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Title:
"METHOD FOR MICROPROPAGATING P. SIDOIDES DC AND P. RENIFORME CURTIS"

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Applicant(s):
STELLENBOSCH UNIVERSITY

Inventor(s):
MAKUNGA, Nokwanda Pearl
COLLING, Janine
KOTZE, Danelle

VS Ref No. P2165ZA01/SC

VS VON SEIDELS
INTELLECTUAL PROPERTY ATTORNEYS
PATENTS, TRADE MARKS, DESIGNS, COPYRIGHT

PO Box 440
Century City , (Cape Town)
7446, South Africa

Tel: +27 (0)21 526-2800
Fax: +27 (0) 86 660 6208
e-mail:
service@vonseidels.com

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
APPLICATION FOR A PATENT AND ACKNOWLEDGEMENT OF RECEIPT
(Section 30(1) – Regulation 22)
The grant of a patent is hereby requested by the undermentioned applicant on the basis of the present application filed in duplicate.

REGISTRAR OF PATENTS, DESIGNS, TRADE MARKS AND COPYRIGHTS
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OFFICIAL APPLICATION NO			AGENT'S REFERENCE
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FULL NAME(S) OF APPLICANT(S)	
71	STELLENBOSCH UNIVERSITY

ADDRESS(ES) OF APPLICANT(S)	
	Admin B, Victoria Street, Stellenbosch, Western Cape Province, 7600 South Africa

TITLE OF INVENTION				
54	METHOD FOR MICROPROPAGATING P. SIDOIDES DC AND P. RENIFORME CURTIS			
x	THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P2 The earliest priority claimed is 29 April 2011 ZA2011/03151			
	THIS APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO.	21	01	
	THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND BASED ON APPLICATION NO.	21	01	

THIS APPLICATION IS ACCOMPANIED BY:

	1a	A single copy of a provisional specification of pages.
x	1b	Two copies of a complete specification of 33 pages.
	2a	Informal drawings of sheets.
x	2b	Formal drawings of 7 sheets.
x	3	Publication particulars and abstract (form P8 in duplicate).
	4	A copy of figure of the drawings for the abstract.
	5	Assignment of invention (from the inventors) or other evidence of title.
	6	Certified priority document(s).
	7	Translation of priority document(s).
	8	Assignment of priority rights.
x	9	A copy of form P2 and a specification of S.A. Patent Application.
	10	A declaration and power of attorney on form P3.
	11	Request for ante-dating on form P4.
	12	Request for classification on form P9.
	13	Statement on the use of indigenous biological resource, genetic resource, traditional knowledge or use
x	14a	Request for delay of acceptance on form P4.
	14b	Request for expedited acceptance on form P4.

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Patent Attorney for Applicant(s)

ADDRESS FOR SERVICE	
74	VON SEIDELS **Docex 16, Century City** P O Box 440, Century City, (Cape Town) 7446, South Africa

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REGISTER OF PATENTS

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71

STELLENBOSCH UNIVERSITY

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ASSIGNEE(S)	DATE REGISTERED
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FULL NAME(S) OF INVENTOR(S)

72

MAKUNGA, Nokwanda Pearl
COLLING, Janine
KOTZE, Danelle

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TITLE OF INVENTION

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METHOD FOR MICROPROPAGATING P. SIDOIDES DC AND P. RENIFORME CURTIS

ADDRESS OF APPLICANT(S) / PATENTEE(S)

Admin B, Victoria Street,
Stellenbosch, Western Cape Province, 7600
South Africa

ADDRESS FOR SERVICE	REF
74 VON SEIDELS Docex 16, Century City P O Box 440, Century City, (Cape Town) 7446, South Africa	P2165ZA01/SC/cds

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REPUBLIC OF SOUTH AFRICA

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COMPLETE SPECIFICATION
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71	STELLENBOSCH UNIVERSITY

FULL NAME(S) OF INVENTOR(S)	
72	MAKUNGA, Nokwanda Pearl COLLING, Janine KOTZE, Danelle

TITLE OF INVENTION	
54	METHOD FOR MICROPROPAGATING P. SIDOIDES DC AND P. RENIFORME CURTIS

METHOD FOR MICROPROPAGATING P. SIDOIDES DC AND P. RENIFORME CURTIS

FIELD OF THE INVENTION

This invention relates to an *in vitro* micropropagation method for pelargonium plants of the species *Pelargonium sidoides* DC and *Pelargonium reniforme* Curtis.

BACKGROUND OF THE INVENTION

Many South African communities rely on traditional medicine for their primary form of healthcare and thus a wide range of medicinal plants, animal products and minerals are exploited for the relevant treatments. *Pelargonium sidoides* DC and *P. reniforme* Curtis, both endemic and harvested mostly in the Eastern Cape of South Africa, are important medicinal species which are used for diarrhoea, dysentery, colds and bronchitis. *Pelargonium sidoides* has also been used for the treatment of tuberculosis (TB), which is caused by *Mycobacterium tuberculosis*, since 1897 as "Stevens Consumption Cure". Nowadays, it is commercially available as a phytopharmaceutical extract (EPs® 7630) for treating colds and influenza plus other respiratory (or bronchial) infections. This product is traded as an over-the-counter preparation under the names Zucol™ ColdCare and Umcka® ColdCare in the United States (http://www.jfponline.com/pages.asp?id=7210). It is also used for the treatment of sick livestock. Almost all of the traditional treatments involve extractions from the tubers. This is also the way in which the commercialised

tincture and other phytopreparations are prepared. The tubers are a rich source of phenolic acids (gallic acid and its methyl derivatives), proanthocyanidins and coumarins. In particular, 7-hydroxy-5,6-dimethoxycoumarin, termed umckalin, has been shown to be produced solely by *P. sidoides* (Franco and Oliveira, 2010). Umckalin has been shown to possess strong antibacterial action, playing a vital role in the efficacy of phytomedicines produced from standardised extracts of this species (Franco and Oliveira, 2010).

The majority of Pelargonium tubers are harvested for the export market (especially to Germany) for the production of a commercial extract. Recent estimates indicate that over 4 million tubers are exported annually, and between the years 2000 and 2008 over 332,8 million plants (in the form of tubers), weighing over 12.8 tonnes, were harvested from the wild for the German herbal market alone (Mayet, 2010).

Field cultivation of *P. sidoides* is virtually negligible and mainly limited to a few growers in the Western Cape of South Africa, and reliance on wild growing populations is thus on the increase. As the foliage of *P. sidoides* and *P. reniforme* is very similar, it is difficult for harvesters to distinguish between these species when the plants are not flowering (it is easier to identify the plants when they are in flower - *P. sidoides* has dark maroon to black flowers and *P. reniforme* has pink flowers) (Figure 1). As a result, although *P. sidoides* is currently more sought after than *P. reniforme*, non-flowering *P. reniforme* plants are often indiscriminately harvested when only *P. sidoides* is required. With the growing demand for these plants, tubers are now also being extracted from plants that have not reached maturity. The wild populations of both species are thus becoming depleted, presenting a biodiversity threat (Lewu et al. 2006a). As a result, there is a move to place *P. sidoides* and *P. reniforme* under Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) and in the national protected species lists.

Cultivation methods are thus urgently needed to alleviate the pressure on these populations and to provide quality stock for the medicinal industry.

SUMMARY OF THE INVENTION

According to a first embodiment of the invention, there is provided an *in vitro* method for micropropagating pelargonium plants of the species *Pelargonium sidoides* DC and *Pelargonium reniforme* Curtis, the method comprising the steps of:

culturing plant material of *P. sidoides* or *P. reniforme* on a first culture medium including an auxin and a cytokinin, wherein the auxin is indole-3-acetic acid (IAA) and the cytokinin is kinetin or the auxin is 1-naphthaleneacetic acid (NAA) and the cytokinin is 6-benzyladenine (BA); and

regenerating plantlets.

The method may additionally comprise one or more of the following steps:

initiating organogenesis of the plant material;

multiplication, elongation and rooting of plantlets;

acclimatising the plantlets; and/or

transferring the plantlets to soil or a potting medium.

The plant material may be a seedling explant, such as a hypocotyl or cotyledon, or a foliage explant, such as leaf material, a petiole, a meristemoid or a meristem explant, or parts thereof.

The auxin and cytokinin in the culture medium may be IAA and kinetin when the plant material is a seedling explant, or NAA and BA when the plant material is a foliage explant.

The IAA and kinetin may be present in the culture medium in a ratio of from about 1: 4 to about 1: 2. For example, the culture medium may include about 5 mg l^{-1} IAA and about 20 mg l^{-1} kinetin or about 10 mg l^{-1} IAA and about 20 mg l^{-1} kinetin.

The NAA and BA may be present in the culture medium in a ratio of about 1:1, about 1:2 or about 2:1. For example, the culture medium may include about 1 mg l^{-1} NAA and about 1 mg l^{-1} BA, about 1 mg l^{-1} NAA and about 2 mg l^{-1} BA, or about 2 mg l^{-1} NAA and about 2 mg l^{-1} BA.

The culture medium may be a Murashige and Skoog (1962) (MS) medium to which the auxin and cytokinin have been added.

The culture medium may be thickened with agar.

The method may result in spontaneous root development, thereby avoiding the need for a specific rooting step.

The method may also be performed without a specific callus stage.

Alternatively, callus may be transferred from the first culture medium to a second culture medium for shoot production, the second culture medium having a different ratio of auxin and cytokinin than the first culture medium. The second culture medium may include about 2 mg l^{-1} NAA and about 1 mg l^{-1} BA.

Regenerating tissue, such as callus or adventitious microplants, may be transferred from the first culture medium or the second culture medium to a further culture medium for root production, the further culture medium not including a plant growth regulator.

The regenerated plantlets or plants may have higher levels of umckalin than plants grown in the wild.

According to a second embodiment of the invention, there is provided a *Pelargonium sidoides* DC or *Pelargonium reniforme* Curtis plantlet or plant which has been propagated by the method described above. The plant or plantlet may have a higher level of umckalin than a wild plant or plantlet.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows A) flowering parts of *P. sidoides* with dark maroon colour petals, a character that is used as a distinguishing feature, whereas those of B) *P. reniforme* are a pink colour; C) leaves of *P. sidoides* are similar to those of D) *P. reniforme,* making it difficult to differentiate between the two sister species when not flowering and resulting in the indiscriminate harvesting of both species in the wild.

Figure 2 shows micropropagation of *P. sidoides* through use of seedling explants.

A) Seedlings were used as explant material for culture induction; **B**) Callus production was prolific for explants tested; **C**) Mass regeneration of hyperhydric shoots when cultured on Gelrite-solidified medium; **D**) Mass production of healthy shoots on agar-solidified medium; **E**) Elongation of adventitious shoots produced in culture. Plantlets were often associated with callus; **F**) Rooting of generated shoots occurred spontaneously on PGR free growth medium; **G**) Plants easily acclimatised to growing in the greenhouse; **H**) Acclimated plantlets were able to flower with the flowers being similar to those of non-propagated *P. sidoides* plants.

Figure 3 shows micropropagation of *P. sidoides* using leaf explants excised from nursery grown plants.

A) Regenerated callus and shoots on medium with 1 mg l^{-1} NAA and 2 mg l^{-1} BA; **B**) Adventitious root formation occurred on PGR-free medium; **C**) Propagated shoots developed thick roots on PGR-free medium; **D**) *In vitro* microplants with a well-formed root mass prior to extraction of coumarins; **E**) Acclimatised plantlet flowering four months after transfer to *ex vitro* greenhouse conditions. Flowers were similar to normal uncultivated plants of *P. sidoides*; **F**) tubers of *in vitro*-derived plants after six months post *ex vitro* transplantation. Two to three tubers were formed per microplant; **G**) Acclimatised plants of *P. sidoides* which adjusted to *ex vitro* growth condition; **H**) A close up of a *P. sidoides* flower from a plant that had been tissue cultured. Flowers were dark in colour and their morphology was similar to that of non-propagated plants.

Figure 4 shows establishment of *P. reniforme* in *in vitro* cultures.

A) An example of a flowering plant of *P. reniforme* which had been purchased. These plants were used as starter material for culture initiation; Callus were successfully regenerated from different explants such as the **B**) leaf, **C**) petiole and **D**) meristem explants; **E**) A close up of regenerated shoots derived from callus; **F**) Mass production of plantlets *in vitro* which rooted on basal MS medium in the absence of PGRs.

Figure 5 shows the principal components analysis of *P. sidoides* extracts prepared from *in vitro* cultures and greenhouse grown plants.

The compounds analysed were quercetin, scopoletin, shikimic acid, 7,8-dihydroxy-6-methoxycoumarin and umckalin. The plant extracts tested were as follows: (**1A**)

Wild foliage extracted with ethanol (EtOH); (**1B**) Wild foliage extracted with water (H_2O); (**1C**) Wild foliage extracted with ethyl acetate; (**1D**) Wild foliage extracted with dichloromethane and methanol (DCM:MeOHl; 1:1; v/v); (**2A**) Wild tuber extracted with EtOH; (**3A**) petiole-derived callus extracted with EtOH; (**3B**) Petiole-derived callus extracted with H_2O; (**3C**) Petiole-derived callus extracted with ethyl acetate; (**3D**) petiole-derived callus extracted with DCM:MeOH; (**4A**) Meristem-derived callus extracted with EtOH; (**4B**) meristem-derived callus extracted with water; (**4C**) Meristem-derived callus extracted with ethyl acetate; (**4D**) Meristem-derived callus extracted with DCM:MeOH; (**5A**) *In vitro* plants derived from leaf explants extracted with EtOH; **(6A)** *In vitro* plants derived from stem plants extracted with EtOH; (**7A**) Tubers from acclimatised plants extracted with EtOH; (**8A**) Foliage of acclimatised plants extracted with EtOH; **(8D)** foliage of acclimatised plants extracted with DCM:MeOH.

Figure 6 shows the micropropagation and acclimatisation protocol of the present invention for *P. sidoides*. *Denotes the favoured explant as it produced the most callus (ANOVA, $p < 0.001$).

Figure 7 shows the micropropagation and acclimatisation protocol of the present invention for *P. reniforme*. *Denotes the preferred explant. Treatment 1 – 1 mg l^{-1} NAA and 2 mg l^{-1} BA; Treatment 2 – 2 mg l^{-1} NAA and 1 mg l^{-1} BA; Treatment 3 – 2 mg l^{-1} NAA and 2 mg l^{-1} BA; Treatment 4 – 1 mg l^{-1} NAA and 1 mg l^{-1} BA.

DETAILED DESCRIPTION OF THE INVENTION

An *in vitro* micropropagation method for pelargonium plants of the species *Pelargonium sidoides* DC and *Pelargonium reniforme* Curtis is described herein.

For many medicinal plants, natural populations exhibit chemical heterogeneity, with some populations being superior to others in terms of their chemical constituents and concentrations thereof. To ensure the preservation of desired or chemo-elite types, *in vitro* culture is a feasible alternative for germplasm conservation. Various *Pelargonium* species have been cultivated for the horticultural and essential oils industries, but there are no protocols that are utilised for *P. sidoides* and *P. reniforme*. Pelargoniums are conventionally

propagated vegetatively through cuttings, planting seeds or splitting of tubers. Although successful, these methods present several problems for the medicinal *Pelargonium* spp., as three to five years may be required before cultivated plants are ready for harvesting as crops. These methods are also labour intensive and unpredictable for these species, as germination of wild collected seed remains largely unknown. Propagules derived from vegetative propagation are highly prone to fungal, bacterial and viral infections increasing costs of disease control. These infections may also alter the chemical components of the plants, which can respond to infection by increasing their biochemical production of secondary metabolites and eliciting production of compounds which may be undesirable for human consumption.

Micropropagation can circumvent the problems eminent from vegetative propagation. Large quantities of plants can be produced from small segments of starting material; *in vitro*-derived 'seedlings' may be utilized to initiate medicinal plant gardens or sold to pharmaceutical companies and/or ethnobotanical markets; and as the materials are relatively disease-free, handling, storage and shipping has fewer restrictions. Access to plant material is not limited by geographical distribution, environmental conditions or seasons and even socio-political instabilities (in cases where these plants grow in developing countries) (Fennell et al. 2006). In addition, tissue cultured-derived plants, due to their clonal nature, are genetically identical to mother plants, and thus extracts prepared from different batches of the same clonal lines would be similar in terms of the types and concentration of compounds detected therein. *In vitro* propagules would be expected to have a more consistent chemistry than those plants collected from natural populations where genetic hybridisation results in population heterogeneity, causing both genetic and chemical variations. Apart from this, natural populations are also exposed to different geographical habitats and climactic conditions and which ultimately leads to unpredictable chemistry of the extracts. All together, these factors precipitate batch-to-batch differences with regards to the commercial syrup. For quality assurance, added labour and highly skilled personnel in chemistry are then required so as to adjust formulations for the standardisation of the final product.

However, tissue culture establishment and plantlet regeneration is species specific and often methods that are appropriate for one member of a family may be unsuitable for other members of the same family, requiring investigation of protocols that facilitate *in vitro* culture. Depending on the species, some explants may be recalcitrant to organogenesis,

requiring the appropriate establishment of phytohormone protocols that may induce this *in vitro* response. The applicant is not aware of any micropropagation methods for *P. sidoides* and *P. reniforme*, even though their medicinal importance in the international phytopharmaceutical industry is growing. Reports of cultivated *P. sidoides* are restricted to clonally propagated plants (Lewu et al. 2006b) and *Agrobacterium*-mediated transformation (Colling et al. 2010).

A micropropagation method has now been developed by the applicant as a conservation and commercialisation tool for *P. sidoides* and *P. reniforme*. According to the method, plant material of *P. sidoides* or *P. reniforme* can be cultured on a culture medium including a combination of at least two plant growth regulators (PGRs) to initiate propagation. The two PGRs are an auxin and a cytokinin, and in particular, are a combination of indole-3-acetic acid (IAA) and kinetin or 1-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA).

The culture medium is typically a Murashige and Skoog (1962) medium, which can be used for initiating organogenesis and/or regeneration of plantlets. Other additives can also be added to the plant medium, such as myo-inositol, sucrose and a thickening agent. Suitable thickening agents are agar or polysaccharide substitutes thereof (e.g. Gelrite™), although in the examples described below, the best results were observed when agar was used.

Suitable plant material for use in regenerating plantlets according to the method of the invention includes seedling explants, leaf explants, petioles, meristemoids and meristem explants. For *P. sidoides* cultivation, preferred material is either seedling explants for culture induction or foliage explants of mature wild mother plants for indirect shoot organogenesis. Preferred seedling explants are hypocotyls and cotyledons or sections thereof, with especially the hypocotyls being most responsive. Foliage explants are the preferred material for *P. reniforme*. For both *P. sidoides and P. reniforme*, petioles are the preferred material from the foliage leaf plants.

The PGRs are typically IAA and kinetin when seedling explants or meristemoids are used, and NAA and BA when foliage explants, such as leaf material, petioles or meristem material, are used.

Generally, the amount of cytokinin present in the culture medium is equal to or greater than the amount of auxin for purposes of initiating propagation. Preferred ratios of IAA: kinetin are 1:4 and 1:2, with a 1:2 IAA: kinetin ratio being most preferred. For example, the culture medium can contain 5 mg l^{-1} IAA and 20 mg l^{-1} kinetin or 10 mg l^{-1} IAA and 20 mg l^{-1} kinetin.

Alternatively, preferred ratios of NAA: BA are 1:1, 1:2 or 2:1 with aims to initiate callus from *P. reniforme* petiole explants (the 1:1 and 1:2 ratios are preferred for culture initiation). The culture medium may contain 1 mg l^{-1} NAA and 1 mg l^{-1} BA, 1 mg l^{-1} NAA and 2 mg l^{-1} BA, 2 mg l^{-1} NAA and 1 mg l^{-1} BA or 2 mg l^{-1} NAA and 2 mg l^{-1} BA.

Other steps which can be performed in the method of the invention include, but are not limited to: initiating organogenesis of the plant material; regenerating plantlets; multiplication; elongation and rooting of plantlets; acclimatisation; and/or transferring the plantlets to soil or a potting medium.

Depending on the plant material used and the auxin: cytokinin combination, spontaneous root development can occur, thereby avoiding the need for a specific rooting step. A specific callus stage can also be avoided.

Alternatively, regenerating tissue (callus or adventitious microplants) can be transferred from the first culture medium containing the PGRs to a culture medium which does not include PGRs for rooting. Optionally, after callus production, the callus material can be transferred to a second culture medium containing a different ratio of PGRs to the first culture medium for promoting shoot production.

Acclimatisation can be performed by applying a seaweed-derived growth stimulating composition containing auxins and cytokinins, such as Kelpak™, to the foliage of the plantlets.

Plantlets can be produced by the method within 30 – 42 days. Higher levels of umckalin can be observed in the plantlets produced according to the method of the invention compared to wild-type plantlets grown in the absence of the tissue culture medium.

Examples

The invention will now be described in more detail by way of the following non-limiting examples.

Materials and methods

Plant material and culture initiation

Cultures were initiated either through seedling explants or from foliage explants.

In vitro plantlet regeneration from P. sidoides seedlings

Seeds of *Pelargonium sidoides* were donated by Parceval Pharmaceuticals (Wellington, Western Cape, South Africa). Seeds were germinated using the protocol described by Colling et al. (2010). Ten days after germination, hypocotyls (1 cm) and cotyledons (1 cm^2) were excised from germinated seeds and used as explants for tissue culture. These explants were placed in tubes (100 mm x 25 mm) filled with 10 ml MS medium containing 4.4 g l^{-1} Murashige and Skoog (1962) salts and vitamins (Highveld Biological, South Africa), 0.1 g l^{-1} myo-inositol, 30 g l^{-1} sucrose and solidified with either 3 g l^{-1} Gelrite® (Sigma, USA) or 10 g l^{-1} agar (Biolab, Gauteng, South Africa). The regeneration medium was supplemented with various ratios of plant growth regulators (PGRs). Different combinations of 1-naphthaleneacetic acid (NAA) (0, 5, 10, 20 mg l^{-1}) and 6-benzyladenine (BA) (10, 20 mg l^{-1}) or kinetin (20 mg l^{-1}) with indole-3-acetic acid (IAA) (5, 10 mg l^{-1}) were added to the MS medium (pH 6.0). Throughout the study, all media were autoclaved at 122 kPa and 120 °C for 20 min and media were poured into culture vessels or test tubes once a temperature of 50 °C or less had been reached. Caps were placed on tubes, which were sealed with 2 cm Parafilm^M strips and incubated at 24 °C ± 2 °C under 24 h light (50 µmol m^{-1} s^{-2} photosynthetic photon flux density, PPFD) which was provided by cool white fluorescent tubes. Ten explants (five hypocotyls and five cotyledons) were placed in separate tubes (1 tube represents 1 replicate). The experiment was repeated twice. The production and number of shoots, roots and/or callus as a result of each treatment, was noted after 28 days. A total number of 45 replicates (N) was used for each combination of PGRs tested.

Generation of plantlets from P. sidoides leaf explants

The use of seedling tissues for culture initiation is not without drawbacks. Natural hybridisation of populations causes genetic variability, ultimately influencing the chemical

composition of regenerants. Direct organogenesis from wild growing leaf explants, particularly from plants with desired traits such as elite chemotypes, is thus sometimes favoured for clonal propagation using tissue culture. Experiments aimed at finding the suitable phytohormone regimen for use with foliage material were thus investigated.

Above-ground sections were excised from mature plants of *P. sidoides* and *P. reniforme* (purchased from Kirstenbosch Gardens, Cape Town, South Africa) after decontamination of tissues. The leaves and stems of *P. sidoides* and *P. reniforme* were washed in running tap water first before incubating in 70% (v/v) ethanol for five min prior to being transferred to a fungicidal solution consisting of 2% (w/v) Dithane WG-45 ™ solution (active ingredient: mancozeb 750 g kg^{-1}; Efekto, South Africa) and Folicur ™ (Tebuconazole 250 g l^{-1} (triazole)) for 20 min. The plant material was then immersed in diluted commercial bleach for 20-30 min with the final concentration of the active ingredient, sodium hyperchlorite, being 1.17% (w/v) for *P. sidoides* and *P. reniforme*. To remove the decontaminating agent, the plant material was washed three times using sterile water for five min at a time.

Excised above-ground sections for both species were used as starter material and were placed on different combinations of NAA (0, 1 or 2 mg l^{-1}) and BA (0, 1 or 2 mg l^{-1}) to induce organogenesis. The MS medium (supplemented with 30 g l^{-1} sucrose, 0.1 g l^{-1} myo-inositol and 10 g l^{-1} agar, pH 5.8; 1 M NaOH or 1 M HCl) was used as the basal medium and PGRs were included in this medium to initiate organogenesis.

Leaves (1 cm^2 in size), petioles (1 cm in length) and meristems (with a small piece of leaf and petiole attached) were placed on MS medium containing 1 mg l^{-1} NAA and 2 mg l^{-1} BA. Medium without PGRs was used as a control. Five replicates per Petri dish (100 mm x 20 mm) were used for the experiment testing organogenesis from leaf and meristem tissue; and, for petiole explants, the number of replicates was increased to eight. Fifteen Petri dishes were used in total and the experiments were repeated once. The response of these explants was monitored for 6 weeks. Thereafter, regenerating tissues (callus or adventitious microplants) from these explants were transferred to the respective medium for regeneration. These cultures were kept in a growth room with an 18 h light cycle (50 µmol m^{-1} s^{-2} photosynthetically active radiation (PAR)) provided by the white fluorescent tubes and the temperature was kept constant at 24 °C ± 2 °C.

Elongation and rooting of *in vitro P. sidoides* plantlets derived from foliage explants

Regenerated shoots were placed in test tubes containing 10 ml of MS medium (with 1 mg l^{-1} IAA: 2 mg l^{-1} kinetin or without PGRs) to determine which medium promotes shoot and root proliferation. Fifteen single shoots or meristemoids (callus clusters with two or more regenerating shoot primordia in a group) were placed on each type of medium to determine which one has the best survival rate and response. The experiment was repeated once (N=30) and the data were recorded after 30 days.

Rooting and acclimatisation of plantlets

For those plants derived from seedling material, rooting occurred spontaneously in culture and these were transferred to a thermostatically controlled glasshouse (Stellenbosch University, South Africa) where the temperature was regulated to a minimum of 15 °C and a maximum of 25 °C with the plants being exposed to natural sunlight (irradiance range between 540 to 810 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) at midday).

Before transfer, the roots (if present) were gently washed under running tap water to remove all traces of medium. These plants were transferred to pots containing a mixture of sand: compost: vermiculite (1:1:1; v/v). Pots were covered with a plastic bag and placed in the glasshouse. Over a period of 30 days, the bag was gradually opened by cutting bigger holes to acclimatise the plants, reducing the relative humidity from 90% to approximately 70% during the acclimatisation period. Plants were watered regularly by hand. For those plants derived from seedling explants, 1% Kelpak (v/v) (2.2 mg l^{-1} auxin and 0.0062% cytokinins; Starke Ayres, South Africa) was sprayed on the leaves every second day to facilitate rapid acclimatisation *ex vitro*. The outdoor temperature conditions ranged between 18 – 33 °C (maximum) during the day and 7 – 13 °C (minimum) at the beginning of the acclimatisation period (April 2010) and average minimum and maximum outdoor temperatures were 24.7 and 12.6 °C during the acclimatisation period (April 2010 to December 2010). During this period, the plantlets were exposed to 12 h 33 min visible light in April, 10 h 56 min visible light in June and 15 h 10 visible light in November 2010.

Secondary tissue culture establishment: germination capacity of seeds from propagated *P. sidoides* plants

From the pool of acclimatised plants, one of the *P. sidoides* plants produced 18 seeds four months after acclimatisation was initiated. These seeds were decontaminated according to Colling et al. (2010), prior to being transferred to Petri dishes containing ¼-strength MS

medium solidified with agar (three seeds per Petri dish; pH 5.8). The seeds from the mother plants were used as a control. All the Petri dishes were placed in a light growth room and the germination response was noted daily for 14 days. Radicle emergence (1-2 mm) from the seed indicated positive germination.

Continuous subculture and commercial potential for the micropropagation protocol

Plantlets were routinely subcultured every four to six weeks and this involved placing sections (2-3 internodes per single explant) of the plantlet on fresh PGR-free RM to facilitate rooting. In vitro plantlets of *P. sidoides* and *P. reniforme* were subcultured to determine the potential amount of plantlets which would arise from the original plantlets. The length of each plantlet, the amount of internodes per plantlet and the "new" plantlets were noted.

Statistical analysis

Data were collected after six weeks with the exception of tissue culture data collected from the seedling-derived microplants of *P. sidoides*, where data analysis occurred after four weeks. The statistical package Statistica Release 7 was used to process all data, unless otherwise stated. All percentage data were arcsine transformed prior to statistical evaluation. Quantitative data were subjected to one-way analysis of variance and when normally distributed followed by a Tukey's multiple HSD range test to separate the means. Otherwise, Kruskal Wallis analysis followed as a posthoc test. For seedling-derived microplants of *P. sidoides* data, analysis was conducted using the SAS Enterprise Guide 3 and for categorical data collected in this instance, M-L chi square analysis. All data collected over a period of time were subjected to a repeated-measures ANOVA and also factorial analyses were conducted. Statistica Release 9 was used for all data collected from foliage-derived *P. sidoides* and *P. reniforme* plantlets.

Extraction of coumarins and antibacterial activity

The coumarins were extracted and analysed according to Colling et al. (2010) with the inclusion of 7-hydroxy-5,6-dimethoxycoumarin, hereafter referred to as umckalin, as a standard. Other standards included in the analysis were scopoletin, shikimic acid, quercetin and 7,8-dihydroxy-6-methoxycoumarin (synonymous with fraxetin) (purchased from Sigma Blochemicals, Germany). Ground material was extracted twice with sonication using water, ethanol, ethyl acetate and a dichloromethane-methanol (DCM: MeOH; 1:1; v/v) solution. Extracts were prepared in a similar fashion except for those prepared from water. Dried residues were resuspended in the respective solvents, except ethyl acetate and DCM: MeOH

extracts which were resuspended in 1% (v/v) DMSO, to a concentration of 50 mg ml^{-1}. Antibacterial activity of the different extracts produced from different regenerating tissues was tested using a microdilution assay (Eloff 1998). The water extract was made by adding boiling water to ground plant material and was left overnight. The following day, cellular debris was removed by filtration using Whatman no. 1 filter paper. Thereafter, samples were freeze-dried (Vertis Benchtop K; 20mTorr vacuum at -60 °C) for two days and the lyophilised material was resuspended in water (50 mg ml^{-1}). Activity was tested against two Gram-positive (*Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 12600) and two Gram-negative bacteria (*Escherichia coli* ATCC 11775 and *Klebsiella pneumoniae* ATCC 13883). Bacterial suspensions, established from cultures grown overnight, using sterile Mueller-Hinton (MH) broth as a diluent (1:50 (ml); bacteria to MH) were set up. Hundred microlitres of each 50 mg ml^{-1} extract was diluted using a two-fold serial dilution in a 96-well-microplate using sterile water for each bacterium. This was done in triplicate. Similarly, 0.1 mg ml^{-1} streptomycin (Sigma) was diluted as a positive control. Several negative controls were set up against each test bacterium and these included using bacteria-free broth, distilled water and solvent controls (water, ethanol and 1% (v/v) DMSO). These solvent controls were respective to the type of extractant used to generate and resuspend extracts. The DMSO was thus used to resuspend ethyl acetate and DCM: methanolic extracts. The microplates were incubated overnight at 37°C and the following day, 50 µl of 0.2 mg ml^{-1} *p*-iodonitrotetrazolium chloride (INT) was used to detect bacterial growth. The plates were further incubated for one hour and those wells that were clear indicated the ability of the extracts to inhibit bacterial growth whereas all wells that were pink showed presence of bacterial survival. The MIC value was recorded as the lowest concentration that could inhibit bacteria growth indicated by clear wells.

Results and discussion

Micropropagation from seedling explants of *P. sidoides*

Upon successful germination (Figure 2A), the hypocotyls, cotyledons and petioles were investigated as target tissue for induction of *P. sidoides* cultures (Table 1). Direct adventitious shoot formation, which is most desired in tissue culture due to its low capacity for generation of somaclonal variants was achieved on media with BA at 10 mg l^{-1}, 5 mg l^{-1} IAA and 20 mg l^{-1} kinetin with 10 mg l^{-1} IAA at a frequency of 50%, 77.8% and 57.1% when using hypocotyls. Most of the treatments used were able to generate callus with all the

tissue converting to callus masses (Figure 2B) when cultured on 5 mg l^{-1} NAA and 10 mg l^{-1} BA irrespective of explant type or PGR combination (Table 1). When comparing NAA and BA combinations to the use of IAA and kinetin combinations, it is evident that the 5 mg l^{-1} IAA: 20 mg l^{-1} kinetin combination (Table 1) is far more effective in inducing prolific shoot regeneration from the hypocotyls at a frequency of 77.8% ($p < 0.05$). The organogenic potential for shoot regeneration is significantly dependent on the type of explant (hypocotyl) ($p = 0.0037$) compared to cotyledons (33.3%). This is the first time that IAA and kinetin have been used in combination for this particular species for microplant production.

Indirect organogenesis as a route for plantlet regeneration is not always beneficial as it can produce between 12 to 99% somaclonal variation in the progeny. The direct shoot organogenesis from hypocotyls as per the present invention circumvents the pre-requisite for a callus stage as part of the micropropagation regime and thus reduces the waiting time for plantlet multiplication and elongation for *P. sidoides*, also avoiding production of genetic variation. The use of meristem tip cultures for the production of disease-free plants is an important objective for the horticultural industry (Mithila et al. 2001). However, there are drawbacks associated with meristem culture as it is labour intensive, time consuming and requires highly skilled personnel. Initiation of tissue cultures with hypocotyls as the starting material requires less finesse and technical expertise as compared to meristem culture making this strategy easier to adopt for commercial propagation. The system described herein results in expedient production of healthy plants, without apparent signs of infection, with plantlets (>10) being produced within a month.

Although plantlet production was efficient on medium solidified with Gelrite®, hyperhydricity became apparent (Figure 2C). Hyperhydricity often results in unhealthy, malformed plantlets and this phenomenon is well known to restrict growth and multiplication *in vitro*, further limiting establishment and survival of plants *ex vitro* (Debergh et al. 1992). Agar proved beneficial for reducing the appearance of hyperhydric tissues in culture without affecting the multiplication rate especially on medium with 10 mg l^{-1} IAA and 20 mg l^{-1} kinetin. This ratio induced a higher regeneration rate on medium solidified with agar (91.7%; Table 2). Healthy plantlets produced *en masse* on agar-solidified medium validated the use of this solidifying agent instead of Gelrite for routine propagation (Figure 2D and 2E). Regeneration was thus notably higher compared to the respective Gelrite-medium where a 51.7% shoot induction response was recorded. Moreover, spontaneous root development was a highly desired product of maintaining plantlets on the 10 mg l^{-1} IAA with 20 mg l^{-1} kinetin medium

(Figure 2F), eliminating the need to develop a rooting regimen for *P. sidoides*. This shortened the time period in culture prior to acclimatisation, an added advantage for establishing a commercially viable regime.

Table 1: The effect of auxins and cytokinins on the response of different *P. sidoides* explants on Gelrite®-solidified regeneration medium

PGR Treatment		Explant Type	% Shoot production	% Callus production
NAA (mg l^{-1})	**BA (mg l^{-1})**			
0	10	Cotyledon	0.0	0.0
		Hypocotyl	50.0	50.0
		Petiole	0.0	0.0
5	10	Cotyledon	0.0	100.0
		Hypocotyl	0.0	100.0
		Petiole	0.0	100.0
10	10	Cotyledon	0.0	0.0
		Hypocotyl	0.0	50.0
20	10	Cotyledon	0.0	16.7
		Hypocotyl	0.0	28.6
0	20	Cotyledon	0.0	0.0
		Hypocotyl	0.0	66.7
		Petiole	0.0	0.0
5	20	Cotyledon	0.0	0.0
		Hypocotyl	0.0	100.0
		Petiole	0.0	66.7
10	20	Cotyledon	0.0	14.3
		Hypocotyl	0.0	50.0
20	20	Cotyledon	0.0	0.0
		Hypocotyl	0.0	16.7
IAA (mg l^{-1})	**Kinetin (mg l^{-1})**			
5	20	Cotyledon	33.3	0.0
		Hypocotyl	77.8	66.7
10	20	Cotyledon	0.0	0.0
		Hypocotyl	57.1	42.9

[a] PGR denotes plant growth regulator
Data were analysed using the M-L Chi square test, $p < 0.05$ indicated significant differences

Table 2: Effect of auxins and cytokinins on the regeneration response of different *P. sidoides* explants on agar-solidified (10%, w/v [pH 6]) regeneration medium

PGR Treatment		Explant Type	Shoots Production (%)	Callus Production (%)	Root Production (%)
NAA (mg l^{-1})	**BA (mg l^{-1})**				
5	20	Cotyledon	0.0	20.0	0.0
		Hypocotyl	0.0	66.7	0.0
IAA (mg l^{-1})	**Kinetin (mg l^{-1})**				
5	20	Cotyledon	16.7	0.0	0.0
		Hypocotyl	71.4	71.4	0.0
10	20	Cotyledon	0.0	0.0	0.0
		Hypocotyl	91.7	58.3	25.0

[a] PGR denotes plant growth regulator
Data were analysed using the M-L Chi square test, $p < 0.05$ indicated significant differences

All of the rooted plantlets (Figure 2F) were successfully acclimatised within a month. During this time, plants were sprayed with Kelpak (a growth stimulant containing auxins and cytokinins) which alleviates transplantation stress and has been shown to reduce plantlet mortality (Madubanya et al. 2006). Part of the high acclimation rate was thus noted was attributed to use of this seaweed concentrate as a foliar spray. Plantlets were healthy with thick broad leathery fleshy leaves (Figure 2G) and they were generally morphologically similar to *P. sidoides* plants occurring in the wild. These plants flowered six to eight months after *in vitro* cultivation and flowers were dark maroon in colour (Figure 2H).

In vitro regeneration from leaf explants for *P. sidoides* and *P. reniforme*

The use of foliage as a starter material is preferred in cases where related taxa hybridise easily or where heterogeneity is a common occurrence amongst different populations of the same taxa, resulting in genetically hybrid seed material. This is particularly important for conservation of unique genotypes. Although *P. sidoides* cultures at the point of initiation were prone to fungal and bacterial contamination, at a frequency of 33.52% for the NAA: BA supplemented medium and 34.63% for the controls, callus was easily established (Table 3). Vigorous callus production on NAA: BA-supplemented media was evident after three weeks and as expected, the control showed no organogenic response. Out of the explants tested, petiole explants responded best (Table 3; $p < 0.001$).

This callus was generally friable with a high growth rate increasing its biomass rapidly (data not shown). From this callus, shoots were formed rapidly after 6 to 10 weeks on 3% and 9% of the meristem and petiole explants, respectively (e.g. Figure 3A). On transfer of the regenerating shoots onto PGR-free conditions, a further 13.33% adventitious shoot organogenesis rate was recorded (Table 4). This shoot regeneration capacity could not be maintained under prolonged culture in the absence of growth regulators, as only one of the original 30 single shoots survived throughout the culture period of 30 days. This may indicate the dependency for adventitious shoot formation on the combination of cytokinins and auxins.

Preferred protocols developed according to the present invention are shown in Figures 6 and 7.

Table 3: Establishment of *in vitro* cultures from leaf, petiole and meristem explants of *P. sidoides* on medium with NAA and BA (mg l^{-1}). The frequency of organogenesis and culture response is indicated by percentages (%)

Explant Type	Treatment (mg l^{-1})	Callus Production (%)	Shoot Regeneration (%)	Root Formation (%)
Leaves	No PGR[a]	0.00	0.00	0.00
	1 NAA: 2 BA	0.67	0.00	0.00
Petioles	No PGR	0.00	0.00	0.00
	1 NAA: 2 BA	**19.17***	2.50	0.00
Meristem	No PGR	0.00	0.00	0.00
	1 NAA: 2 BA	4.00	1.33	0.00

[a]PGR denotes plant growth regulator
*Significant callus formation; $p < 0.001$ (ANOVA)

Table 4: Adventitious shoot formation and rooting of regenerated *in vitro* cultures of *P. sidoides* on medium supplemented with 1 mg l^{-1} IAA and 2 mg l^{-1} kinetin

Explant Type	Treatment (mg l^{-1})	Callus Production (%)	Shoot regeneration (%)	Root formation (%)
Single shoots	No PGR	3.33	13.33	0.00
	IAA: Kinetin	6.67	3.33	0.00
Meristemoids	No PGR	0.00	53.33	16.67
	IAA: Kinetin	3.33	46.67	0.00

[a]PGR denotes plant growth regulator
*Significant regeneration response; $p < 0.001$ (ANOVA)

On the other hand, the use of meristemoids was highly efficacious, as meristemoids had a better survival rate (6.67%) upon transfer to PGR-free medium. The meristemoidal callus tissue was also composed of non-friable callus clumps with many microshoots developing from it. A shoot regeneration rate of 53.33% was recorded accompanied by a 16.67% rooting capacity without growth regulators (Table 4). This may indicate that high levels of endogenous auxins were produced by these microplants *in vitro* and these then facilitated spontaneous rooting (Figure 3B) after 30 days. However, on medium with 1 mg l^{-1} IAA: 2 mg l^{-1} kinetin, more shoots were regenerated by using meristemoids (46.67%) but spontaneous rooting was lowered for *P. sidoides*. A vigorous continuous culture system (Figure 3C) could be maintained with *in vitro* plantlets having an extensive root network (Figure 3D) which facilitated acclimatisation (Figure 3E-H).

Similarly for *P. reniforme*, petiole explants responded better to MS containing PGRs than the leaf or meristem explants (Table 5: $p < 0.001$). All the explants tested converted easily to callus (Figure 4B-D). Shoots were formed after 10 weeks on subcultured callus (Figure 4E). Contamination was recorded for MS with PGRs (30.74%) and the control (27.78%) throughout the culture period. Both fungal and bacterial infections were present but these were not identified. Overall, the levels of contamination were low and all contaminated cultures were then discarded. On the NAA: BA medium (1:2 mg l^{-1}), most of the shoots lost their organogenic ability, with them fast becoming brown, necrotic and eventually dying, with the exception of five of the leaf-derived shoots. Those shoots that maintained growth elongated fast and also regenerated new shoots.

Table 5: Induction of callus cultures from *P. reniforme* foliage explants on medium supplied with 1 mg l^{-1} NAA and 2 mg l^{-1} BA

Explant	Treatment (mg l^{-1})	Callus regeneration (%)	Contamination (%)
Leaves	No PGR	0.00	22.67
	1:2	0.00	34.00
Meristem	No PGR	0.00	32.00
	1:2	0.00	30.00
Petioles	No PGR	0.00	28.33
	1:2	24.17*	29.17

[a]PGR denotes plant growth regulator
*Significant regeneration response; $p < 0.001$ (ANOVA)

For this reason, other phytohormone combinations which could potentially facilitate prolific shoot regeneration were investigated and these included four phytohormone treatments (2:2; 1:2, 2:1, 1:1 (v/v) NAA: BA (mg l^{-1})) which were important for callus establishment from petiole explants (Table 5). As petiole explants produced the most abundant masses of callus, further experimentation involved using this explant type for microplant production. Overall, the MS medium with 2 mg l^{-1} NAA and 2 mg l^{-1} BA resulted in expedient callugenesis but could not support adventitious organ formation directly from explants (Table 6). In this case, an additional step to convert this callus to shoots is thus necessary. Protocols mainly use callus as a means to generate plantlets for Pelargonium and related taxa (Mithila et al. 2001), with many reports indicating little to no production of somaclonal variants. Direct organogenesis is more difficult with the tissues tested for initiation of plant tissue cultures from this species. It is thus important to test the extent of somaclonal variation with microplants derived from this system and to gain an understanding of potential chemical changes due to genetic variation. Although medium supplemented with equal ratios of NAA and BA (at 1 mg l^{-1}) was able to induce callus production, the frequency was less when these were doubled. Otherwise, shoot regeneration via a callus phase is possible with these shoots growing vigourously and multiplying rapidly in culture (Figure 4F).

Table 6: Effect of varying NAA: BA combinations on callus regeneration from petiole explants of *P. reniforme*

PGR combination	Callus production (%)	Contamination (%)
No PGR (control)	0.00	25.00
1:0	0.00	58.75
2:0	0.00	10.00
0:1	0.00	42.50
1:1	23.75	48.75
2:1	30.00	27.50
0:2	0.00	2.50
1:2	28.75	6.25
2:2	37.50	3.75

Acclimatisation of leaf-derived *in vitro* propagules of *P. sidoides*

Plantlets derived from leaf explants were able to adjust to out of culture conditions with a moderate survival rate (67.31%). These plants had an ability to flower (Figure 3E) and form underground storage organs (tubers) (Figure 3F). These microplants quickly regenerated more petioles (Figure 3G) and developed tubers (Figure 3F), adopting normal growth and developmental patterns similar to plants growing *ex vitro*. Each plant had 2-3 tubers which were 2.873 ± 0.77 cm in length and 0.82 ± 0.137 cm. The tubers extracted from those plants that had been growing in the greenhouse weighed 1.001 ± 0.313 g (fresh mass) and 0.221 ± 0.077 grams (dry mass) six months after acclimatisation. Survival rates were lower than those plants that came from seedling material. The lowered rate of acclimatisation for those plants derived from leaf sections was likely due to these microplants being acclimatised without the application of Kelpak at regular intervals. Even so, plantlet mortality was not viewed as being problematic.

Antibacterial analysis and phytochemical of foliage-derived microplants

Suboptimal plant tissue culture conditions may elicit undesired genetic (somatic) variation generating phenotypes that have an unpredictable morphological, physiological and biochemical nature. It was thus important to test for differences in pharmacological activity to validate the effectiveness of the micropropagation method. Extracts were prepared from the

tissues of the *in vitro* cultures, acclimatised plants and uncultivated plants ('wild') plants to ascertain possible pharmacological activity. Out of the solvent extracts, ethanol extracts proved to be the most effective against test bacterial strains (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*) (with the minimum inhibitory concentration (MIC) values ranging between ≤1 and 6 mg ml-1) but tuber, foliage, petiole derived (*P. sidoides*) and acclimatised plant (*P. sidoides*) extracts were more potent. Propagated plants of *P. sidoides* were similar to wild foliage extracts in their bacterial inhibition with the lowest recorded MIC value being 0.39 mg ml-1 (Table 7). The different extracts were screened against fungal species (*Candida albicans* and *Cryptococcus neoformans*). However, the acclimatised microplants were the best of the propagated stock because of their good antifungal activity (MIC ≥ 0.3125 mg ml-1). The overall activity of the propagated and wild foliage extracts suggests that a more sustainable usage of these plants is possible as leaf material appears to be a good candidate for use as a substitute for tubers for commercial preparations.

Metabolite profiling was conducted using different chromatographic techniques. The coumarins, apart from phenolic acids, are thought to be responsible for pharmacological activity and the presence of umckalin may be an important reference or marker compound for standardization of commercial extracts (Figure 5). The amount of coumarins analysed via LCMS are indicated in Table 8. The presence of similar compounds in the foliage of *P. sidoides* and tubers has previously been shown by Lewu et al. (2007), with these researchers advocating the use of leaf material instead of tubers. Tuber extracts produced from *in vitro* derived plantlets had the highest levels of umckalin. Tubers from microplants derived from *in vitro* culture had over 85.77 µg ml^{-1} of umckalin (detected as an outlier upon PCA (Figure 5)) whereas tubers removed from purchased plants that were then maintained under greenhouse conditions (indicated here as wild plants) had lower levels of umckalin. This particular compound is regarded as an important marker to identify *P. sidoides* plants, as it is solely produced by this species and does not accumulate at all in *P. reniforme*.

Synthesis of coumarins (which are used as biomarker compounds) in propagated and nonpropagated clones confirmed that the micropropagation method of the present invention does not negatively affect the chemistry of the Pelargoniums examined. The markedly elevated capacity for accumulation of umckalin by micropropagated plants within the roots makes them desirable as an alternative source for producing the medicinal syrup of *P. sidoides*. Shikimic acid was found in all extracts tested. This is an important precursor for

coumarin synthesis and it may be shunted into a variety of different secondary pathways for the production of other secondary metabolites apart from coumarins. The highest levels of shikimic acid were detected in the foliage of acclimated microplants (Table 8). Superior concentrations of this compound in the leaves clearly indicates its importance as a biosynthetic precursor for coumarin metabolism which is likely synthesised in the leaves but stored as reserves in the underground parts (tubers). Shikimic acid may thus be shunted to a variety of secondary metabolic pathways prior to translocation of those metabolites to storage organs.

Table 7: Anti-bacterial activity of non-propagated and propagated individuals of *P. sidoides*

Plant parts used	Extract	Anti-bacterial activity (MIC) after 24 hours (mg ml^{-1})			
		B. subtilis	***S. aureus***	***K. pneumoniae***	***E. coli***
Wild plants (foliage)	Water	12.5 ± 0.00	12.5 ± 0.00	12.5 ± 0.00	12.5 ± 0.00
	Ethanol	**0.39 ± 0.00**	**0.39 ± 0.00**	**0.39 ± 0.00**	**0.39 ± 0.00**
	Ethyl acetate	12.5 ± 0.00	12.5 ± 0.00	12.5 ± 0.00	ND
	DCM: MeOH	12.5 ± 0.00	12.5 ± 0.00	12.5 ± 0.00	10.42 ± 3.61
Wild plants (tubers)	Ethanol	6.25 ± 0.00	6.25 ± 0.00	4.687 ± 2.71	6.25 ± 0.00
Petiole-derived callus	Water	[1]ND	ND	ND	ND
	Ethanol	3.125 ± 0.00	3.125 ± 0.00	1.56 ± 0.00	1.56 ± 0.00
	Ethyl acetate	6.25 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	6.25 ± 0.00
	DCM: MeOH	12.5 ± 0.00	3.125 ± 0.00	3.125 ± 0.00	3.125 ± 0.00
Meristem-derived callus	Water	10.42 ± 3.61	12.5 ± 0.00	12.5 ± 0.00	ND
	Ethanol	3.125 ± 0.00	3.125 ± 0.00	3.125 ± 0.00	3.125 ± 0.00
	Ethyl acetate	ND	ND	12.5 ± 0.00	12.5 ± 0.00
	DCM: MeOH	ND	12.5 ± 0.00	12.5 ± 0.00	12.5 ± 0.00
Leaf-derived plantlets	Ethanol	1.56 ± 0.00	1.56 ± 0.00	1.56 ± 0.00	1.56 ± 0.00
Petiole-derived plantlets	Ethanol	**0.78 ± 0.00**	**0.78 ± 0.00**	1.56 ± 0.00	1.56 ± 0.00
Acclimatised plants (foliage)	Ethanol	**0.520 ± 0.225**	**0.39 ± 0.00**	**0.39 ± 0.00**	[2]NT
	DCM: MeOH	6.25 ± 0.00	1.56 ± 0.00	1.56 ± 0.00	1.56 ± 0.00
Acclimatised plants (tubers)	Ethanol	1.56 ± 0.00	1.56 ± 0.00	1.56 ± 0.00	1.56 ± 0.00

Values in bold are those with MIC ≤1 mg ml^{-1} which is regarded as significant
[1]ND denotes not detected and [2]NT denotes not tested

Table 8: Coumarin levels (µg ml^{-1}) in various *in vitro* propagated and non-propagated tissues of *P. sidoides*

Plant material	Extraction Solution	Quercetin	Scopoletin	Shikimic acid	Fraxetin[a]	Umckalin[b]
Wild plant foliage	Water	0.00	7.02			
	Ethyl acetate	0.00	0.91	1.18	0.34	0.01
	Ethanol	0.00	0.05	2.36	0.10	0.01
	Dichloromethane: Methanol	0.00	2.33	78.25	0.00	0.00
Wild plant tubers	Water	0.00				
	Ethyl acetate	0.00				
	Ethanol	0.00	4.05	3.90	0.00	61.61
	Dichloromethane: Methanol	0.00				
Acclimated microplants	Water					
	Ethyl acetate					
	Ethanol	0.96	0.12	72.49		0.55
	Dichloromethane: Methanol		0.61	**2388.01**	0.00	2.59
Acclimated tuber	Water	0.00				
	Ethyl acetate	0.00				2.59
	Ethanol	0.00	0.73	13.37	3.21	85.77
	Dichloromethane: Methanol	0.00				
***In vitro* culture derived from stem explants**	Water	0.00				
	Ethyl acetate	0.00				
	Ethanol	1.43	0.27	15.35	2.81	1.31
	Dichloromethane: Methanol	0.00				
***In vitro* culture derived from leaf explants**	Water	0.00				
	Ethyl acetate	0.00				
	Ethanol	0.00	0.17	36.39	1.03	0.45
	Dichloromethane: Methanol	0.00				
Callus derived from stem explants	Water	0.00				
	Ethyl acetate	0.00	0.03	3.56	0.03	
	Ethanol	0.00	0.00	15.35	0.00	0.00
	Dichloromethane: Methanol	0.00	0.18	27.46	0.51	0.19
Callus derived from meristem explants	Water	0.00				
	Ethyl acetate	0.00	4.49	46.23		1.12
	Ethanol	0.00	0.00	7.14	0.00	0.00
	Dichloromethane: Methanol	0.00	0.11	52.37	0.00	0.00

[a]Fraxetin is used as a term here and this is synonymous to 7,8-dihydroxy-6-methoxycoumarin

[b]Umckalin is used as a term here and this is synonymous to 77-hydroxy-5,6-dimethoxycoumarin

Values in bold indicate superior levels of a chemical compound

Conclusions

With regards to culture induction using seedling explants of *P. sidoides*, seeds germinated best in the light irrespective of the scarification method used. Transfer of hypocotyl sections from seedlings onto a medium with a ratio of 10: 20 (mg l^{-1}) IAA: kinetin (v/v) produced healthy shoots with a high proliferation frequency of 91.7%. This medium can be used as a continuous culture system and plants root spontaneously, circumventing the need for an additional rooting step. Even so, to increase the incidence of rooting, transfer to a PGR-free medium is an option. The use of Gelrite ™ as a solidifying agent is not advised as shoots become hyperhydric, even though a 77.8% regeneration rate is possible in the presence of 5 mg l^{-1} IAA and 20 mg l^{-1} kinetin as phytohormone additives. Rooted plants are acclimatised by reducing the humidity and spraying with Kelpak ™ (a seaweed concentrate) to increase survival rates out of culture. Acclimatisation occurred at a high percentage, with over 90% survival.

Indirect shoot organogenesis from *P. sidoides* foliage explants was optimal with a medium supplemented with 1: 2 NAA: BA (mg l^{-1}). Petioles are the preferred foliage explant for plantlet production. Regenerated shoots rooted on basal Murashige and Skoog (1962) medium without phytohormone supplementation. Again, rooted propagules were acclimatised successfully to glasshouse conditions.

For *P. reniforme*, foliage explants produced the best results, especially when the petiole is used as starter material. A ratio of 1: 2 NAA: BA (mg l^{-1}) was found to be optimal, although callus regeneration was much less than for *P. sidoides*. Direct shoot organogenesis was also less frequent with *P. reniforme*, requiring an additional phytohormone step to facilitate shoot generation and multiplication. The 1 NAA: 2 BA (mg l^{-1}) combination is preferred for callus induction and this is followed by a shoot multiplication phase which combines 2 mg l^{-1} NAA and 1 mg l^{-1} BA for promoting plantlet regeneration.

In all cases, micropropagation produced phenotypically normal plants which were able to survive acclimatization. Those that flowered had typical floral parts of respective species.

The applicant has thus developed a tissue culture regime which may be adopted for commercial micropropagation of *P. sidoides* and *P. reniforme* plants on a massive scale

(hundreds of new plantlets can be manufactured from one *P. sidoides* plantlet (125 - 1 728 new plants) or *P. reniforme* plantlet (216 – 2 744 new plants) over three months). The plantlets have a superior or similar capacity for the synthesis of umckalin and other bioactive coumarins ensuring a uniform, high-yielding genetic stock within a short time period. Extracts prepared from clonally propagated plant materials will result in less variation in the final commercial product and thus reduce the need for labour for adjusting product formulations so as to have a medicinal syrup with defined concentrations of key active compounds. Also, through commercialisation of this *in vitro* strategy, reliance on wild crafted tubers by commercial producers of phytopharmaceutics produced from *P. sidoides* will be reduced, ensuring sustainable exploitation of both *P.sidoides and P. reniforme.*

Acknowledgements

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CLAIMS:

1. An *in vitro* method for micropropagating pelargonium plants of the species *Pelargonium sidoides* DC and *Pelargonium reniforme* Curtis, the method comprising the steps of:

culturing plant material of *P. sidoides* or *P. reniforme* on a first culture medium including an auxin and a cytokinin, wherein the auxin is indole-3-acetic acid (IAA) and the cytokinin is kinetin or the auxin is 1-naphthaleneacetic acid (NAA) and the cytokinin is 6-benzyladenine (BA); and

regenerating plantlets.

2. A method according to claim 1, which additionally comprises one or more of the following steps:

initiating organogenesis of the plant material;

multiplication, elongation and rooting of plantlets;

acclimatising the plantlets; and/or

transferring the plantlets to soil or a potting medium.

3. A method according to either of claims 1 or 2, wherein the plant material is a seedling explant or foliage explant.

4. A method according to claim 3, wherein the seedling explant is a hypocotyl or part thereof.

5. A method according to claim 3, wherein the seedling explant is a cotyledon or a part thereof.

6. A method according to claim 3, wherein the foliage explant is leaf material, a petiole, a meristemoid or a meristem explant.

7. A method according to claim 6, wherein the foliage explant is a petiole.

8. A method according to any one of claims 3 to 5, wherein the auxin and cytokinin in the culture medium are IAA and kinetin when the plant material is a seedling explant.

9. A method according to any one of claims 3 and 6 to 7, wherein the auxin and cytokinin in the culture medium are NAA and BA when the plant material is a foliage explant.

10. A method according to any one of claims 1 to 8, wherein the IAA and kinetin are present in the culture medium in a ratio of from about 1: 4 to about 1: 2.

11. A method according to claim 10, wherein the ratio of IAA: kinetin in the culture medium is about 1: 2.

12. A method according to claim 10, wherein the culture medium includes about 5 mg l^{-1} IAA and about 20 mg l^{-1} kinetin.

13. A method according to claim 11, wherein the culture medium includes about 10 mg l^{-1} IAA and about 20 mg l^{-1} kinetin.

14. A method according to any one of claims 1 to 7 and 9, wherein the NAA and BA are present in the culture medium in a ratio of about 1:1, about 1:2 or about 2:1.

15. A method according to claim 14, wherein the culture medium includes about 1 mg l^{-1} NAA and about 1 mg l^{-1} BA.

16. A method according to claim 14, wherein the culture medium includes about 1 mg l^{-1} NAA and about 2 mg l^{-1} BA.

17. A method according to claim 14, wherein the culture medium includes about 2 mg l^{-1} NAA and about 2 mg l^{-1} BA.

18. A method according to any one of claims 1 to 19, wherein the culture medium is a Murashige and Skoog (1962) (MS) medium to which the auxin and cytokinin have been added.

19. A method according to any one of claims 1 to 18, wherein the culture medium is thickened with agar.

20. A method according to any one of claims 1 to 19, which results in spontaneous root development, thereby avoiding the need for a specific rooting step.

21. A method according to any one of claims 1 to 20, which is performed without a specific callus stage.

22. A method according to any one of claims 1 to 19, wherein callus is transferred from the first culture medium to a second culture medium for shoot production, the second culture medium having a different ratio of auxin and cytokinin than the first culture medium.

23. A method according to claim 24, wherein the second culture medium includes about 2 mg l^{-1} NAA and about 1 mg l^{-1} BA.

24. A method according to any one of claims 1 to 19 or 23, wherein regenerating tissue is transferred from the first culture medium or the second culture medium to a further culture medium for root production, the further culture medium not including a plant growth regulator.

25. A method according to claim 24, wherein the regenerating tissue is callus or adventitious microplants.

26. A method according to any one of claims 1 to 25, wherein the regenerated plantlets or plants have higher levels of umckalin than plants grown in the wild.

27. A *Pelargonium sidoides* DC or *Pelargonium reniforme* Curtis plantlet or plant which has been propagated by the method according to any one of claims 1 to 26.

28. A plant or plantlet according to claim 27, wherein the plant or plantlet has a higher level of umckalin than a wild plant or plantlet.

29. A method according to any one of claims 1 to 26, substantially as herein described with reference to the examples.

30. A *Pelargonium sidoides* DC or *Pelargonium reniforme* Curtis plantlet or plant according to either of claims 27 or 28, substantially as herein described with reference to the examples.

Dated this 27th day of July 2012

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VON SEIDELS Intellectual Property Attorneys

for the applicant

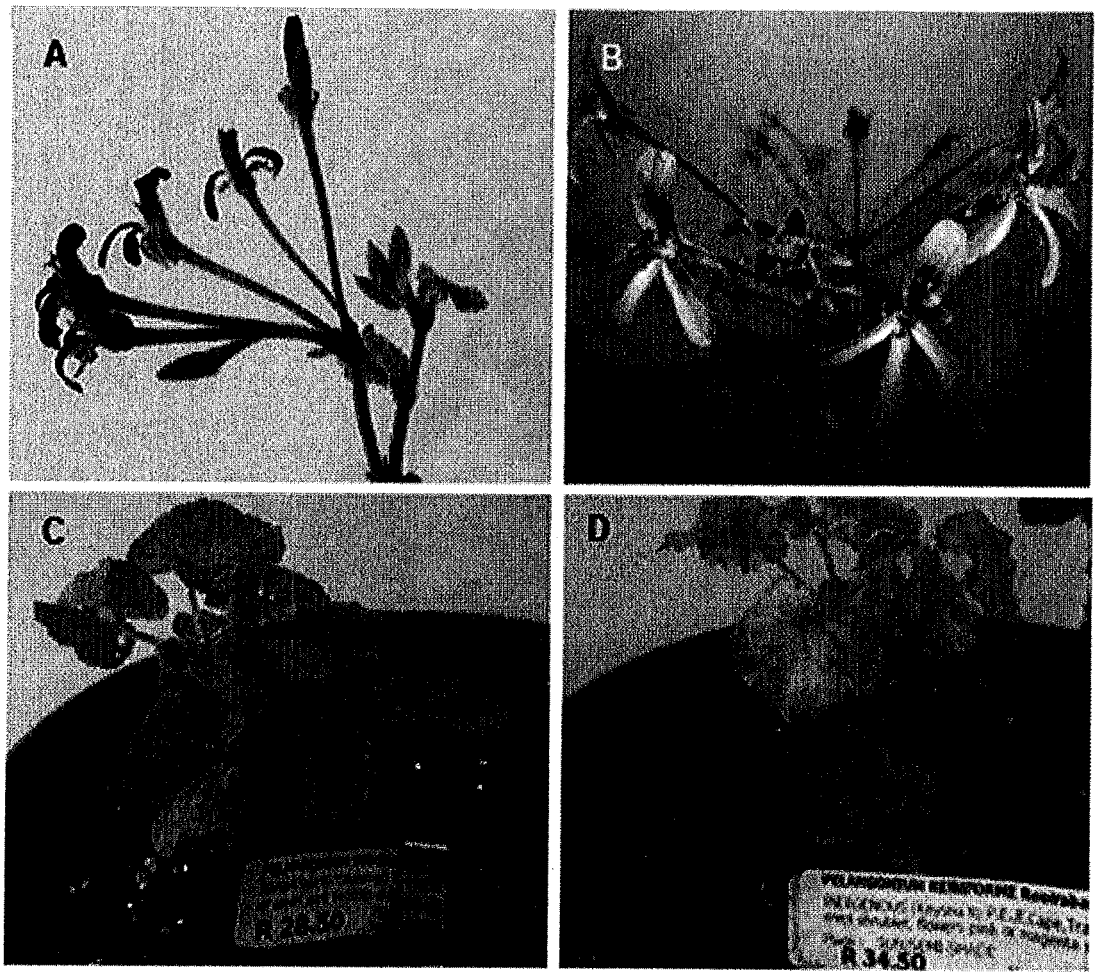

Figure 1

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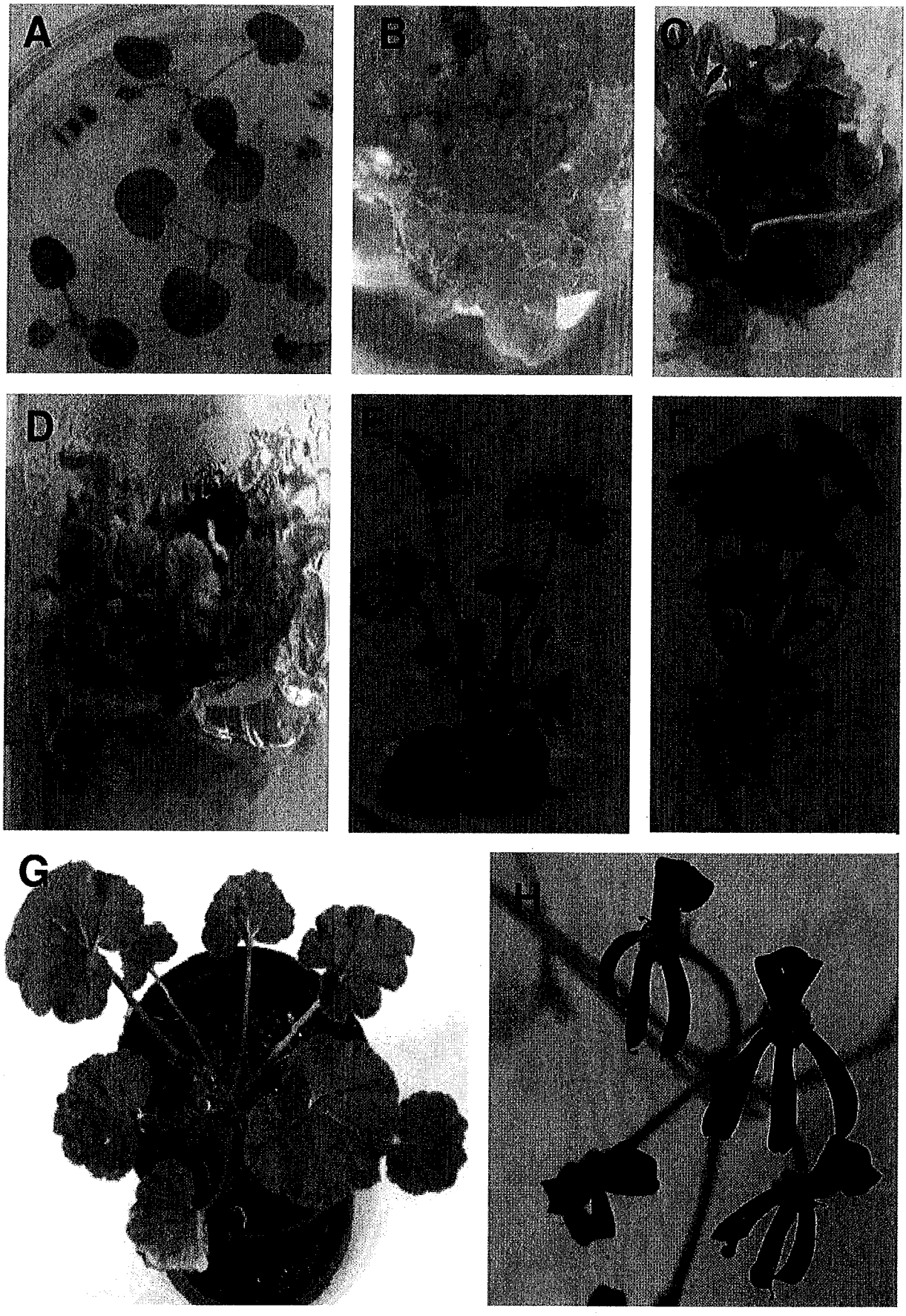

Figure 2

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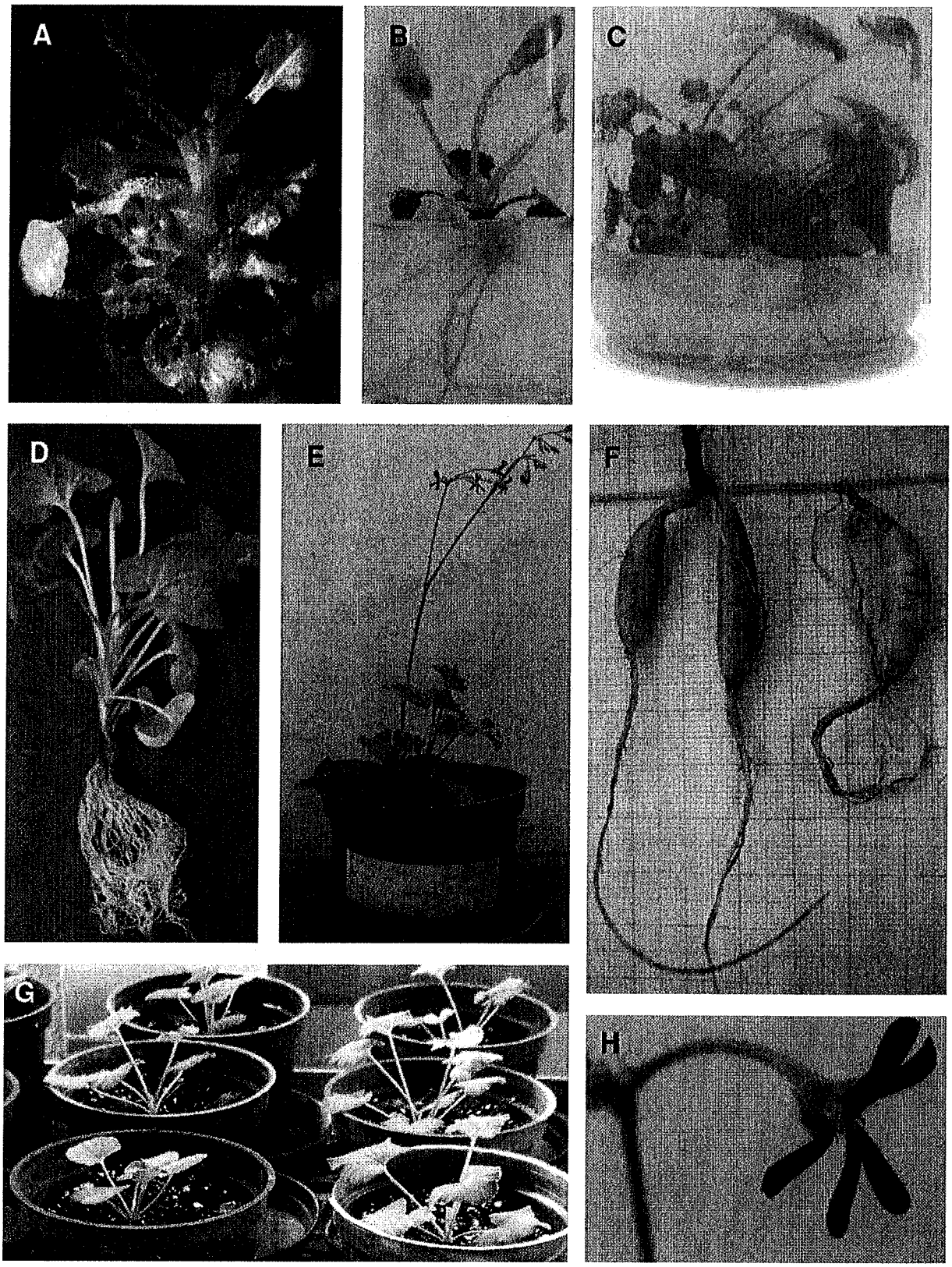

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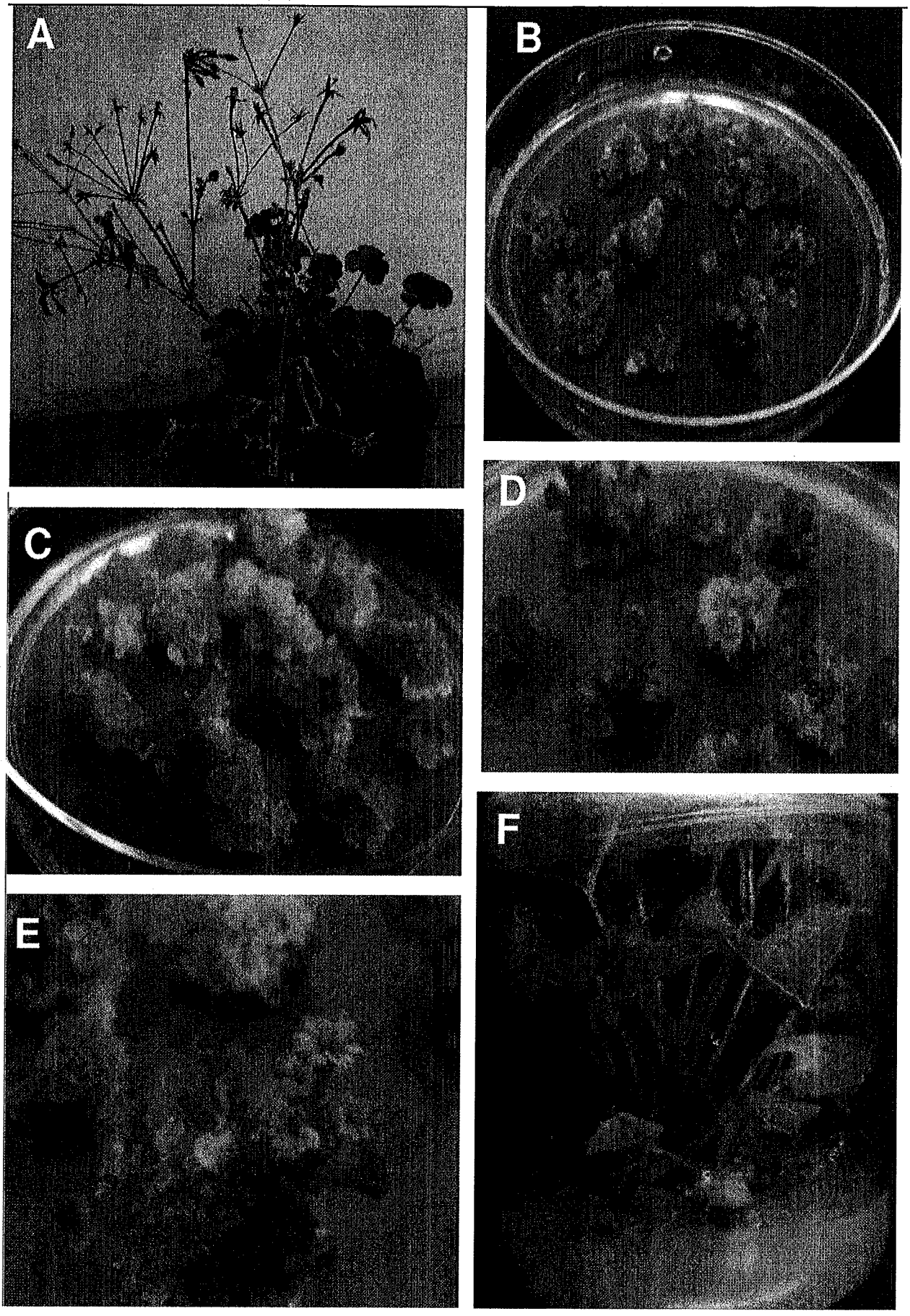

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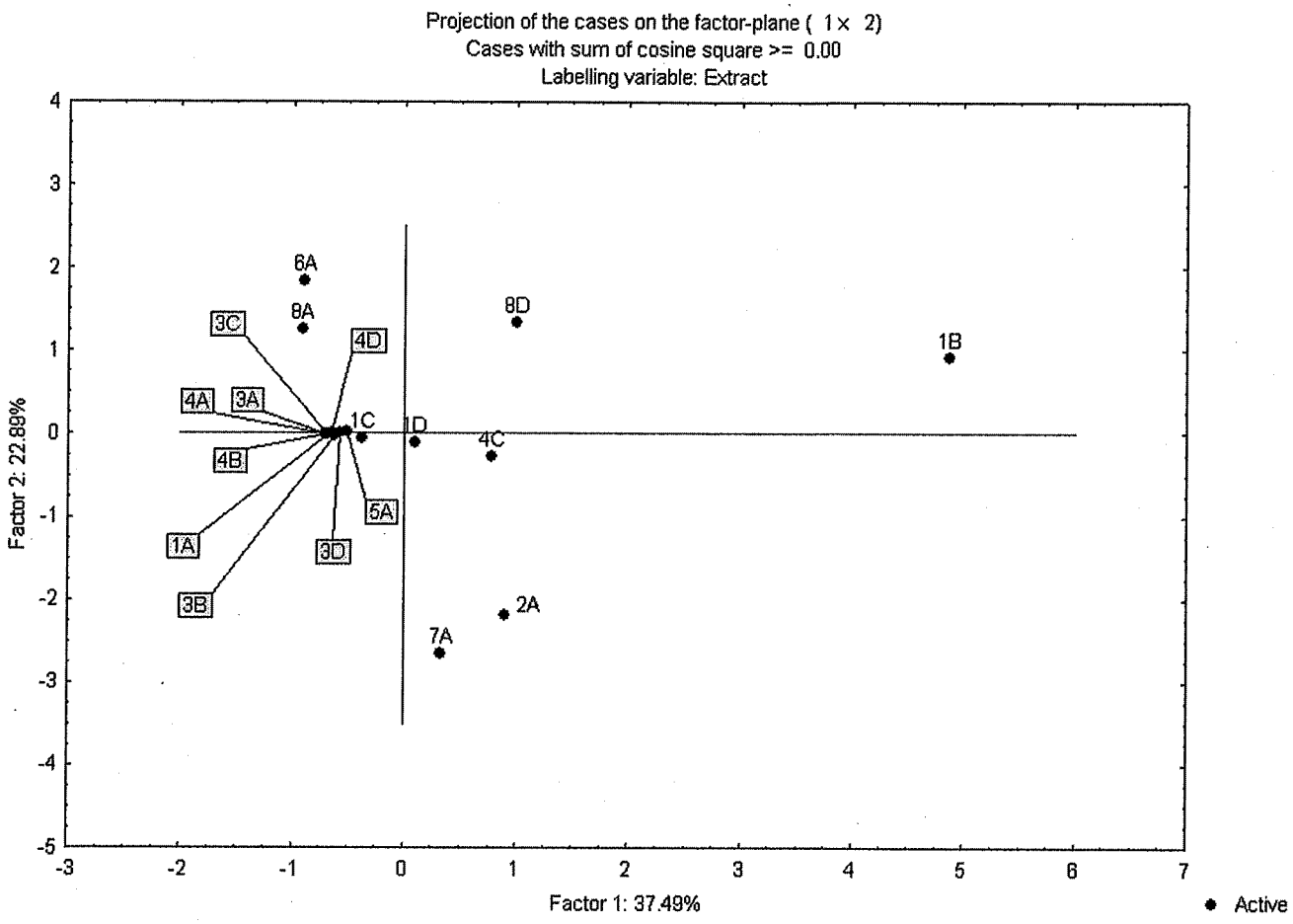

Figure 5

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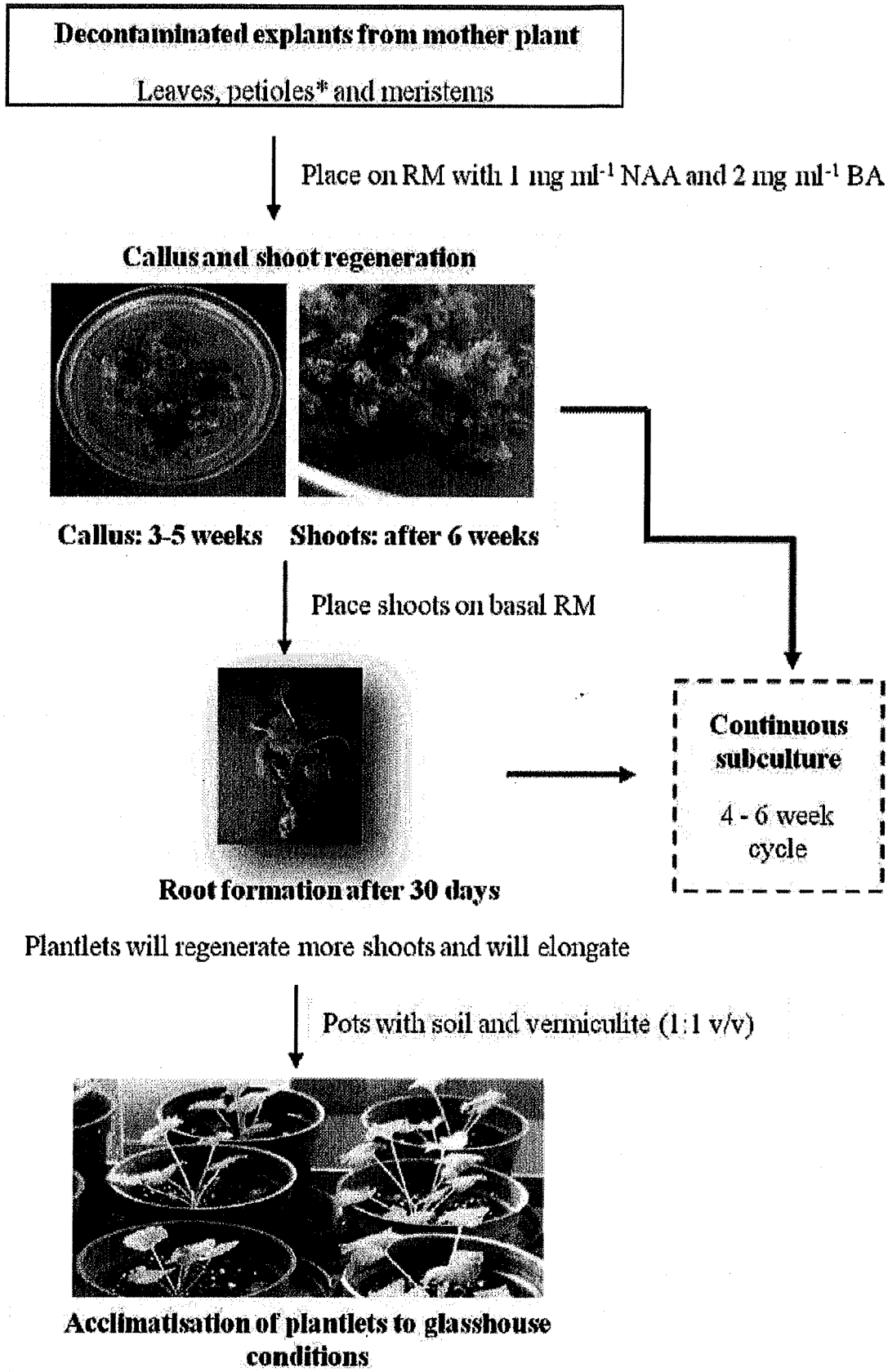

Figure 6

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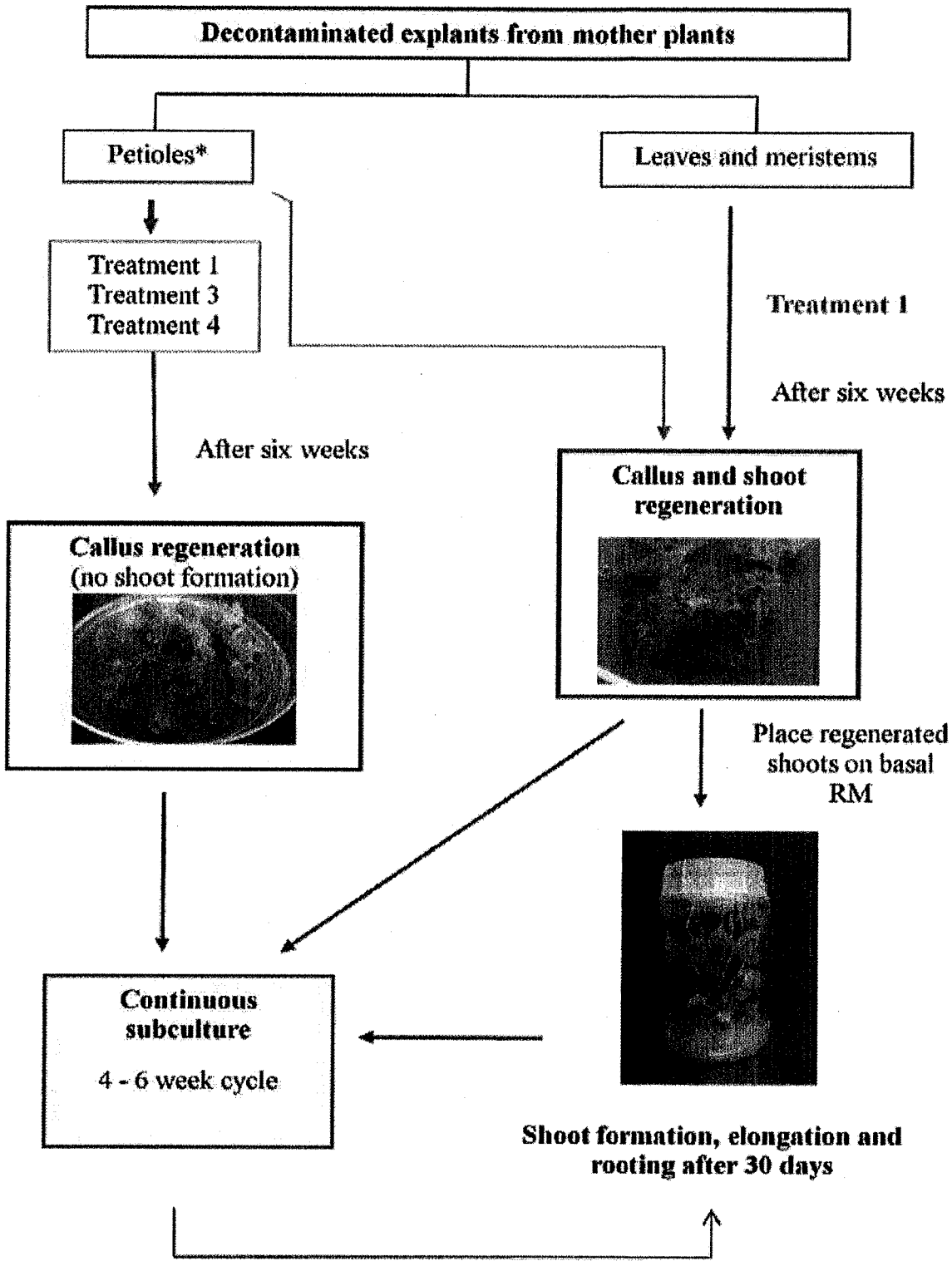

Figure 7

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