the bacterial endophytes' secondary metabolites crude extracts. Five pathogenic bacterial strains (Gram-negative strains *Escherichia coli*, ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 13182; Gram-positive strains *Staphylococcus aureus* NCTC 6571 and *Bacillus cereus* ATCC 10876), were grown overnight at 37°C on Mueller-Hinton (MH) broth which was adjusted to 0.5 McFarland standard. The cultures were further spread plated on MH agar plates and sterilized circular paper discs (6 mm) were placed on the MH agar containing bacterial lawn culture of the test pathogenic strains. The crude extract of endophytes was dissolved in ethyl acetate and a 10 µL of each endophyte crude extracts were aseptically placed on each disc. 10 µL of 1 mg/mL Streptomycin (Sigma, Aldrich, Switzerland, BCBP5897V) was also aseptically placed on one of the paper disk in each plate as a positive control. The plates were incubated at 37°C for 48 to 72 h and the anti-bacterial activity was assessed by measuring the diameter of the zone of inhibition in mm. The anti-bacterial tests were performed in triplicates.

Statistical analysis

The antibacterial data was reported as mean ± standard deviations (SD). Data obtained was analysed using Two-way analysis of variance (ANOVA). The analysis was carried out using the Microsoft Excel 2010 ANOVA. P values < 0.05 were considered statistically different.

RESULTS AND DISCUSSION

Morphological identification of endophytes from *C. macowanii*

C. macowanii bulb was surface-sterilized prior to isolation of bacterial endophytes. The surface sterilization method was adequate as all control plates did not have any microbial growth. Five putative bacterial endophytes were isolated and identified. The bulb was dominated with four Gram positive bacteria and one additional Gram negative bacterial endophyte. The Gram stain reaction further indicated that three of the bacterial endophytes were cocci shaped and the remaining two were rod shaped. The bacterial endophyte shapes were further confirmed by the SEM electron micrographs (results not shown). Bacterial endophytes have been previously reported in

Table 2. Relationship of endophytic bacteria isolates with highly similar genera found in NCBI.

Bacterial isolate codes	Bacterial name	Highly similar genus	NCBI blast homology % of dominant genus	Accession number	Size of 16S rRNA gene (basepairs)
C2	<i>Staphylococcus</i> spp.	<i>Staphylococcus</i>	99	MF085046	199
C3	<i>Staphylococcus</i> spp.	<i>Staphylococcus</i>	100	MF085048	500
C4	<i>Bacillus</i> spp.	<i>Bacillus</i>	100	MF509594	517
C5	<i>Acinetobacter</i> spp.	<i>Acinetobacter</i>	99	MF509593	551
C6	<i>Staphylococcus</i> spp.	<i>Staphylococcus</i>	99	MF509595	505

other medicinal plants including *Catharanthus roseus*, *Ocimum sanctum* and *Mentha arvensis* (Anjum and Chandra, 2015), *Lonicera japonica* (Zhao et al., 2015) and *Ferula songorica* (Liu et al., 2016); however, no bacterial endophytes have been reported in *C. macowanii*.

Medicinal plants including *C. macowanii* have been considered valuable sources of bioactive compounds for drug development. However, due to their destruction and loss, these can no longer be utilised for drug development as there is a high decline in plant populations caused by overexploitation in drug development and other industrial applications (Tomita, 2003). Because endophytes are known to produce similar secondary metabolites as their host plant, these can be isolated, identified and further investigations performed on their produced secondary metabolites for drug development. Even though *C. macowanii* bulbs had varying antimicrobial results (Sebola et al., 2016), it continues to be utilised as traditional medical plant. In order to explore the potential applications of the bacterial endophytes from *C. macowanii*, it was necessary to isolate and identify its bacterial endophytes.

Molecular identification and phylogenetic analysis

The 16S rRNA gene sequence results were used to confirm the bacterial endophyte identification. The BLAST search results indicate that bacterial isolate C2 was closely related to bacterial species belonging to *Staphylococcus* genus, C3 to *Staphylococcus* genus, C4 to *Bacillus*, C5 *Acinetobacter* and C6 *Staphylococcus* genus as indicated in Table 2. *Staphylococcus* was the dominant genus with three species, followed by one of *Bacillus* and *Acinetobacter*. The isolated putative bacterial endophytes grouped with closely related bacterial species on the delineated phylogenetic tree Figure 1. The two *Staphylococcus* C3 and C6 had a sister relationship with 100% bootstrap value. These two species also had a monophyletic relationship with the *Bacillus* C4 isolate. Another monophyletic relationship was observed between *Acinetobacter* spp. C5 and *Acinetobacter johnsonii* PVB6L3 with the *Gamma proteobacterium* PM20.

Bacillus and *Acinetobacter* spp. are part of the 16

genera that have been identified as endophytes (Sekhar and Thomas, 2015; Gouda et al., 2016). These species have been isolated from roots, stem and leaves of *L. japonica* (Zhao et al., 2015) and sterilized roots of *Beta vulgaris* (Shi et al., 2011). Although *Staphylococcus* spp. are established members of the human micro-flora, some species such as *Staphylococcus epidermidis* have been previously reported as plant endophytes (Berg et al., 2005; Kai et al., 2007, 2008; Vendan et al., 2010). Chaudhry and Patil (2016) have indicated that this *Staphylococcus* species are adapted in various hosts and have shown protection and development characteristics to its plant host. These are characteristics of endophytes within their plant hosts.

The bulb of *C. macowanii* was chosen for isolation of bacterial endophytes as it was expected to have more endophytes isolates than the above ground tissues. However, only five bacterial endophytes were isolated in this study, providing limited diversity of bacterial endophytes as compared to other similar studies. These results could be associated with factors other than the selected plant tissue. Jasim et al. (2014) stated that the number of endophytes isolates could differ due to the plant used, age, seasonal collection or the environment. The isolation method used also play a role in number of bacterial endophytes yielded. For this study a maceration of plant tissue was used. Huang et al. (2015) indicated that isolation method whereby plant material is cut into small pieces and placed on growth medium yield highly numerous endophytes.

Phytochemical analysis of *C. macowanii* and bacterial endophytes

The results of phytochemical screening showed that *C. macowanii* bulb constitutes alkaloids, saponins and tannins and contained no flavonoids and steroids. This plant is found in Amaryllideaceae family which contain abundance of various alkaloid components (Tram et al., 2002). Numerous alkaloids were reported by Fennell and Staden (2001) in *Crinum* species. Maroyi (2016) further indicated alkaloids are abundant in bulbs of *Crinum* spp. Sebola et al. (2016) reported that *C. macowanii* bulb contains high amount of alkaloids which confirmed that

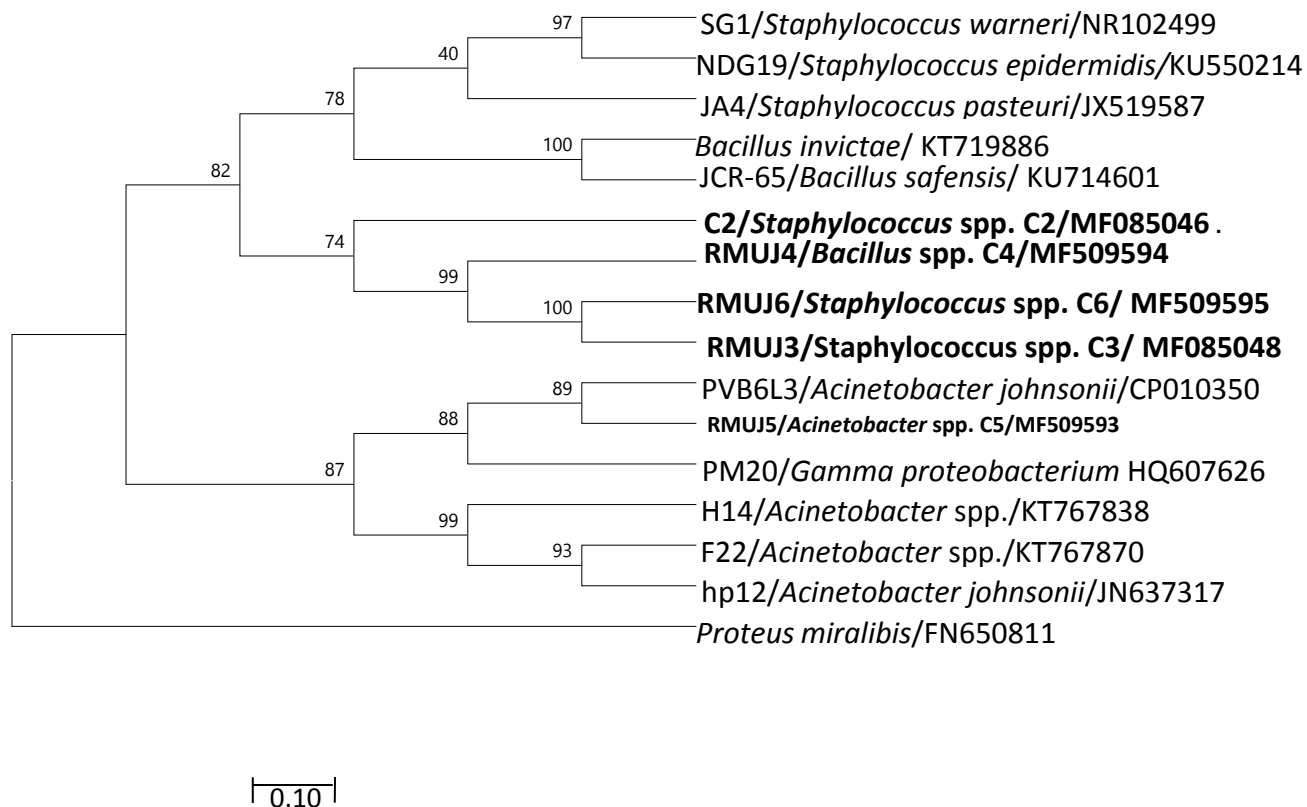


Figure 1. Phylogenetic tree analysis based on 16S rDNA sequences of five endophytic bacteria isolates, with *Bacillus* spp. C4 closely related to *Staphylococcus* species isolated and the *Acinetobacter* spp. C5 unrelated to the *Staphylococcus* species isolates by neighbour-joining method showing an ancestral group of *Proteus mirabilis*.

this species is rich with this kind of metabolites. The alkaloids in *Crinum* spp. have been reported to have therapeutic properties, hence *C. macowanii* and other *Crinum* spp. are used as medicine to treat illness (Fennell and Staden, 2001).

However, the bacterial endophytes isolated from *C. macowanii* have shown different chemical constituents to their host. Bacterial endophyte isolates, *Acinetobacter* spp. C5 and *Staphylococcus* spp. C6 indicated the presence of alkaloids, while *Staphylococcus* spp. C2 and C3 indicated the presence of flavonoids which was not found in *C. macowanii* bulb. *Bacillus* spp. C4 was the only species which contained tannins compounds. The saponins were not found in all the bacterial endophytes, but were present in the plant. There were no steroids in both the plant and their bacterial endophytes.

Antibacterial activity of endophytes extracts against pathogenic strains

The bacterial endophytes showed anti-bacterial activity against selected pathogenic strains as shown in Figure 2. All of the bacterial endophytes had antimicrobial activity

against pathogenic strains (Gram-negative strains *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 13182; Gram-positive *S. aureus* NCTC 6571 and *B. cereus* ATCC 10876). *Bacillus* spp. C4 and *Acinetobacter* spp. C5 had the highest antibacterial activity against *S. aureus*, *E. coli* and *B. cereus*. None of the bacterial endophytes had antibacterial activity against *K. pneumoniae*; this could be due to the resistance mechanism by *K. pneumoniae* such as development of extended-spectrum β -lactamases (ESBLs) (Carlet, 2012; Lin et al., 2016). The ANOVA statistics analysis indicated that p value was less than 0.05 ($p < 0.05$), therefore there was a significant difference between all five bacterial endophytes antimicrobial activity.

Antibacterial activity of endophytes extracts have shown variation as compared to antibacterial activity of *C. macowanii* extracts reported by Sebola et al. (2016). The antibacterial activity of *C. macowanii* had broad inhibition spectrum against pathogenic strains such as *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *S. epidermidis*. Therefore, there is a need to improve the extraction method of extracts from endophytes or increase the concentration of endophytes extracts in

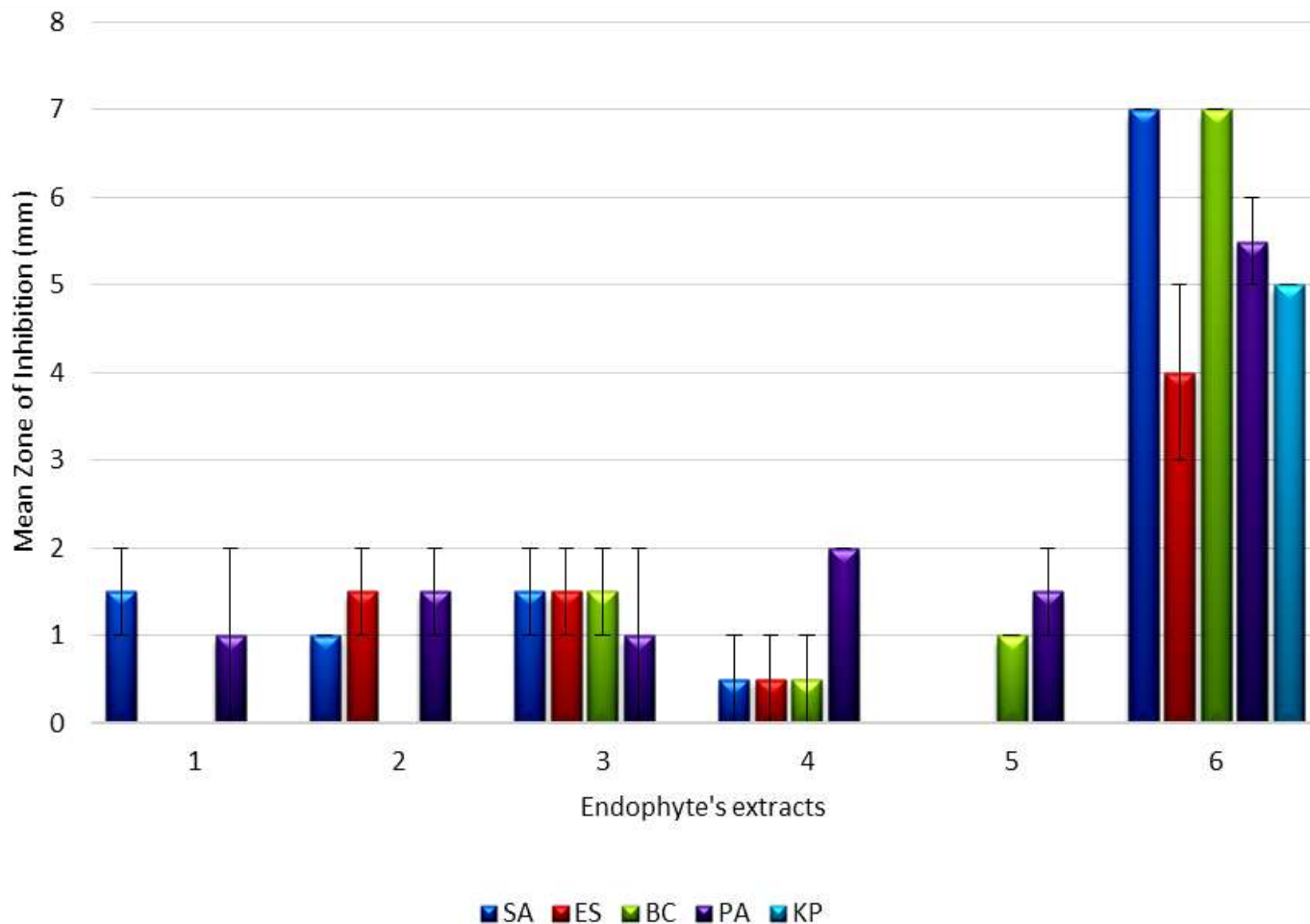


Figure 2. Antibacterial activity of endophytes extracts and streptomycin (positive control) against pathogenic strains. Antimicrobial activity is measured by zone of inhibition (mm) of the pathogenic strains. SA: *Staphylococcus aureus*, EC: *Escherichia coli*, BC: *Bacillus cereus*, PA: *Pseudomonas aeruginosa*, KP: *Klebsiella pneumoniae*. 1, *Staphylococcus C2*; 2, *Staphylococcus C3*; 3, *Bacillus C4*; 4, *Acinetobacter C5*; 5, *Staphylococcus C6*; 6, streptomycin.

antimicrobial activity.

According to Kumar et al. (2015), media composition and culture conditions enhance antibacterial producing ability of microorganisms. The production media must provide components which are source of energy for microorganisms to synthesize bioactive metabolites (Costa et al., 2002). In this study, Nutrient Broth (NB) was used as a growth media for endophytes and the antibacterial activity was low against the tested pathogenic strains. In comparison to a study conducted by Kumar et al. (2015), it was shown that extracts from microorganisms grown in Trypticase Soy Broth (TSB) had significant antibacterial activity, followed by Luria Broth (LB) based extracts with high activity and lastly extracts of microorganisms grown in Nutrient Broth (NB) had low activity. In another study by Malash et al. (2016), the extracts from *Bacillus* spp. grown in LB had higher antibacterial activity, while the extracts of the same species grown in NB had lower activity. It was also shown

that *Pantoea agglomerans* grown in Nutrient Broth produced low amount of bioactive metabolites (Costa et al., 2002). It was stated that yeast extract is a good source of carbon and nitrogen for many microorganisms (Costa et al., 2002; Narayana and Vijayalakshmi, 2008); therefore, LB was seen as the best production medium since it contains yeast extract. The low antibacterial activity in this study was associated with the media used.

The potential of endophytes to inhibit growth of pathogenic strains have shown that these microorganisms have potential in development of therapeutic drugs, furthermore Gram negative and Gram positive test microorganisms were both inhibited in the current study. Strobel and Daisy (2003) have indicated that endophytes are potential source for bioactive compounds which can be used in medical, agriculture and other industries. In the study reported by Sandhu et al. (2014), the significant number of endophytic bacteria isolated from medicinal plants is of great importance due to presence of bioactive

extracts which can be used against pathogenic strains.

Conclusion

The identified endophytes were the first endophytes isolated from *C. macowanii*. *C. macowanii* and its bacterial endophytes have shown slightly similar phytochemical analysis; only two species, *Acinetobacter* spp. C5 and *Staphylococcus* spp. C6 contains alkaloids constituents. The antimicrobial activity of endophytes has shown inhibition effects against the selected pathogenic strains. From these results, it can be concluded that the endophytic bacteria isolated from medicinal plant, *C. macowanii* produces potential bioactive compounds which can be explored further for other biological activities. For future purpose, this study or study similar to this can be improved by using alternative methods for isolation of bacterial endophytes to achieve numerous endophytes with great diversity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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