

TITLE OF THE INVENTION

Multiplication and *in vitro* Flowering of Rose Cultivars

TECHNICAL FIELD OF THE INVENTION

The present invention relates to the fields of plant husbandry, plant cloning and horticulture. Specifically, this invention relates to the vegetative propagation of a rose plant or tissue culture and *in vitro* induced flowering of rose plantlets derived from the plant or tissue culture. More particularly, the invention is directed to media formulations and efficient methods for the *in vitro* micropropagation, flower bud induction and flowering of roses and rose plants.

BACKGROUND OF THE INVENTION

The rose plant belongs to the plant family Rosaceae Juss. This family is very large, with over 100 genera and 2000 herbaceous to woody species of plants as members. Many important food and ornamental plants are in the Rosaceae family, including for example strawberry, apple, almond, cherry, peach and blackberry. The exact species involved in the development of the present-day rose is not known. Most rose species are found in the temperate parts of the Northern Hemisphere, especially from southern China and the far East, to the Himalayas and Bengal into Ethiopia and west to North America from the Arctic Circle to New Mexico. Recurrent or perennial flowering rose plants introduced into Europe from the Far East at the end of the eighteenth century were the results of countless generations of breeding in China, India, and Japan. The rose is one of the world's most popular flowers. Roses are commonly grown as cut flowers, potted plants, or specimen plants in home gardens. There are numerous types of cut roses, including for example long stem standards, short stem standards, small flowered sweethearts and multi flowered bunch roses.

Since most of the current commercial rose varieties are hybrids, propagation from seeds is not a feasible option, mainly due to segregation of traits among progenies. Commercial propagation of hybrid cut roses is normally carried out by budding or grafting of a specific shoot cutting, or scion, onto an established wild rose rootstock. For pot roses of small sizes, field asexual propagation of plant cuttings in green houses is the common practice. An alternative approach to the above methods is micropropagation, also known as axillary bud propagation or *in vitro* asexual propagation, which is the method of propagating plants from axillary buds under sterile aseptic conditions. Micropropagation has the advantage of fast propagation of a desirable

plant into many genetically uniform plantlets. Plantlets derived from micropropagation are also free of bacterial, fungal and viral infections and also are free of insect pests. Micropropagation procedures involve preparation of explants from the relevant plant, culture of the explant on a medium supplemented with phytohormones, incubation, and recovery of true to type shoots (or shoots with roots) [Douglas, In: Methods in Molecular Biology, Vol. 6, W. Pollard, J. M. Walker, eds. (1990); George and Sherrington, Exegetics, Ltd. U.K., p.3 (1984); and Brown and Thorpe, In: Cell Culture and Somatic Cell Genetics of Plants, p49-65, I. K. Vasil, ed. (1986)]. Micropropagation provides a potential cost effective means for the mass propagation of certain plants. An important consideration of this technology is the ability to add significant economic value to the plants being propagated. Examination of the relevant literature reveals that present propagation protocols are in general very inefficient at inducing the flowering of plantlets *in vitro*. Efficiency is dependent on the variety of plant used but is nevertheless low (about 10%) even for the best cultivars.

In recent years, potted rose varieties with sizes ranging from 7.5 cm to 20 cm tall have become very popular due to their compact size and longer shelf life as compared to cut roses. Also, the ability to produce multiple flowers at reasonable cost has further expanded the popularity of potted roses. Breeding for miniature rose hybrids has also been carried out around the world. By way of example, there have been more than 300 miniature rose hybrids registered in the United States alone. The most prominent breeders for commercial miniature rose hybrids are Poulsen Roser ApS in Denmark (with registered trademark PARADE®) and De Ruiter's New Rose International in Netherlands. Miniature pot roses are displayed in gardens, patios or even in homes for decorative effect. A miniature potted rose, however, is somewhat limited for use as an indoor decoration, mainly due to the requirement for soil in the pot. Also, considering a total average height between 15-30 cm (including the pot), even the height of miniature potted plants is still too high for placement on tables, for example. Furthermore, due to the increased urbanization around the world, more and more people spend more quality time indoors in the home or office, for example. Thus there presently exists a demand for decorative rose plants with multiple flowers with long shelf life that are both clean and compact.

Commercial propagation of roses is normally carried out by budding or grafting of a specific shoot cutting onto an established wild rose root stock. In recent years numerous publications have dealt with the propagation of roses through tissue culture. Examination of the

literature reveals that different varieties and types display greatly varying nutrient and hormone requirements. Most commercial growers of roses still propagate rose plants by traditional means. Media developed for the climbing rose Improved Blaze allowed significant growth of three miniature rose varieties but not for all hybrid tea roses (Hasegawa, *J. Amer. Soc. Hort. Sci.* 1980, 105:216-220). Furthermore, plant growth regulator requirements for multiplying *R. hybrida* cultivars Tropicana and Bridal Pink differ considerably from those of two old world species *R. damascena* and *R. canina* (Khosh-Khui and Sink, *J. Hort. Sci.* 1982 57, 315-319; Khosh-Khui and Sink, *Scientia Horticulturae* 1982 17, 371-376).

To optimize growth conditions most studies have focused on variations in the concentrations of certain growth regulators, Hasegawa, *supra*. A few studies have investigated the effect of certain other inorganic or organic components, bud position, temperature, hours of light per day, Murashige and Skoog ingredient concentrations, and weeks in culture between transfers. Khosh-Khui and Sink, *supra* Bressan *et al.*, *J. Amer. Soc. Hort. Sci.* 1982 107, 979-990. U.S. Patent 5,843,782 to Dobres *et al.* discloses a method of micropropagation of a rose plant comprising culturing a stem bearing a node in a first culture medium comprising a cytokinin, an auxin, and gibberellin and then in a second culture medium comprising a cytokinin, an auxin, and gibberellin to produce a flowering rose plant capable of being transferred to soil.

The present invention is directed to the development of novel compositions and methods useful for the propagation of miniature rose plantlets and the efficient induction of *in vitro* flowering for the production of miniature roses without transfer to soil and the need for a pot. In a preferred embodiment, the invention provides the roses flowered in an enclosed container on media for commercial and decorative purposes.

The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice are incorporated by reference.

SUMMARY OF THE INVENTION

In one aspect, the invention provides methods for the vegetative propagation and multiplication of a rose plant or rose plant tissue culture material.

In another aspect, the invention provides a method for the *in vitro* induced flowering of multiplied plantlets derived from a rose plant or rose plant tissue culture material.

In another aspect, the invention provides compositions for the micropropagation and *in vitro* flowering of multiplied plantlets derived from a rose plant or rose plant tissue culture material.

In one embodiment, rose shoots from potted plants are cultured on a first culture medium comprising inorganic nutrients, vitamins, a cytokinin, an auxin, and sucrose as a carbon source until buds form from the shoots. Buds are cut from the shoots and transferred to an enclosed vessel containing the first culture medium for about fifty (50) days to produce plantlets and new buds. The newly formed buds are cut and transferred to an enclosed vessel containing the first culture medium and cultured to multiply the plantlet and bud yield. Following the multiplication step, rose buds are excised and cultured again to produce plantlets on the first medium.

In one preferred embodiment, the plantlets are then transferred to a second culture medium comprising inorganic nutrients, vitamins, sucrose as a carbon source, the phytohormone thidiazuron, the cytokinin kinetin, an auxin and optionally myo-inositol to induce flower buds. In this protocol, after flower bud induction plantlets are transferred to an elongation media comprising inorganic nutrients, vitamins, sucrose as a carbon source, a cytokinin, an auxin, and myo-inositol. Following elongation, plantlets are then cultured on a culture medium comprising inorganic nutrients, vitamins and ampicillin for *in vitro* flowering.

In another embodiment, once the buds are multiplied, plantlets are then transferred to a culture medium comprising inorganic nutrients, vitamins, sucrose as a carbon source, the cytokinin Zeatin, and inositol to induce flower buds. In this protocol, plantlets are then transferred directly to medium comprising inorganic nutrients, vitamins and ampicillin for *in vitro* flowering.

BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawings will be provided by the Patent and Trademark Office upon request and payment of the necessary fee. Figure 1 depicts a flow chart showing the time required for completion of the various steps involved in the propagation and *in vitro* flowering of miniature rose cultivars. Figure 2 shows a full view of *in vitro* rose plantlet flowering in an enclosed vessel. Figure 3 shows a full view of enclosed vessels on shelf with rose plantlets flowering.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the invention provides methods for the vegetative propagation and multiplication of a rose plant or rose plant tissue culture material.

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In another aspect, the invention provides compositions for the micropropagation and *in vitro* flowering of multiplied plantlets derived from a rose plant or rose plant tissue culture material.

In one embodiment, rose shoots from potted plants are cultured on a first culture medium comprising inorganic nutrients, vitamins, a cytokinin, an auxin, and sucrose as a carbon source until buds form from the shoots. Buds are cut from the shoots and transferred to an enclosed vessel containing the first culture medium for about fifty (50) days to produce plantlets and new buds. The newly formed buds are cut and transferred to an enclosed vessel containing the first culture medium and cultured to multiply the plantlet yield. Following the multiplication step, rose buds are excised and cultured again to produce additional plantlets on the first medium.

We have discovered that including a step for flower bud induction, in which a phytohormone is added to the culturing media following multiplication, greatly improves the flower efficiency for rose plant cultivars over the previously disclosed methods. We have also observed that the increased efficiency of *in vitro* flowering does not require phytohormones in the final culture medium. Finally, we have discovered that in certain instances that adding an elongation step (which includes *inter alia* phytohormones as a component of the elongation media) after flower bud induction and prior to *in vitro* flowering also increases the efficiency of *in vitro* flowering over the prior art. In one preferred embodiment, after multiplication of plantlets in a first media comprising a cytokinin, an auxin and sucrose as a carbon source, the plantlets are transferred to a second culture medium comprising inorganic nutrients, vitamins, sucrose as a carbon source, the phytohormone thidiazuron, the cytokinin kinetin, an auxin and myo-inositol to induce flower buds. In this protocol, after flower bud induction plantlets are transferred to an elongation media comprising inorganic nutrients, vitamins, sucrose as a carbon source, a cytokinin, an auxin, and myo-inositol. Following elongation, plantlets are then cultured on a culture medium comprising inorganic nutrients, vitamins and auxin for plant elongation followed by transfer of elongated plantlets to culture medium comprising inorganic nutrients, vitamins and ampicillin for *in vitro* flowering.

In another embodiment, once the buds are multiplied in the first media, plantlets are then transferred to a culture medium comprising inorganic nutrients, vitamins, sucrose as a carbon source, the phytohormone zeatin, an auxin and myo-inositol to induce flower buds. Plantlets are then transferred directly to medium comprising inorganic nutrients, vitamins and ampicillin for *in vitro* flowering.

In each of these methods, the rose plant derived from vegetative propagation and multiplication may be a flowering rose that is capable of being stored and displayed on the media of the present invention. Unflowered plantlets can be used in an additional propagation procedure.

A culture medium for tissue culture micropropagation of a rose plant is also an embodiment of the present invention and comprises about 1.0 to about 2.0 mg/L benzyladenine, 0.05 to about 0.1 mg/L indole acetic acid or naphthalene acetic acid and about 1.5 % to 2.0 % sucrose as a carbon source. Preferably the culture medium contains about 2.0 mg/l benzyladenine, 0.1 mg/L of an auxin, and 2 % sucrose. The media of the present invention do not contain gibberellic acid.

A rose plant, including a flowering rose, produced by the method is also within the scope of the present invention. The rose is preferably Rose, and most preferably a cultivar selected from the group consisting of Orange PARADE®, Fiesta PARADE®, Scarlet PARADE®, Bianca PARADE® and Frosty PARADE®.

The present invention is also directed to a rose tissue culture comprising a rose stem bearing a node contained in an enclosed vessel containing the culture medium of the present invention. The rose stem may comprise at least one shoot, the rose tissue culture capable of producing a rose, for example, a flowering rose. A rose explant is also within the scope of the present invention. The rose explant may be selected from a rose stem bearing a node, a rose stem bearing a node and at least one shoot, and a rose shoot. The rose explant may be contained in an enclosed vessel comprising culture medium and may be cultured until a rose plant is produced, a flowering rose plant, for example.

The present invention is directed, *inter alia*, to a flowering rose plant which may remain in culture without feeding or watering for a period of time which permits the plant to continue to be sustained. Once the rose flowers in the enclosed vessel, it typically may be maintained for more than one month. The only limitation on the enclosed vessels contemplated for use in the present invention is that they must be able to maintain without leaking the solid culture medium

containing water and nutrients such as minerals, salt and vitamins. In a particular embodiment, the culture medium may also contain one or more dyes which are specially selected to enhance the attractiveness of the *in vitro* cultured flowering plant growing in the container. In one embodiment, a cover and base together comprise the enclosed vessel and the cover and base are attached so that they do not separate in normal handling and transit and so that moisture is retained inside the container. The cover, and desirably the entire container, is made of high transparency, high clarity material and the interior of the container is optionally coated with chemical to prevent water condensation on the inner surface of the container, giving a clear view of the *in vitro* flowering plantlet inside. Suitable materials include polycarbonate, poly (methyl methacrylate) and glass. Any other material that can withstand high heat (e.g. >160°C) and pressure ($\geq$ atmospheric pressure) with high clarity can also be used. Examples of antifogging chemicals which can be used for this purpose are Sicanett®, product of Anfora (SV), Italy, and Siclair® (Nettoyant universal), an anti-statique manufactured by SI-International S.A. France. Antifogging agent is sprayed directly onto inner surface of the container. The present invention is also directed to each individual step of the two steps of the method of tissue culture micropropagation set forth above. Within this general framework, and in addition to the aforementioned components benzyladenine and either indole acetic acid or naphthalene acetic acid, the culture medium of the present invention may further comprise a nutritive medium. Preferably the medium is a modified Murashige and Skoog (MS) basal medium that has been described elsewhere (Murashige et al., *Physiol. Plant.*, Vol. 15, pp. 473-97 (1962). Modified MS medium is MS medium containing Gamborg's B5 vitamins (final concentration of 10 mg/L thiamine hydrochloride, 1 mg/L nicotinic acid, 1 mg/L pyridoxine, 100 mg/L myo-inositol), pH 5.8, with 2% sucrose and gelled by 0.30% phytigel. In the preferred embodiment, this is the medium that is supplemented with the various additional phytohormones and other additives as disclosed herein.

The auxins used in the practice of the preferred embodiments of the invention include indole acetic acid and naphthalene acetic acid. The cytokinin include thidiazuron, kinetin 6-benzyladenine and zeatin.

Benzyladenine may be substituted with another natural or synthetic cytokinin selected from the group consisting of 6-benzylaminopurine riboside; 6-(γ - γ -dimethylallylamino)purine; DL-Dihydrozeatin; t-zeatin riboside; zeatin; N-(2-Chloro-4-pyridyl)-N'-phenylurea; N-benzyl-9-(2-tetrahydropyranyl)adenine; kinetin; kinetin riboside; and the like, wherein the cytokinin may

be used alone or in combination with one or more other cytokinin. Indole acetic acid may be substituted with another natural or synthetic auxin selected from the group consisting of naphthalene acetic acid, indole butyric acid, picloram, dicamba, and the like, usually at a concentration of about 0.03 to about 0.3 mg/l, wherein the auxin may be used alone or in combination with one or more other auxin. The media may be modified such that hormonal constituents are provided for specific varieties of plants. Nutrient levels may be modified in the basal media when inducing flowering. For example, Murashige and Skoog (MS) medium, which provides the general nutritional and growth requirements of tissue culture plant cells, may be substituted with other conventional culture or growth medium such are known in the art. It will readily be appreciated by the skilled artisan that the precise amount of each component to be employed will depend upon the type of rose to be cultured. Typically, the compounds are initially employed at the lower levels, and the amounts increased as necessary to achieve the desired effect.

Optimally, the concentration of thidiazuron when added to the composition and method for flower bud induction is between 0.4-0.5 mg/L.

Optimally, the concentration of zeatin is between 0.5 and 1.0 mg/L.

When benzyladenine is added to the media for the multiplication and vegetative processes, the optimal concentration is about 2 mg/L. When benzyladenine is added to the media used for the elongation of plantlets with induced flower buds, the optimal concentration is about 0.1 mg/L.

When naphthalene acetic acid is the auxin added to the media of the present invention, it is added at a concentration of less than 2 mg/L. Optimally, the concentration of naphthalene acetic acid is between 0.05 and 0.1 mg/L.

When indole acetic acid is the auxin added to the media of the present invention, it is added at a concentration between 0.05 and 0.1 mg/L. Optimally, the concentration of indole acetic acid is 0.1 mg/L for multiplication of plantlets. When indole acetic acid is added to the media used for the elongation of plantlets with induced flower buds, the optimal concentration is about 1.0 mg/L.

Various alterations to the micropropagation conditions will be readily apparent to those skilled in the art and include choosing donor tissues for culture initiation which come from plants of appropriate genotype and physiological and development states. In addition, variation of explant source, plant variety, and physical environment in which the cultures are grown are

contemplated by the present invention. For example, gas environment, temperature and light conditions may be varied to provide for an improved rose plant contained in an enclosed vessel. In general, the optimal temperature for flower bud induction will be $\leq 25^{\circ}\text{C}$. Any suitable gelling agent such as and not limited to PHYTAGEL.TM. comprising gellan gum, GEL-RITE.TM. comprising gellan gum; AGARGEL.TM. comprising gellan gum; agar such as and not limited to agar types A, E, and M, High Gel Strength, Purified, Bacteriological Flake; AGARGEL.TM. a blend of agar and PHYTAGEL.TM.; agarose, such as and not limited to Type VII; alginic acid; carrageenan; transfergel or hydroxyethylcellulose; and the like.

A rose explant is an embodiment of the present invention. The rose explant may be contained in an enclosed vessel comprising the aforementioned culture medium, and may be cultured until a rose plant is produced, a flowering rose plant, for example. The explants may be obtained from a plant, such as and not limited to the genus *Rosa*, including and not limited to the hybrid rose *Rosa hybrida*, such as and not limited to the cultivars Orange PARADE®, Fiesta PARADE®, Scarlet PARADE®, Bianca PARADE® and Frosty PARADE® and the like; *Rosa damascena*, *Rosa multiflora*, and *Rosa gallica*, and the like. The tissue culture techniques of the present invention comprise culturing a rose explant in an enclosed vessel such as and not limited to a petri dish, a test tube, a flask, an eppendorf tube, a baby food jar, a canning jar, and any other enclosed container capable of supporting the growth of the rose plant of the present invention in accordance with the methods of the present invention.

A rose, including a flowering rose, produced by the methods of the present invention, is also within the scope of the present invention. The rose plant may be contained within the enclosed vessel. Rose tissue culture or plantlet is also within the scope of the present invention. The rose tissue culture is capable of producing a rose, such as a flowering rose.

The rose in an enclosed vessel may be used to screen new cultivars for flower color and form. For example, plants may be subjected to mutational treatment during tissue culture of rose callus, explants or axillary bud. Mutated rose plants may then be grown in the culture of the present invention and screened for those with altered flower color, form, disease resistance, growth vigor, cold and heat sensitivity, and any other valuable trait. In addition, the present invention also permits performing cross pollination of rose plants in culture. For example, since flowers contain visibly developed male and female organs, such flowers may be pollinated with pollen derived from the same flower or from a plant of any other source. Alternatively, flowers

may be propagated *in vitro* and stored or displayed in a clear, transparent container for decorative purposes.

General Methods

In the description the following terms and abbreviations are employed.

Benzyladenine is a plant hormone of the cytokinin type.

Zeatin is a plant hormone of the cytokinin type.

Naphthalene acetic acid or 2-naphthylacetic acid is a plant hormone of the auxin type.

Indole acetic acid or 3-indoleacetic acid is a plant hormone.

The term cytokinin refers to plant hormones. In low concentrations, these organic substances promote elongation of root cells.

The term auxins refers to plant hormones. In low concentrations, these organic substances promote elongation of plant shoots and control other specific growth effects. Auxins include 3-(3-indolyl)-propionic acid (IPA), abscisic acid (ABA), and 2,4-D, Napthalene acetic acid and GA3.

The term "cultivar" refers to a commercially valuable, horticulturally derived, variety, as distinguished from a naturally occurring variety.

As used herein "micropropagation" refers to *in vitro* asexual clonal reproduction of plants wherein large numbers of new shoots may be obtained in a short time period from the induced buds of parental plants by culture in a medium containing plant hormones, minerals, vitamins and carbohydrates in the appropriate concentrations.

The following media were utilized in the practice of the present invention:

RMS₀: Modified MS media containing full strength Murashige Skoog inorganics nutrients and Gamborg's B5 vitamins (final concentration of 10 mg/L thiamine hydrochloride, 1mg/L nicotinic acid, 1mg/L pyridoxine, 100 mg/L myo-inositol), pH 5.8, with 2% sucrose and gelled by 0.30% phytagel. At different developmental stages, the medium was supplemented with different phytohormones.

RMS₁: RMS₀ supplemented with 2 mg/L 6-benzyladenine and 0.05 mg/L naphthalene acetic acid.

RMS₂: RMS₀ with 3% sucrose and supplemented with 0.4-0.5 mg/L thidiazuron, 0.05 mg/L naphthalene acetic acid, 0.1 mg/L kinetin and 400 mg/L inositol.

RMS₃: RMS₀ with 3% sucrose and supplemented with 0.5-1.0 mg/L zeatin, 0.05 mg/L naphthalene acetic acid, and 400 mg/L inositol.

RMS₄: RMS₀ with 3% sucrose and supplemented with 0.1 mg/L benzyladenine, 1.0 mg/L indole acetic acid and 400 mg/L myo-inositol.

RMS₅: RMS₀ with 2% sucrose and supplemented with 2 mg/L benzyladenine and 0.1 mg/L indole acetic acid.

Obtaining Explants From Mother Stock

In a preferred embodiment, young shoots with axillary buds are used as explants. To ensure that the explant is free of bacteria and fungi infections (contaminants) in the medium, the explant is surface sterilized before use. Many sterilizing techniques are available in the art for the purpose of preparing explant for culture. Such techniques involve dipping the explant in the solution containing at least one sterilizing agent. Such sterilizing agents include, sodium hypochlorite, calcium hypochlorite, mercuric chloride, ethyl alcohol etc.

In the preferred method of the invention, young shoots of 10-12 cm long from mature plants are stripped of the outer leaves and washed under running water first, and then sterilized with 0.1% HgCl₂ for 5-10 minutes and then washed completely with running water for more than 30 minutes. The shoots are then immersed in 10-15% CloroxR (containing 5.25% sodium hypochlorite) solution for 10-15 minutes. Finally, the shoots are rinsed 4-6 times with sterile distilled water. Young shoots can alternatively be derived from rose plant tissue culture using techniques well known within the art. It is thus contemplated that young shoots obtained from tissue culture may be used in place of the young shoots prepared from mature plants without departing from the scope of the present invention.

Plantlet formation

The explants are placed on solid media and cultured under 16 hr photo periods with a light intensity of 4500-5500 lux (provided by daylight type lights). When the plantlets are exposed to the above lighting conditions, the temperature is maintained at 23°C. Alternatively, when the light source is removed, the plantlets are maintained at 19°C. Whole shoots are cultured on RMS₁ [RMS₀ supplemented with 2 mg/L 6-Benzyladenine, 0.05 mg/L Naphthalene acetic acid]. The explants turn green gradually and will usually form new buds after 10 to 15

days. The buds are cut from shoots at this point in time in preparation for multiplication of the plantlet.

Multiplication

Multiplication of plantlets is the process where whole plant material is obtained from a juvenile or rejuvenated growing plant, typically at a growing point or area of rapidly dividing cells at the tip of a root or shoot, referred to as meristem material. The shoot material is placed in a predefined hormonal and nutritional medium to produce explants which gradually form plantlets having new buds. Individual buds are then removed from such explants and cultured in the same predefined hormonal and nutritional medium as above to produce multiple plantlets from a single whole shoot. Such asexual reproduction of plants from a single parent allows cloning of plant progeny having identical genetic characteristics to those of the parent. Cut buds are transferred to RMS₁ medium and cultured for about 50 days. Utilizing the above procedures, buds grow into plantlets and new buds form on these plantlets. The newly formed buds are transferred when ready to be cut onto new RMS₁ medium for propagation and multiplication of plantlets. The multiplied ratio will usually range from 4 to 7 times the original starting number of explants.

Flower Bud induction

Flower bud induction is critical for *in vitro* flowering at commercial scale. There are two alternative approaches to flower bud induction contemplated by the present invention.

In one embodiment, following multiplication flower buds are induced by culturing the multiplied plantlets on RMS₂ medium in an enclosed vessel. The induction culturing lasts about fifty (50) days. Following this step, the plantlets are transferred to an enclosed vessel containing RMS₄ for an elongation step which lasts about fifteen (15) to thirty (30) days. After elongation, plantlets are transferred to MS₀ with 20 mg/L to 50 mg/L ampicillin. Flowers usually open within ten (10) to twenty (20) days.

In an alternative but still preferred embodiment, following multiplication flower buds are induced by culturing the multiplied plantlets on RMS₃ medium in an enclosed vessel for a period of about fifty (50) days. Following induction, plantlets are transferred directly to MS₀ with 20 mg/L to 50 mg/L ampicillin. Flowers usually open within fifteen (15) to twenty (20) days.

EXAMPLES

The present invention is further detailed in the following examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described *infra* are utilized.

EXAMPLE 1

This example demonstrates the induction of flower buds on medium augmented with thidiazuron. Young shoots with axillary buds from mature plants were used as explants. Rose explants were placed on solid media and cultured under 16 hr photoperiods with a light intensity of 4500-5500 lux (provided by daylight type lights) and a temperature of 23°C with lighting and 19°C without lighting. Whole shoots were cultured on RMS₁. The explants turned green gradually and formed new buds after 10 to 15 days. The buds were cut from shoots. Multiplied plantlets (> two times multiplication) were cultured on RMS₂ to induce floral buds. After flower bud induction, plantlets were transferred onto elongation media for in vitro flowering. Plantlets were cultured on the elongation media, RMS₄ for 15-30 days before transferring into MS₀ with ampicillin at concentrations between 20-50 mg/L. Flowers opened within 10-20 days following transfer to MS₀ plus ampicillin. Forty to fifty percent plantlets yielded flowers and more than 80% of the flowers were normal in appearance. Unflowered plantlets could go back to propagation. The shelf life of flowers cultured was more than 1 month at less than 25 °C.

EXAMPLE 2

This example demonstrates the induction of flower buds on medium augmented with zeatin. Young shoots with axillary buds from mature plants were used as explants. Rose explants were placed on solid media and cultured under 16hr photoperiods with a light intensity of 4500-5500 lux (provided by daylight typelights) and a temperature of 23°C with lighting and 19°C without lighting. Multiplied plantlets (> two times multiplication) were cultured on RMS₃. After flower bud induction, plantlets with flower buds were transferred directly into MS₀ with ampicillin 20-50 mg/L for flowering. Flowers would be opened within 15-20 days. Forty to fifty percent plantlets yielded flowers and more than 80% were normal. Unflowered plantlets could go back to propagation. The shelf life of flowers cultured was more than 1 month at less than 25°C.

EXAMPLE 3

Rose plants of the cultivars Fiesta PARADE®, Scarlet PARADE®, Bianca PARADE® and Frosty PARADE® were used as explants and were grown using the above compositions and methods. Multiplication media RMS₅, which contains indole acetic acid in place of naphthalene acetic acid was used in place of RMS₁. More than thirty-percent of the plantlets multiplied and subjected to the *in vitro* flowering methods of the present invention yielded plantlets with normal flowers.

It should be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

CLAIMS

What is claimed is:

1. A method for micropropagation of a rose plant and flowering of roses from the plant comprising:

- (a) preparing young shoots from a mature rose plant or rose plant tissue culture under aseptic conditions;
- (b) culturing the shoots on a first culturing medium in a closed vessel, the first culturing medium comprising a cytokinin, an auxin and sucrose as a carbon source to produce explants with buds;
- (c) removing the buds from the explants;
- (d) propagating the buds on the first culturing medium in an enclosed vessel to produce plantlets with newly formed buds;
- (e) multiplying the plantlets on the first culturing medium in an enclosed vessel;
- (f) culturing the multiplied plantlets to induce floral buds on a second culture medium in an enclosed vessel, the second culture medium comprising at least one cytokinin, and an auxin;
- (g) culturing the plantlets with floral buds on a third culture medium in an enclosed vessel, the third culture medium comprising sucrose as a carbon source.

2. A method for micropropagation of a rose plant and flowering of roses from the plant comprising:

- (a) preparing young shoots from a mature rose plant or rose plant tissue culture under aseptic conditions;
- (b) culturing the shoots on a first culturing medium in a closed vessel, the first culturing medium comprising benzyladenine, an auxin selected from the group consisting of naphthalene acetic acid and indole acetic acid and sucrose as a carbon source to produce explants with buds;
- (c) removing the buds from the explants;
- (d) propagating the buds on the first culturing medium in an enclosed vessel to produce plantlets with newly formed buds;
- (e) multiplying the plantlets on the first culturing medium in an enclosed vessel;

(f) culturing the multiplied plantlets to induce floral buds on a second culture medium in an enclosed vessel, the second culture medium comprising thidiazuron, kinetin, sucrose as a carbon source, myo-inositol and naphthalene acetic acid;

(g) culturing the plantlets with floral buds to induce elongation and *in vitro* flowering on a third culture medium in an enclosed vessel, the third culture medium comprising sucrose as a carbon source, benzyladenine, indole acetic acid, and myoinositol; and

(h) culturing the elongated plantlets on a fourth culture medium in an enclosed vessel wherein the fourth culture medium comprises sucrose as a carbon source and an antibiotic.

3. A method as in claim 2 wherein the first culture medium has a concentration of benzyladenine between about 1.0 mg/L and about 2.0 mg/L and the concentration of sucrose is about 2%.

4. A method as in claim 3 wherein the auxin in the first culture medium is about 0.05 mg/L to about 0.2 mg/L naphthalene acetic acid.

5. A method as in claim 3 wherein the auxin in the first culture medium is about 0.1 mg/L indole acetic acid.

6. A method as in claim 2, 3, 4 or 5 wherein the second culture medium has a concentration of thidiazuron between about 0.1 mg/L and about 0.3 mg/L, a concentration of about 0.1 mg/L kinetin, a concentration of myo-inositol between 250 mg/L and 500 mg/L and a concentration of sucrose between 1.5% and 3%.

7. A method as in claim 6 wherein the second culture medium has a concentration of thidiazuron between about 0.4 mg/L and about 0.5 mg/L, a concentration of myo-inositol at about 400 mg/L and a concentration of sucrose at about 3%.

8. A method as in claim 6 or claim 7 wherein the third culture medium has a concentration of benzyladenine between 0.5 and 0.2 mg/L, a concentration of indole acetic acid of 1 mg/L, a concentration of myo-inositol between 250 mg/L and 500 mg/L, and about 3% sucrose.

9. A method as in claim 8 wherein the fourth culture medium has a concentration of sucrose of about 2% and the antibiotic is ampicillin at a concentration between 20 mg/L and 50 mg/L.

10. A method for micropropagation of a rose plant and flowering of roses from the plant comprising:

(a) preparing young shoots from a mature rose plant or rose plant tissue culture under aseptic conditions;

(b) culturing the shoots on a first culturing medium in a closed vessel, the first culturing medium comprising a benzyladenine, an auxin selected from the group consisting of naphthalene acetic acid and Indole acetic acid and sucrose as a carbon source to produce explants with buds;

(c) removing the buds from the explants;

(d) propagating the buds on the first culturing medium in an enclosed vessel to produce plantlets with newly formed buds;

(e) multiplying the plantlets on the first culturing medium in an enclosed vessel;

(f) culturing the multiplied plantlets to induce floral buds on a second culture medium in an enclosed vessel, the second culture medium comprising zeatin, sucrose as a carbon source, myo-inositol and naphthalene acetic acid;

(g) culturing the plantlets with floral buds to induce elongation and *in vitro* flowering on a third culture medium in an enclosed vessel, the third culture medium comprising sucrose as a carbon source and an antibiotic.

11. A method as in claim 10 wherein the first culture medium has a concentration of Benzyladenine between about 1.0 mg/L and about 2.0 mg/L.

12. A method as in claim 11 wherein the auxin in the first culture medium is about 0.05 mg/l to about 0.2 mg/L Naphthalene acetic acid.

13. A method as in claim 11 wherein the auxin in the first culture medium is about 0.1 mg/L Indole acetic acid.

14. A method as in claim 10, 11, 12 or 13 wherein the second culture medium has a concentration of Zeatin between 0.5 mg/L and 1.0 mg/L, a concentration of myo-inositol between 250 mg/L and 500 mg/L and a concentration of sucrose between 1.5% and 3%.
15. A method as in claim 14 wherein the second culture medium has a concentration of myo-inositol at about 400 mg/L and a concentration of sucrose at about 3%.
16. A method as in claim 15 wherein the third culture medium has a concentration of sucrose of about 2% and the antibiotic is ampicillin at a concentration between 20 mg/L and 50 mg/L.
17. A method as in claim 1, 2 or 10 wherein each culture medium further comprises a nutritive medium.
18. The method of claim 17 wherein the nutritive medium comprises MS medium in combination with Gamborg's B5 vitamins.
19. The method of claim 1, 2 or 10 wherein the rose plant is *Rosa hybrida*.
20. The method of claim 19 wherein said *Rosa hybrida* is selected from the cultivars consisting of the PARADE® rose varieties.
21. The method of claim 20 wherein said *Rosa hybrida* is selected from the cultivars consisting of Orange PARADE®, Fiesta PARADE®, Scarlet PARADE®, Bianca PARADE® and Frosty PARADE®.
22. A culture medium for tissue culture micropropagation of a rose plant comprising about 1.0 mg/L to about 2.0 mg/L benzyladenine and about 1.0 mg/L to about 2.0 mg/L indole acetic acid and about 2% sucrose as a carbon source.
23. A culture medium for the multiplication of rose plantlets comprising about 2 mg/L benzyladenine, about 0.05 mg/L naphthalene acetic acid and about 2% sucrose as a carbon source.

24. A culture medium for the induction of floral buds from a rose plant comprising:
at least one cytokinin selected from the group consisting of thidiazuron, kinetin and zeatin; naphthalene acetic acid at a concentration of about 0.5 mg/L;
3% sucrose as a carbon source; and
about 400 mg/L myo-inositol.
25. A culture medium as in claims 22, 23 or 24 further comprising a nutritive medium.
26. A culture medium as in claim 25 wherein said nutritive medium comprises MS medium in combination with Gamborg's B5 vitamins.
27. A culture medium for the induction of floral buds as in claim 26 comprising thidiazuron at a concentration of about 0.4 to 0.5 mg/L, naphthalene acetic acid at a concentration of about 0.5 mg/L, kinetin at a concentration of about 0.1 mg/L, 3% sucrose as a carbon source and about 400 mg/L myo-inositol, wherein zeatin is not present in the medium.
28. A culture medium for the induction of floral buds as in claim 26 comprising zeatin at a concentration of about 0.5 to 1.0 mg/L, naphthalene acetic acid at a concentration of about 0.5 mg/L, 3% sucrose as a carbon source and about 400 mg/L myo-inositol, wherein thidiazuron and kinetin are not present in the medium.
29. The culture medium of claim 24 wherein the rose plant is *Rosa hybrida*.
30. The culture medium of claim 29 wherein the *Rosa hybrida* is selected from the cultivar consisting of Orange PARADE®, Fiesta PARADE®, Scarlet PARADE®, Bianca PARADE® and Frosty PARADE®.

31. A method for micropropagation of a rose cultivar and flowering of roses from the plant comprising:

- (a) preparing young shoots from a mature rose plant or rose plant tissue culture under aseptic conditions;
- (b) culturing the shoots on a first culturing medium in a closed vessel, the first culturing medium comprising a cytokinin, an auxin and sucrose as a carbon source to produce explants with buds;
- (c) removing the buds from the explants;
- (d) propagating the buds on the first culturing medium in an enclosed vessel to produce plantlets with newly formed buds;
- (e) multiplying the plantlets on the first culturing medium in an enclosed vessel; and
- (f) culturing the multiplied plantlets on the first culturing medium in an enclosed vessel.