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GENETIC TRANSFORMATION OF *CERATOTHECA TRILOBA* FOR THE PRODUCTION OF ANTHRAQUINONES FROM HAIRY ROOT CULTURES

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Abstract

Background: *Ceratotheca triloba* was found to contain three anthraquinones (9, 10-anthracenedione, 1-hydroxy-4-methylanthraquinone and 5, 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione [DTP]) in its roots. Inhibition of the human topoisomerase II enzyme is the basis of some currently used cancer drugs such as doxorubicin which is shown to be cardio-toxic. For this reason we decided to investigate anthraquinones from *C. triloba* as a possible anticancer drug, however the main limitation was the large quantities of roots that are required to obtain a good yield of the active compound. Therefore the aim of this research was to obtain a higher yield of anthraquinones in hairy roots cultures than the parent plant as well as to compare yields of hairy root, cell suspension and shoot cultures.
Materials and Methods: Protocols for seed sterilization, seed germination, shoot cultivation, callus induction, *A. rhizogenes* mediated-transformation and hormone supplementations of hairy roots were developed.
Results: The results revealed that stem explants was susceptible to transformation by *Agrobacterium rhizogenes* at a low optical density of 0.2. Induced hairy roots were decontaminated by exposure to cefotaxime at 500mg.l^{-1} for five days and then 200mg.l^{-1} for eight days. Visualization of culture extract profiles by TLC revealed anthraquinones were present in all cultures. Analysis of the culture extracts by HPLC showed the highest yield of anthraquinones was produced in hairy root cultures supplemented with 1-Naphthaleneacetic acid [NAA] (8 mg).
Conclusions: This was a 17 fold increase compared to field roots (0.47 mg). Therefore *C. triloba* hairy root cultures are the preferable biological system for anthraquinones production over shoot (0.13 mg) and cell suspension cultures (0.70 mg).

Keywords: anthraquinones, *Agrobacterium rhizogenes*, *Ceratotheca triloba*, topoisomerase II, hairy root cultures, hormone supplementations

Introduction

C. triloba is an annual plant that is found in the summer rainfall areas of South Africa. The root extract of the plant was found to contain three anthraquinones; 9, 10-anthracenedione, 1-hydroxy-4-methylanthraquinone and 5, 8-dimethoxy-2, 3, 10, 10a-tetrahydro-1H, 4aH-phenanthrene-4, 9-dione (DTP), and one steroid; androst-5-ene-3, 17, 19-triol (Mohanlall et al., 2011). Derivatives of the anthraquinones molecule have shown to exhibit various pharmacological and biological activities which include: anticancer, antibacterial, antitrypanosomal and antineoplastic activities (Baguley, 1991; Monneret, 2001; Dzierzbicka and Kolodziejczyk, 2005; Preobrazhenskaya et al., 2006). Experiments in our lab have revealed that DTP can inhibit the human topoisomerase II enzyme which transforms supercoiled DNA to linear DNA. This mechanism is the basis of some currently used cancer drugs. For example: doxorubicin is used to treat tumors of the mammary gland and gynaecological and haematological malignancies (Preobrazhenskaya et al., 2006). However the use of this compound can be life threatening due to its cardiotoxicity (Hsiao et al., 2008). Recent research has also shown that anthracenedione derivatives induce apoptosis in the human cervical cancer cell line, CaSki (Amin et al., 2009). Until now there has been no effective compound against this type of cancer.

Due to the applications of anthraquinones and the need for alternative anticancer molecules, we decided to conduct further research on the anthraquinones from *C. triloba*. There are certain limitations to extracting this anthraquinone from field grown plants. A large quantity of roots is required to obtain a reasonable yield of the active compound. The plant growth is negatively affected by cold winter conditions and is sensitive to pathogens and insects. Therefore we turned our attention to the *in vitro* production of anthraquinones. In a previous study, cell suspension cultures of *C. triloba* were cultured to elicit an overproduction of anthraquinones, but only 0.02µg.ml^{-1} and 0.75 µg.ml^{-1} were produced in the control and elicited cultures, respectively (Naicker, 2011). This was due to the undifferentiated state of the plant cells which synthesize low concentrations of secondary metabolites (Palazon et al., 2006). Hence in this study we cultured hairy root cultures. These are differentiated plant tissues which are cultured by infecting stem explants with the Gram negative bacterium, *Agrobacterium rhizogenes* which habours the Ri plasmid. The genes on this plasmid direct the transfer of tDNA into the plants genome. This DNA contains genes that are responsible for the hairy root phenotype and auxin and cytokine production that allow for growth without exogenous hormones. These cultures have been successfully employed towards producing important anticancer plant compounds (taxol, vinblastine and vincristine) due to several advantages (Rowinsky and Donehower, 1995; Kim et al., 2009; Ataei-Azimi et al., 2008; Schmelzer and Gurib-Fakim, 2008). Hairy root cultures can synthesize metabolites at a higher level than the parent plant (Pietrosiuk et al., 2007). They can produce metabolites specific to the parent plant as well as novel metabolites that cannot be detected in the mother plant or other plant tissue cultures (Nader et al., 2006). When compared to other *in vitro* cultures they have a higher growth rate and secondary metabolite yield than untransformed cultures (Charlwood and Charlwood, 1991; Flores et al., 1999). Therefore aims of this research were to obtain a higher yield of anthraquinones in hairy roots cultures than the parent plant as well as to compare yields of hairy root, cell suspension and shoot cultures.

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Materials and Methods

Plant Material

Seed pods and roots of *C. triloba* were collected from wild plants in Durban, Kwa-Zulu Natal, South Africa. The plant was identified by using taxonomic keys and a voucher specimen was deposited in the Ward Herbarium at the University of Kwa-Zulu Natal (Westville campus). The seed pods were broken to remove the seeds which were stored in a closed bottle at room temperature. The roots were dried in the laboratory at room temperature for 5-8 days or until they broke easily by hand. These were then ground to a fine powder using a Wareing blender and stored in a closed bottle at room temperature until required.

Seed Germination and Shoot Cultivation

In order to determine the optimum time for effective surface sterilization the seeds were exposed to 30% NaCIO for 0 (control), 5, 10, 15 and 20 minutes. The seeds were then washed with sterile distilled water and placed in a Petri dish containing solid MS medium (5 seeds per plate) (Murashige and Skoog, 1962). A replicate of 5 Petri dishes were prepared for each exposure time. The Petri dishes were incubated under visible light at room temperature. The seeds were examined for contamination after two weeks and the treatment regime that produced the highest percentage of sterile seeds was used for germination seeds to produce shoot cultures for the *in vitro* transformation experiment.

The germinated seedlings (3 weeks old) were cultivated into shoot cultures in 12 cm culture vessels containing MS medium which was supplemented with 1 $mg.l^{-1}$ of 6-Benzylaminopurine [6-BAP] (Sigma-Aldrich, Inc.) and 0.5 $mg.l^{-1}$ of Indole-3-acetic acid [IAA] (Sigma-Aldrich, Inc.). The seedlings were incubated for 45 days in a growth chamber at 26˚C under standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}m^{-2}$ and a 16 h photoperiod.

Call us Induction and Preparation of Cell Suspension Cultures

The leaves of *in vitro* shoot cultures were removed and cut into 1 cm square disks by using a scalpel. They (5 leaf disks per plate) were placed on MS medium supplemented with 1 $mg.l^{-1}$ of 2.4-D and 1 $mg.l^{-1}$ of 6-BAP (Sigma-Aldrich, Inc.). These plates were then placed in a cardboard box and incubated in the dark phase at 26˚C for 4 weeks. Induce calli were transferred onto fresh medium and maintained by sub-culturing at 3 week intervals.

Inoculum for the cell suspension cultures were initiated by transferring approximately 1 g of callus (three weeks old) from the second sub-culture into four 1000 ml Erlenmeyer flasks containing 50 ml of MS liquid medium which was supplemented with 1 $mg.l^{-1}$ of 2.4-D and 1 $mg.l^{-1}$ of 6-BAP. The flasks were agitated on a shaker (Infors Ecotron, Polychem supplies cc) at 100 rpm at 26˚C in the dark phase for one week. Thereafter, 150 ml of MS medium was added to the inocula and the flasks were incubated for a further 2 months. At the end of cultivation period the cell mass of suspension cultures was determined by centrifuging 50 ml volumes at 4000 rpm for 10 minutes at 20˚C. The total wet weight of the cell suspension cultures was determined by the following equation: (mass of beaker + cell mass) – (mass of beaker) = total wet weight of cell suspension cultures from four flasks.

Production of Hairy Root Cultures

Cultivation of *A. Rhizogenes*

A. Rhizogenes strain 15834 was provided on yeast extract peptone (YEP) agar plates by the Council of Scientific and Industrial Research (CSIR), South Africa. Plate cultures of *A. rhizogenes* were prepared by streaking the culture onto YEP agar (An *et al*., 1988). The strain was cultivated for 24 hours at 30°C. A loopful of this culture was used to inoculate a 250 ml Erlenmeyer flask containing 50 ml of YEP liquid medium. The flasks were placed on a shaker and incubated for 24 hours at 30˚C. The optical density (OD) of the culture was determined spectrophotometrically at 600 nm and the culture was diluted with YEP medium to an OD of 1, 0.5 and 0.2 for the transformation experiment.

Hairy Root Induction in *C. Triloba* Using *A. Rhizogenes*

Shoots of the *in vitro* grown plantlets were cut at the stems and the leaves and stems were used as explants. These were inoculated with a sterile scalpel dipped in three concentrations (OD of 1, 0.5 and 0.2) of the *A. rhizogenes* culture. Leaf explants were inoculated by pricking and stem explants were cut. Explants inoculated with a scalpel dipped in YEP medium served as the control. The infected and control explants were placed on MS medium (containing no hormones) and incubated for 3-4 weeks at 26˚C under standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}m^{-2}$ and a 16 h photoperiod. The response of the explants to Agrobacterium in terms of hairy root emergence was measured by determining the transformation efficiency: (number of hairy roots/number of explants) X 100. Induced hairy roots were excised and then decontaminated by treating them with 500 $mg.l^{-1}$ of cefotaxime for five days, and thereafter with 200 $mg.l^{-1}$ of cefotaxime for eight days. The sterility of the hairy roots was confirmed by placing 1 cm of the root onto a YEP plate which was incubated at 30˚C for three days. Once the roots were confirmed to be sterile the hairy root cultures were placed on MS medium plates and maintained by sub-culturing onto fresh medium at three week intervals.

Effect of Auxins on Hairy Root Biomass Production

In order to improve the growth of hairy roots two auxins were used, NAA at 1 $mg.l^{-1}$and IAA at 1 $mg.l^{-1}$. Inoculum cultures were prepared

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by transferring approximately 200 mg hairy roots into 1 L flasks containing 30 ml of MS medium. Three flasks containing medium with no hormone served as the control cultures. All cultures were incubated at 26˚C in a shaker under standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}m^{-2}$ and a 16 h photoperiod. An aliquot of 70 ml of medium was added to the inoculum cultures after 3 weeks and a final volume of 100 ml of medium was added to the flasks after a further 3 weeks. Cultures were then incubated for 4 weeks. Thereafter the biomass was removed from the medium using a forceps and the increase in wet weight was determined by the following equation: (final wet weight – initial weight = increase in wet weight).

Examination of the Morphological Characteristics of Adventitious Roots and Hairy Roots

Shoot cultures were sub-cultured on Petri plates containing MS medium supplemented with 1 $mg.l^{-1}$ of IBA to induce adventitious roots. Induced hairy roots were transferred onto plain MS medium and MS medium supplemented with 1 $mg.l^{-1}$ of NAA. The plates were incubated at 26˚C under standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}m^{-2}$ and a 16 h photoperiod. The morphology of adventitious roots and hairy roots were examined according to color and branching of the lateral roots.

Extraction and Analysis of Anthraquinones

The preparation of the field roots, shoot, cell suspension and hairy root cultures for extraction varied but the preparation of the different extracts was the same. This is outlined below:

Field roots: the ground material of the field roots was extracted with hexane by agitation on a shaker at 180 rpm for 24 hours at room temperature.

Shoot cultures*:* the shoot cultures were dried at 30˚C in an oven and crushed to a fine powder in a Waring blender. The powder was extracted in 100 ml of hexane at room temperature by agitation on a shaker at 180 rpm for 24 hours.

Cell suspension cultures*:* The cell mass was harvested by centrifuging 50 ml volumes of suspension culture at 4000 rpm for 10 minutes at 4˚C. The cell mass pellet was disrupted by sonication (Virsonic, Virtis) at 4 psi for 10 minutes. The cell mass (used to obtain the intra-cellular extract) and the supernatant (used to obtain the extra-cellular extract) were agitated in 100 ml of hexane in 1 L flasks on a shaker at 180 rpm for 24 hours at room temperature.

Hairy root cultures*:* The hairy root biomass was macerated using a scalpel. The macerated root mass (used to obtain the intra-cellular extract) and the supernatant (used to obtain the extra-cellular extract) were extracted in 200 ml of hexane on a shaker at 180 rpm for three days at room temperature.

Preparation of the intra-cellular extracts involved using the following protocol. Filtration was used to separate the powdered plant material, cells and macerated hairy roots from the hexane extract which was then concentrated by using a roto-evaporator (Heidolph Laborota 400 efficient) with the water bath set at a temperature of 50°C and the flask rotated at 60 rpm. The residue was dissolved in 10 ml of hexane and transferred to a glass bottle which was covered with foil to prevent light from degrading the compounds. The extract was dried under an air current for 3 days and used for chromatographic analyses. For the extra-cellular extract the hexane fraction was slowly poured out into a flask and concentrated as described above.

Profile of Compounds and Detection of Anthraquinones in the Field Root Extract and in Plant Cell and Tissue Culture Extracts by TLC

Thin layer chromatography (TLC) was performed to compare the compounds and detect anthraquinones in *C. triloba* field root extract and plant cell and tissue culture extracts by using commercial standards; 9,10-Anthracenedione and 1-Hydroxy-4-methylanthaquinone (Sigma-Aldrich, Inc) (1 $mg.ml^{-1}$ dissolved ethyl acetate). Approximately 10 µl of each standard solution, 50 µl of the field root extract and plant cell and tissue culture extracts were applied to the TLC silica gel plate (Merck TLC F_{254}). Hexane: ethyl acetate (90:10) was used as the mobile phase. The developed TLC plate was visualized under ultraviolet light at 312 nm and 264 nm (Camag Universal UV lamp TL-600). The TLC plate was also sprayed with p-anisaldehyde solution (13.31 ml of anisaldehyde in 250 ml of ethanol and 2.5 ml of H_2SO_4) and heated at 120˚C in an oven for 15 minutes as described by Wagner *et al*. (1984)

Quantification of Anthraquinones by HPLC

HPLC analysis was carried out according to the method described by Fernand *et al.* (2008) using commercial standards. A standard curve was generated with 10, 20, 50, 5, and 2 $\mu g.ml^{-1}$ of 9, 10- anthracenedione and 1-hydroxy-4-methylanthaquinone (Sigma-Aldrich, Inc.) which were dissolved in ethyl acetate. The field root extract, hairy root, shoot and cell suspension culture extracts were dissolved in ethyl acetate and filtered for HPLC analysis. Separation and quantitative analyses of anthraquinones were performed on a Merck- Hitachi LaChrom system (Darmstadt, Germany) consisting of a D7000 system controller, four pumps (D7400), a Merck- Hitachi LaChrom (L-7200) auto injector and an Merck- Hitachi LaChrom (L-7200) UV-VIS detector (λ = 260 nm). Separation of the analytes was performed at 40 ˚C on a Licrospher C18 (2) column, 100 ˚A pore size, 5µm particle size, 250×4.6 mm i.d.column containing a guard column (Merck, Darmstadt, Germany). The analytes were eluted isocratically at a flow rate

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of 0.4 ml.min^{-1} using an acetonitrile/methanol/buffer (25:55:20, v/v). The buffer used as 10 mM ammonium acetate at pH 6.8. The injection volume was 10 µL.

Results and Discussion

Seed Germination and Shoot Culture

The optimum exposure time to NaClO for maximum seed germination and minimum contamination was determined by exposing the *C. triloba* seeds to 30% NaClO for 0, 5, 10, 15 and 20 minutes. It was found that the percentage of seed germination decreased as the exposure time of the seeds to NaClO increased (**Fig 1**). This could be due to damage of the seed by NaClO. The best exposure time for sterilization of *C. triloba* seeds with 30 % NaClO was 10 minutes as 26 % of seeds germinated and only 6 % was contaminated. The sterilized seeds germinated on MS medium within two to three weeks. The seedlings developed into shoots after 45 days.

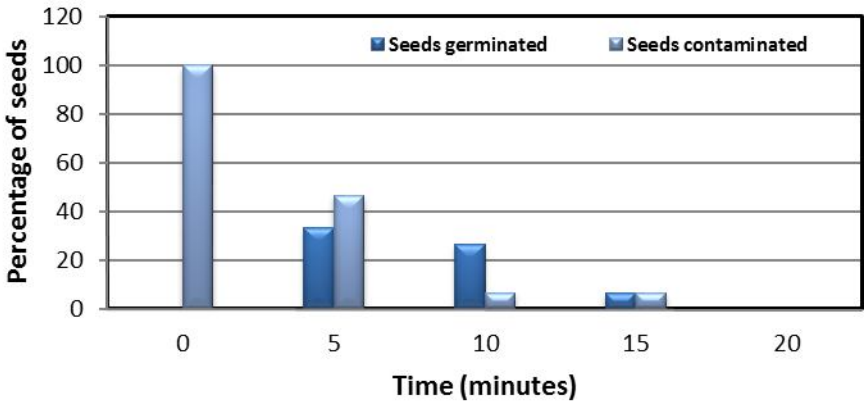

Figure 1: Percentage of seeds that germinated and the percentage of seeds that were contaminated after treatment with 30% NaClO at different time intervals.

Call us and Cell Suspension Cultures

Callus initiation was observed on the surface or cut ends of the leaf explants after 2-3 weeks of inoculation. Callus cultures induced from *C. triloba* leaf explants on MS medium were orange-yellow in color. When callus cultures were transferred into flasks containing MS liquid medium, the friable callus tissue dispersed into small aggregates after the flasks were agitated on the shaker. This allowed for semi-homogenous cell suspension cultures to form for scaling up to 200 ml. After two months of cultivation the cell suspension cultures turned from orange to brown in color. This phenomenon was observed in *Morinda elliptica* cells which turned brown when anthraquinones were produced (Abdullah *et al*., 1998). Thus the browning of these plant cell cultures can indicate the time to harvest the cells from suspension cultures for extraction of secondary metabolites.

Induction of Hairy Roots

The transformation of *C. triloba* to produce hairy roots was dependent on the type of explant used (young tissues of sterile plantlets, hypocotyl segments, cotyledons, petioles and young stems and leaves are normally used). In this study stem and leaf explants of shoot cultures were used and it was found that stem explants gave the highest transformation efficiency (73 %) while leaf explants were not responsive to transformation by *A. rhizogenes* (**Table 1, Fig 2A and 2B**). The concentration of *A. rhizogenes* used was also significant as it influenced the survival of the explants. A low concentration of *A. rhizogenes* (culture with an optical density of 0.2) transformed the stem explants whereas high concentrations (culture with an optical density of 0.5 or 1) killed the explants (**Table 1**). The concentration of cefotaxime used to decontaminate the induced hairy root cultures was also significant. Induced hairy roots were disinfected by transferring onto MS medium containing 500 mg.l^{-1} of cefotaxime. Hairy roots kept on this medium for more than five days resulted in browning and they stopped growing. Therefore, they were transferred onto MS medium containing a lower concentration of cefotaxime (200 mg.l^{-1}). After eight days on this medium, the sterility test showed that the hairy roots were sterile as no bacterial colonies grew on YEP solid medium.

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Table 1: Concentration of *A. rhizogenes* used to transform *C. triloba* and the transformation efficiency of leaf and stem explants.

Explant	Concentration of bacteria (OD)	No. of explants transformed	Transformation efficiency (%)
Leaf	Control	None	0
	1	None	0
	0.5	None	0
	0.2	None	0
Stem	Control	None	0
	1	None	0
	0.5	None	0
	0.2	11	73.33

Figure 2: *C. triloba* hairy roots were induced on MS medium and became highly branched after 4 weeks (A). The hairy roots emerged from stem explants (B).

Morphological Characteristics of Adventitious and Hairy Roots

Hairy roots cultured on MS medium containing no hormone were slow growing. Therefore, the morphology of adventitious roots (non-transformed roots) and hairy roots were examined to ensure that the hairy roots induced were as result of Agrobacterium transformation. Adventitious roots were induced in *C. triloba* shoot cultures using MS medium containing 1 $mg.l^{-1}$ of IBA Morphologically, these roots were highly branched and were white in colour. In contrast, the hairy roots cultured with no hormone or with NAA were less branched and turned brown as the biomass increased in the plate. Hence, based on the morphological features it can be assumed that in this study the induced hairy roots resulted from Agrobacterium transformation.

The Effect of Auxins Hairy Root Biomass

Hairy root cultures containing no hormone did not grow well as the entire hairy root biomass turned brown after four weeks. This result contradicted the findings of Estruch *et al*. (1991) who indicated that hairy roots can growth rapidly in hormone free medium. The mean doubling time of hairy roots after inoculation varies from 24 to 90 hours, but in some cultures this time is even longer, for example the doubling time of *Galphimia glauca* hairy roots was 6 days (Nader *et al*., 2006) and as long as 15 days has been reported for *Cinchona* hairy roots (Geerlings *et al*., 1999). Thus great variations exist from one species to another. It has been reported that the improvement of the culture medium can increase the growth rate of slow growing hairy roots as well as the yield of secondary metabolites. In our study we added auxins; IAA or NAA into the medium. Cultures containing either one of these hormones turned brown while their lateral roots were yellow in color and continued growing). Moreover the mean biomass of these cultures increased by 23.21 and 44.79 times, respectively in comparison to the control culture. Thus MS medium containing NAA produced the highest mean biomass (46.13 g) (**Fig 3**). A study by Balvanyos *et al*. (2001) also showed that the addition of NAA into the medium increased the biomass production of *Lobelia inflata* L. hairy root cultures.

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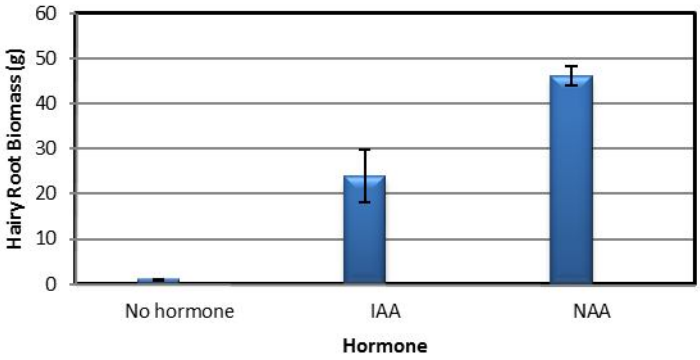
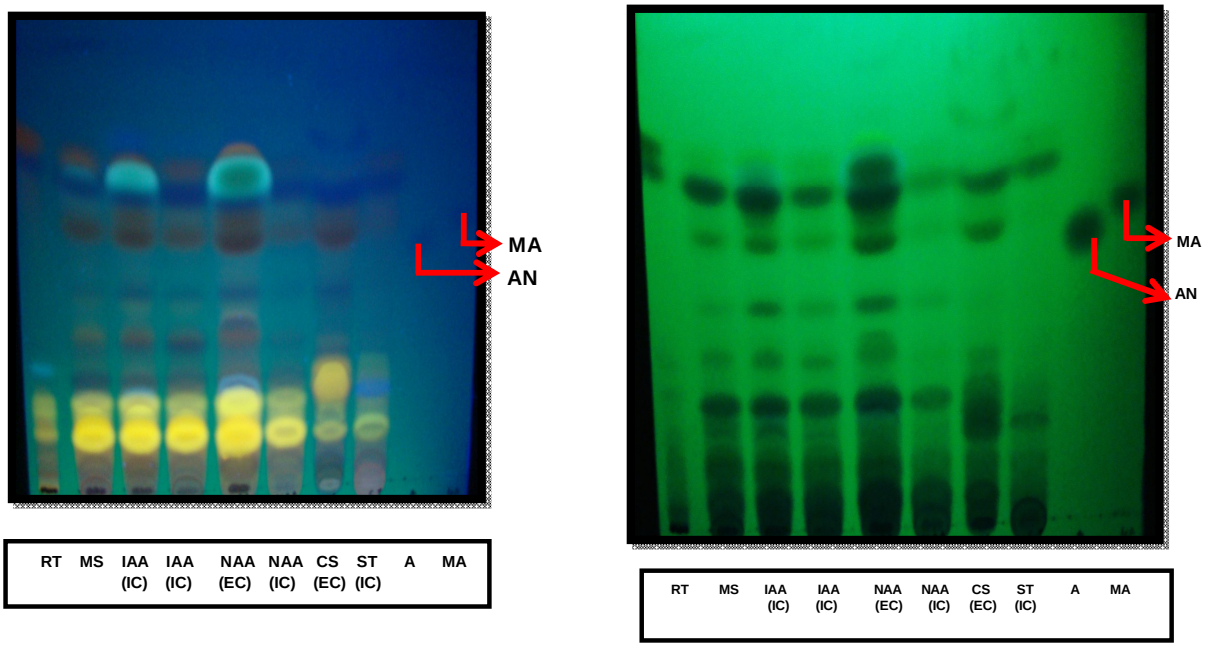


Figure 3: The biomass of hairy root cultures were cultivated in MS medium containing no hormone (control) and 1 mg.l^{-1} of IAA or 1 mg.l^{-1} of NAA



A

B

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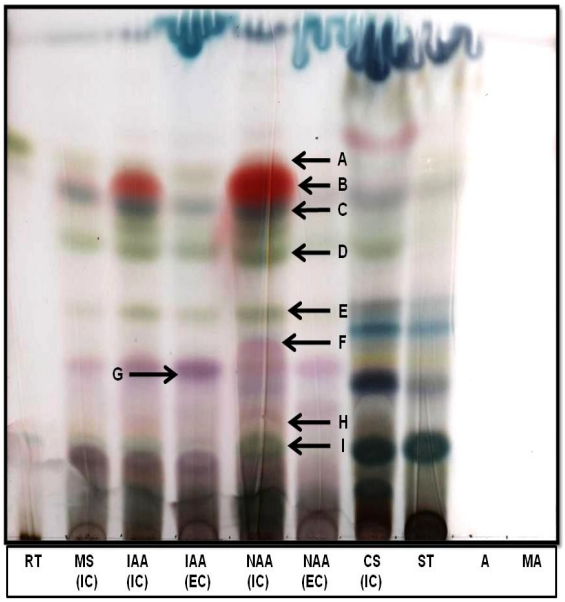

C

Figure 4: Separation of compounds and detection of anthraquinones from the field root extract and plant cell and tissue culture extracts of *C. triloba*. The TLC profiles of the field root extract and hairy root, shoot and cell suspension culture extracts of *C. triloba* were compared under UV light at 312 nm (A) and 264 nm (B) and by using p-anisaldehyde spray reagent (C). Field root extract (RT) (lane 1). Hairy root extract from the cultures cultivated with no hormone (lane 2), hairy root extracts from the cultures supplemented with IAA or NAA (lane 3-6). Cell suspension culture extract (CS) (lane 7). Shoot culture extract (ST) (lane 8). 9, 10-Anthracenedione (AN) (lane 9), 1-Hydroxy-4-methylanthaquinone (MA) (lane 10). Intra-cellular extract (IC), Extra-cellular extract (EC).

Profile of Compounds and Detection Of Anthraquinones in the Field Root Extract and Plant Cell and Tissue Culture Extracts by TLC

The TLC and R*f* value profiles of the field roots and hairy root, shoot and cell suspension cultures are shown in **Fig 4**. Different visualization methods were used as not all bands were visible when only one method was used. For instance band A was only shown clearing on the TLC viewed under UV light at 312 nm. The TLC viewed at 264 nm showed the standards clearly. This allowed for the Rf values of 9, 10-Anthracenedione (0.55) and 1-hydroxy-4-methylanthaquinone (0.60) to be compared to the R*f* values of the bands in the extracts. The R*f* value of band D (0.55) in the hairy root and cell suspension culture extracts correlated to R*f* value of 9,10-Anthracenedione. There were three other bands that had a R*f* value similar to1-hydroxy-4-methylanthaquinone. These were: band A (0.71) (**Fig 4A**) which was present in field root and hairy root extracts, band B (0.70) (**Fig 4B**) which was present only in hairy root culture extracts and band C (0.64) (**Fig 4B**) which was present in the field root and hairy root, shoot and cell suspension culture extracts. The TLC sprayed with p-anisaldehyde showed most of the bands present the extracts, however the standards were not visible (**Fig 4C**). Thus the TLC results gave indication that anthraquinones were present in the extracts.

Quantification of Anthraquinones by HPLC

HPLC analysis showed that the 9, 10-Anthracenedione and 1-Hydroxy-4-methylanthaquinone standards eluted at retention times (R_t) of 3.72-3.75 minutes and 4.07-4.12 minutes, respectively (**Table 2**). The field root extract showed peak at 3.57 minutes. The hairy root culture extracts showed a peak between 3.60-3.70 minutes. The shoot and cell suspension cultures extracts showed a peak at 3.55 and 3.68 minutes, respectively. Thus anthraquinones were present in the plant cell and tissue culture extracts. However, individual anthraquinones were not identified due to co-elution and therefore, the total amount of anthraquinones was calculated.

Table 2: Retention times of anthraquinones from *C. triloba* field root, plant cell and tissue culture extracts

Sample/Standard	9.10-Anthracenedione Retention Time (R_t) – minutes	1-Hydroxy-4-methylanthaquinone Retention Time (R_t) - minutes
Standard 9.10-Anthracenedione	3.72	
Standard		4.07

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1-Hydroxy-4-methylanthaquinone		
C. triloba field root extract	3.57	-
Hairy root (cultivated in MS medium without hormone) intra-cellular extract	3.60	-
Hairy root (cultivated in MS medium supplemented with IAA) intra-cellular extract	3.62	-
Hairy root (cultivated in MS medium supplemented with NAA) intra-cellular extract	3.70	-
Shoot culture extract	3.55	-
Cell suspension culture intra-cellular extract	3.68	-

The concentration of anthraquinones in *C. triloba* field root extract and plant cell and tissue culture extracts were calculated by using the standard curve of 9,10-Anthracenedione (Table 3). Hairy root cultures supplemented with IAA (125.03 µg.mg^{-1} [intra-cellular extract]) or NAA (98.25 µg. mg^{-1} [intra-cellular extract]) produced a higher concentration of anthraquinones compared to the control culture (13.33 µg.mg^{-1} [intra-cellular extract]). This was a 9.38 and a 7.37 fold increase, respectively. Also hairy root cultures supplemented with IAA or NAA produced a higher concentration of anthraquinones compared to the field roots of *C. triloba* (33.51 µg. mg^{-1}). This was a 3.73 and a 2.93 fold increase, respectively. This is possible as there is evidence that the secondary metabolites produced by hairy roots in culture are same as those usually synthesized in the intact parent roots and have similar or higher yields. For instance similar results were obtained in *C. roseus* hairy root cultures (Pietrosiuk *et al*., 2007). Studies show that the biosynthesis of secondary metabolites in hairy root cultures is directed by the effect of the oncogenes (the four *rol* genes A, B, C and D) (Schmülling *et al*., 1988; Petersen *et al*., 1989; Estruch *et al*., 1991). For example, it has been reported that a correlation exists between the expression of the *rol*C gene and the production of *C. roseus* alkaloids (Palazon *et al*., 1998). Furthermore a study reported by Bulgakov *et al*. (2002) showed that an association exists between the *rol* genes and anthraquinone production. In this study callus cultures of *Rubia cordifolia* were transformed with *rol* genes and the level of anthraquinones (purpurin and munjistin) was as high as 4.8% of the dry weight extract. This was much higher than the yields reported by Mischenko *et al.* (1999) for *R. cordifolia* field roots (0.2% of the dry weight extract) and non-transformed callus cultures (0.62-1.22% of the dry weight extract). The *rol* genes have also been considered to be potential activators of secondary metabolism in transformed plants as shown in Solanaceae, Araliaceae, Rubiaceae, Vitaceae and Rosaceae families (Bulgakov, 2008). Thus, the effect of the *rol* genes on secondary metabolism may probably be the reason for the higher anthraquinone yields in hairy root cultures of *C. triloba*.

Shoot (3.23 µg.mg^{-1}) produced a lower concentration of the anthraquinones in comparison to the field roots and hairy roots (supplemented with hormones). According to literature shoot cultures can produce high yields of secondary metabolites for example; *Frangula alnus* and *Frangula rupestris* shoot cultures (Kovačević and Grubišić, 2005). However, some shoot cultures produce lower quantities of secondary metabolites than the parent plant for example; *Gentianella austriaca* shoot culture (Vinterhalter *et al*., 2008).

Cell suspension cultures (13.17 µg.mg^{-1}) also produced a lower concentration of the anthraquinones in comparison to the field roots and hairy roots (supplemented with hormones). A major limitation of cell suspension cultures is their lack of ability to synthesize secondary metabolites at the same level as the intact parent plant. This could be due to the lower degree of differentiation and organization of cell suspension cultures compared to organ cultures, and the location of key enzymes involved in the biosynthetic pathways (Palazon *et al*., 2006). There are certain genes and proteins that are required to produce the secondary metabolites of interest. For example, the expression *pmt* gene is essential for the synthesis of scopolamine and the expression of this gene is specific to the pericycle of the roots (Suzuki *et al*., 1999). In the case of *C. triloba* the anthraquinones of interest are predominantly synthesized in the roots and thus root differentiation of the plant tissue is required to express certain genes in order for high yields of anthraquinones to be synthesized.

Table 3: Concentration and yield of anthraquinones from *C. triloba* field root and plant cell and tissue culture extracts

Plant material	Hormones	Extract (mg)	Anthraquinones (µg.mg^{-1})	Yield of anthraquinones in the extract (mg)	Fold increase (comparison to field roots)
Natural roots		14	33.51	0.47 ± 0.11*	-
Hairy roots	No hormones (control) (IC)	37.7	13.33	0.50 ± 0.09	-
	IAA (IC)	38.8	125.03	4.85 ± 0.17	3.73
	IAA (EC)	13.3	0.0037	0.00005 ± 0.05	-
	NAA (IC)	81.4	98.25	8.00 ± 0.25	2.93
	NAA (C)	5.3	117.23	0.62 ± 0.13	3.50
Cell suspension	2,4-D and 6-BAP (IC)	53.1	13.17	0.70 ± 0.14	-
Shoot culture	6-BAP and IAA	39.6	3.23	0.13 ± 0.09	-

IC (Intra-cellular extract), EC (Extra-cellular extract). Fold increase is representative of the increase in the concentration of anthraquinones found in hairy roots cultures compared to *C. triloba* field roots. * n=3

In this study, we established the *C. triloba* hairy root system that exhibits the biosynthetic capacity for anthraquinones production. This was

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achieved by obtaining the following objectives. Protocols have been developed for the surface sterilization and germination of *C. triloba* seeds for the cultivation shoot cultures to serve as a source of explants for transformation. Hairy root cultures were induced by employing a low cell density inoculum of *A. rhizogenes* and antibiotic decontamination to induced cultures. Improvement of the biomass yield of the hairy root cultures by hormone supplementation; allowed for the comparison of the TLC profiles of the hairy root culture extracts to the field roots, shoot and cell suspension culture extract profiles. Also the highest yield of anthraquinones was produced in hairy root cultures supplemented with NAA (8 ± 0.25 mg). This was a 17 fold increase compared to field roots (0.47 ± 0.11 mg). Therefore *C. triloba* hairy root cultures are the preferable mode of anthraquinones production over shoot and cell suspension cultures.

Conclusion

This is the first study that shows the induction of *C. triloba* hairy root cultures and compares concentration of anthraquinones from *C. triloba* hairy root extracts to the field root, shoot and cell suspension culture extracts. The levels of anthraquinones produced by hairy root cultures showed a 17 fold increase as compared to the filed roots. Further optimization of culture parameters (the selection of *A. rhizogenes* strain, medium composition, influence of pH and temperature and the effect of elicitors) can allow for the scale-up of the cultivation of *C. triloba* hairy roots for the mass production of anthraquinones in bioreactors. For example, *Catharanthus trichophylleus* hairy roots were cultivated at a 20 L scale and 17 monomeric indole alkaloids were isolated including vindoline, ajmalicine, lochnericine and tabersonine (Pietrosiuk *et al*., 2007). Advances in bio-techniques and plant tissue culture technology have provided new means for commercial processing of medicinal plants and their valuable phytochemicals. For example, the German company ROOTec has specialized in the large-scale cultivation of hairy root cultures for the production of valuable secondary metabolites such as camptothecin and podophyllotoxin (anticancer drugs) (Shuler and Kargi, 1992).

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