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Genetic transformation of the medicinal plant *Salvia runcinata* L. f. using *Agrobacterium rhizogenes*

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ABSTRACT

Two different agropine strains (A4T and LBA 9402) of *Agrobacterium rhizogenes* were tested for their capacity to induce hairy roots on *Salvia runcinata* L. f. explants. Both nodal and leaf explants were examined and hairy roots formed 3 to 4 weeks after inoculation. Although the LBA 9402 strain produced significantly more roots on each explant compared with the A4T strains, at the time of transformation, stable cultures could only be established using the A4T strain at a frequency of 4.17%. Putative transgenic roots exhibited a faster growth pattern when compared with wildtype roots. Out of the roots established, we continued the study with two clones that showed typical features of the hairy root syndrome. Amplification of the *rol* A, *rol* B, *rol* C and *ags* gene (showing 300, 400, 600 and 1600 bp bands after electrophoresis, respectively) was positive using the polymerase chain reaction (PCR) and reverse-transcriptase-polymerase chain reaction (RT-PCR). Moderate antifungal activity against two *Fusarium* strains was exhibited by extracts generated from transgenic clones. We were also able to show the presence of some caffeic acid metabolites. These biochemicals are known to exhibit various biological activities, and therefore, the transgenic hairy roots of *S. runcinata* may be an alternative source of these industrially useful chemicals.

1. Introduction

Hairy root cultures have many advantages which have promoted their use as potential sources of secondary metabolites (Cônsolli et al., 1995; Christey, 2001). These transformed root cultures generated after integration of *rol* genes, typically transferred by *Agrobacterium rhizogenes*, are often phenotypically and genetically stable (Giri and Narasu, 2000). They may display a rapid growth rate independent of any source of exogenous hormone and are characterised by their lack of geotropism (Giri and Narasu, 2000; Christey and Braun, 2004; Georgiev et al., 2007). At times, hairy roots of some plants may produce novel secondary metabolites that are not normally present in plants, and therefore, represent a possibility for the discovery of new biologically important compounds (Dhakulkar et al., 2005). These transgenic root cultures are easy to maintain and exhibit about the same or a greater biosynthetic capacity for secondary metabolite production compared to non-transgenic mother plants (Kim et al., 2002; Ashraf et al., 2015). For these reasons, researchers have utilised them as a study tool as they hold higher potential for commercial production purposes for secondary metabolites. The strategy of hairy root production may facilitate the move from laboratories to large-scale industrial production through the use of bioreactor systems (Guillon et al., 2006).

Hairy root cultures of *Salvia* plant species using *A. rhizogenes* were initially established in plants such as *Salvia miltiorrhiza* (Zhi and Alfermann, 1993), *Salvia officinalis* (Grzegorczyk et al., 2006), *Salvia sclarea* (Kuźma et al., 2006; Walencka et al., 2007) and *Salvia tomentosa* (Marchev et al., 2011) for the production of mainly diterpenes, phenolic acids and flavonoids. However, more studies have focused on the Chinese species, *S. miltiorrhiza* for producing tanshinones as these chemicals are implicated in treating a variety of health problems such as cancers and cardiovascular diseases (Zhi and Alfermann, 1993) and they accumulate in the roots. Recent reports indicate successful transformations in other species such as *Salvia austrica* (Kuźma et al., 2011); *Salvia sclarea* (Vaccaro et al., 2014); *Salvia castanea* (Li et al., 2016) and *Salvia wagneriana* (Ruffoni et al., 2016). In our laboratories, we have an interest in the African *Salvia* species and *Salvia runcinata* is one of 26 indigenous African sages that are found scattered within the southern African region. The plants are utilised as traditional medicines to treat a variety of diseases. These plants are known to possess antioxidants and they may have strong antimicrobial and anti-inflammatory activities (Kamatou et al., 2008). They are also effective against several important fungal pathogens (including *Botrytis cinerea* affecting many plant species;

Abbreviations: ANOVA, analysis of variance; CTAB, cetyltrimethylammonium bromide; GC–MS, gas chromatography–mass spectrometry; MIC, minimum inhibitory concentration; MS, Murashige and Skoog (1962); PCR, polymerase chain reaction; PGR, plant growth regulator; rpm, revolutions per minute; RT-PCR, reverse transcriptase polymerase chain reaction; YEP, yeast extract peptone.

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Candida albicans having potential to cause oral thrush and systemic infections in humans; *Fusarium* species detrimental to both plants and animals, just to mention a few) (Dulger and Hacioglu, 2008). There is thus potential for use of these extracts for human disease management, but exploitation in agriculture as agrochemicals is also possible as *Fusarium* spp. cause major losses to various crops that are utilised as staples in African diets. In cases where the immune system is highly compromised by diseases such as HIV/AIDS, which has been a major killer in South Africa, a patient's susceptibility to *Fusarium*-related diseases increases (Esnakula et al., 2013). *Fusarium* infections are also linked to development of oesophageal and stomach cancers as they produce mycotoxins (Nkomo et al., 2014).

Most of the South African *Salvia* species are confined to the Cape region (Kamatou et al., 2008) but *S. runcinata*, a perennial erect herb that grows from 0.15 to 0.5 m tall is found in South Africa; and also in the neighbouring countries of Botswana and Lesotho (Codd, 1985). Kamatou et al. (2010) studied the essential oil chemistry of several different populations of *S. runcinata* and related sister species and found chemical variability. *S. runcinata* produces a volatile oil that contains the commercial metabolite, epi-α-bisabolol, amongst other terpenoids. Epi-α-bisabolol is important as it is included in skin care products. Other compounds that are of significant interest in this species, as they accumulate at higher levels, are caffeic acid derivatives. These phenolic acids (for example caffeic acid, rosmarinic acid, salvianolic acids, sagecoumarin, lithospermic acids, sugernic acid and yunnaneic acids; amongst others) (Lopresti, 2017) are known to possess antibacterial and antifungal activities (Nkomo et al., 2014; Rus et al., 2015; Medjahed et al., 2016; Lopresti, 2017). Because these plants produce terpenoids and phenolics, that include tannins and various flavonoids, which are responsible for the biological activity (Drewes et al., 2006), interest on their pharmacological potential is high (Kamatou et al., 2008). Of the flavonoids, salvigenin 6-hydroxyflavones characterise the species of *Salvia* and they include a variety of 6-hydroxylated flavonones such as apigenin, luteolin and cirsimaritin and their derivatives (Lu and Foo, 2002; Kamatou et al., 2006). Chemicals such as salvigenin, carnosol, rosmadial, ursolic acid, carnosic acid and carnosol have well established antimicrobial activity (Hussein et al., 2007). They are all usually prominent in solvent extracts generated from *S. runcinata* and we were interested in establishing a hairy root culture system to monitor the effects of genetic transformation on these particular chemicals.

The simplicity of *A. rhizogenes* transformation is useful as it may be employed in studying signal transduction mechanisms that control secondary metabolism and for the over-expression of genes that are in fact responsible for secondary metabolites. Furthermore, transgenic clones offer a platform for the biomanufacture of interesting metabolites that can easily be manipulated *in vitro* to study metabolic flux, providing a better understanding of the mode of operation of biosynthetic pathways. Transformation for the generation of hairy roots is strain, explant and species dependent and several parameters need to be tested for successful transformation. The use of *A. rhizogenes* agropine strains (for example: LBA 9402; ATCC 1583; A4; A4 1027) is favoured for successful production of transgenic hairy roots with *Salvia* species. The contribution of transgenic techniques may assist in providing new routes of metabolite synthesis and these transgenic lines may be brought into a commercial production line (Moyo et al., 2011). Nkomo et al. (2014) analysed various populations of *Salvia africana-lutea* that showed chemical variation that was correlated to the geographic location of populations. Their study included an analysis of the extracts for activity against *Fusarium*, and plants from different regions had variable bioactivity against this fungus. Chemotypic variation associated with natural populations is often an incentive for using a biotechnology platform as an alternative resource for acquiring secondary metabolites because production is usually better controlled and more reliable. The establishment of superior transgenic culture lines that are maintained in controlled environments that produce interesting metabolites is one solution to circumvent a heavy reliance on wild plant populations.

With these ideas in mind, we report on the transformation and induction of hairy roots in *Salvia runcinata* using two agropine strains, *A. rhizogenes* strains A4T and LBA 9402. This work was followed with the analysis of antifungal properties and gas chromatography–mass spectrometry profiling of the extracts focusing on two clonal lines that were grown for several years in culture. This study may be used as a platform for the genetic manipulation of biosynthetic pathways that produce both volatile and non-volatile pharmacologically active compounds of *S. runcinata*.

2. Materials and methods

2.1. Plant material

Salvia runcinata (L. f.) seeds were bought from Silverhill Seeds (Cape Town, South Africa) in May 2010 (http://www.silverhillseeds.co.za/collecting.asp). Seeds were stored in the dark at room temperature before germination. To induce germination, seeds were scarified by incubating in sulphuric acid for 20 min. The seeds were then transferred to 3.5% (w/v) NaOCl for 20 min. Thereafter, seeds were washed three times with sterile water and germinated on medium with smoke solution based on the protocol described by Musarurwa et al. (2010) onto half-strength Murashige and Skoog (1/2 MS) (1962) medium (0.1 g l^{-1} myo-inositol; 0.2 μM smoke solution and 3% (w/v) sucrose solidified with 9 g l^{-1} agar, pH adjusted to 5.8 with 1 M NaOH or 1 M HCl). The MS salts were prepared by Highveld Biologicals (Pretoria, South Africa). Media were autoclaved at 122 kPa and 120 °C for 20 min throughout the study and poured into 100 cm × 2 cm Petri dishes (BD Falcon™). Seeds were transferred onto germination medium under laminar flow conditions. Germination was induced under light conditions (50 μmol m^{-2} s^{-1} photosynthetic photon flux density [PPFD]) at 23 ± 2 °C. Germinated seedlings continued their growth under a 16 h light with 8 h dark cycle. The growth room was fitted with 'cool white' fluorescent lights (L75W/20X Osram). Seedlings were allowed to grow for 60 days before transformation experiments were initiated. Some of the seedlings were used to initiate a stock of plants that would serve as a source of plant material throughout the study that would remain untransformed. Nodal explants (1 to 2 cm) were excised from these plants and each explant contained a pair of axillary buds. The microplants generated rooted and these roots were used to initiate non-transgenic root cultures (further described in detail elsewhere).

2.2. Bacterial strains

Colonies of *Agrobacterium rhizogenes* agropine strains: A4T and LBA 9402 were routinely grown on yeast extract peptone (YEP) agar medium (10 g l^{-1} peptone; 10 g l^{-1} yeast extract; 5 g l^{-1} NaCl solidified with 15 g l^{-1} agar, pH 7.0, pH adjusted to 5.8 with 1 M NaOH or 1 M HCl) supplemented with 250 μM acetosyringone (3′, 5′-dimethoxy-4′-hydroxyacetophenone, Sigma, Germany) and rifampicin (Rimactane®, Sandoz Ltd., South Africa) (100 mg l^{-1}) in the dark, at 28 °C for 24 h using standard microbial inoculation techniques. Liquid cultures were initiated by inoculating a single colony of each of the two strains in 50 ml flasks with YEP medium supplemented with rifampicin (100 mg l^{-1}) and 250 μM acetosyringone. Cultures were shaken in the dark at 28 °C at 130 revolutions per minute (rpm) for two days or until the bacteria had grown to the saturated phase (OD_{600} = 1–2). Prior to transformation, 13 ml of the saturated bacterial solution was centrifuged in a desktop centrifuge (Sigma 302) at 4000 rpm at 20 °C for 15 min. The pellet was washed and resuspended in 15 ml liquid Murashige and Skoog (MS) medium (0.1 g l^{-1} myo-inositol; 3% [w/v] sucrose) to remove the antibiotics. The bacterial solution was centrifuged once more and the pellet was resuspended in 90 ml liquid MS medium (OD_{600} = 0.5–1).