

Chapter 2

Techniques for the Maintenance and Propagation of Phytoplasmas in Glasshouse Collections of *Catharanthus roseus*

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Abstract

Phytoplasma collections are a vital resource for researchers and diagnosticians studying phytoplasma diseases. They provide material as a point of reference and a research tool to increase our understanding of phytoplasmas and the diseases they cause. This chapter describes the techniques required to create and maintain collections of phytoplasma-infected *Catharanthus roseus* (Madagascar periwinkle).

Key words: *Catharanthus roseus*, Cuttings, Glasshouse maintenance, Grafting technique, Phytoplasma

1. Introduction

Catharanthus roseus (L.) G. Don (formerly known as *Vinca rosea*), commonly known as the Madagascar periwinkle has been found to be very susceptible to infections by phytoplasmas, although the reasons for this remain unknown. Because of this it has been used as an experimental host and for the maintenance of phytoplasma collections for research purposes (1–3). Periwinkles are a perennial tropical plant belonging to the family Apocynaceae which are distributed worldwide and widely cultivated (4). *C. roseus* is an evergreen plant with attractive dark glossy foliage and colored flowers meaning it is commonly grown in gardens and has long been used as an ornamental plant (5). However, it also has uses as a medicinal plant due to the discovery during the 1960s that leaf extracts had an antitumoral effect in rats. This is due to the production of numerous monoterpenoid indole alkaloids, two of which (vinblastine and vincristine) are now important commercial cancer therapy drugs (6,7). Interestingly, studies have indicated a significant increase



Fig. 1. Some of the typical symptoms caused by phytoplasma infection of *Catharanthus roseus*; (a) healthy *C. roseus*; (b–f) *C. roseus* infected with various phytoplasma strains showing (b) deformation of petals; (c) virescence of flowers; (d–f) various stages of phyllody.

of vinblastine in the roots of *C. roseus* infected with phytoplasmas compared to healthy plants (3).

Symptoms caused by phytoplasmas in the plant hosts (including *C. roseus*) can include, although are not limited to; virescence (the abnormal development of greening in floral tissue), phyllody (development of floral parts into leaf-like structures), sterility, elongation and etiolation of internodes, flower streaking and malformation, yellowing and upright posture of leaves, excessive branching of axillary shoots, proliferation, witches' broom (proliferation of shoots from a single point, typically in woody plants), general stunting, leaf curl or rolling, small and faintly colored flowers. The set of symptoms in infected plants may vary depending upon the phytoplasma in question, host plant species, the age of plant at infection, and the stage of infection. For example, within the Aster yellows group ('*Candidatus* Phytoplasma asteris') different sub-groups produce distinct subsets of symptoms, and mild strains may induce no obvious symptoms. However, generally the symptoms are deleterious to the plant host (2, 8, 9) (Fig. 1).

The majority of phytoplasmas can be transmitted into *C. roseus* by means of grafting (this Chapter), dodder transmission (see Chapter 4) or insect transmission (see Chapter 5). However, there are notable exceptions, primarily phytoplasmas that are very host specific. In these cases, it is sometimes convenient to maintain the original host in glasshouse collections, for example some of the phytoplasmas which infect grasses such as Napier Grass Stunt (16SrXI).

However in other cases, the stature of the plant prevents this, for example Lethal yellowing diseases (16SrIV, XXII, and XXX) in *Cocos nucifera* (coconut palm), meaning that sources of these isolates are generally only available as natural infections in the environment. Phytoplasma titre (or concentration) is known to vary depending on the plant host, with woody hosts such as trees and grapevines tending to have particularly low levels. *C. roseus* tends to have much higher phytoplasma cell concentrations (10).

Common diseases which may afflict glasshouse grown *C. roseus* plants include (amongst others) red spider mite (*Tetranychus urticae*), powdery mildew, and sciarid fly. Perhaps the most damaging of these is red spider mite due to the slow, hard to detect build up of the disease. Often once the obviously visible symptoms of webbing have appeared any treatments are ineffective and the only option is to discard heavily infested plants. Milder infestations noticed earlier on can be treated with chemical applications or biological control with application of parasitic predators such as *Phytoseiulus persimilis* or *Neoseiulus californicus* (available commercially). However, it is not recommended to treat the plants prophylactically with broad ranging pesticides due to the risk of resistance developing. Sciarid fly infestations tend to result from overly wet compost and can be a more persistent problem if plants are housed on capillary matting in a glasshouse (to enable easier watering). There are no reports of commercially available pesticides or treatments having a derogatory effect on phytoplasmas (see Note 1), so when necessary these can be used to treat any disease outbreaks. However, we would not advocate routine application.

All phytoplasma-infected plant material should be kept in a glasshouse or plant growth rooms within a containment facility licensed by the local governing authority for quarantine pests. Local requirements may vary across countries, but tend to be tightly controlled to prevent the accidental release of a nonnative and/or quarantine disease to the local environment. Generally a license will be required, and the facilities inspected prior to this being granted, which can be a lengthy process—however, strict adherence to local requirements is vital.

To start a collection of phytoplasma-infected plants, plants can be collected from the natural environment (if possible and permitted) or alternatively acquired from existing collections. Many research groups maintain a few isolates which they primarily work on, and they are often willing to provide plants or cuttings to other researchers.

All of the methods detailed here are following standard horticultural procedures for propagation and maintenance of plants. The salient point with regard to phytoplasma-infected plants is the variable distribution within the host plant. This means that when propagating new plants (by whatever means) it is vital that the progeny plants are tested to ensure the phytoplasma has been

transmitted before disposal of the older parent plant. The methods provided here are for grafting within the same species; however in some cases, it is possible to transmit phytoplasmas from the natural host into *C. roseus* by means of grafting across species. To do this, follow the protocol described here, although there is generally a higher success rate if the plant species are more similar in stem type. Often in cross-species grafts the scion will not continue to grow and transmission is achieved by the phytoplasma moving into the rootstock whilst in contact, with the scion later removed.

2. Materials

For maintenance of phytoplasma-infected plants glasshouses or controlled environment rooms, at least one of which meets local quarantine requirements for quarantine organisms, are required. Ideally stock plants of healthy *C. roseus* should be housed in a separate glasshouse or compartment to those infected with phytoplasmas. If this is not possible, then they should be grouped as physically separate as possible, for example on a different bench or at the end of one bench. The size of the glasshouse will determine how many plants can be maintained.

2.1. Growth of Healthy *C. roseus* from Seed

1. Plastic seed tray.
2. Clear plastic propagator lid (with vent) to fit seed tray.
3. Plastic plant pots (approximately 9 cm diameter) (see Note 2).
4. Plant pot saucers to fit pots (see Note 2).
5. Plant labels.
6. Permanent marker pen.
7. Dibber (see Note 3).
8. Proprietary seed and cutting compost.
9. Perlite.
10. Seeds of *C. roseus* (L.) G. Don (see Note 4).

2.2. Maintenance of Established *C. roseus* Plants

1. Plastic plant pots (various sizes) (see Note 2).
2. Proprietary multipurpose compost.
3. Perlite.
4. Secateurs or scalpel blades (e.g., Swann Morton No. 10A or 22).
5. 100% ethanol (if using secateurs).
6. Sharps disposal vessel (if using scalpel blades).
7. Plant fertilizer.



Fig. 2. The equipment required to take cuttings and conduct grafting, including seed tray and vented propagator lid, plant saucers, clear plastic bags, plant canes, plant labels, parafilm®, elastic bands, sterile scalpel blades, and permanent marker pen.

2.3. Propagation by Cuttings (Fig. 2)

1. Secateurs or sterile scalpel blades (e.g., Swann Morton No. 10A or 22).
2. 100% ethanol (if using secateurs).
3. Sharps disposal vessel (if using scalpel blades).
4. Proprietary seed and cutting compost.
5. Perlite.
6. Plastic plant pots (approximately 9 cm diameter) (see Note 2).
7. Plastic seed tray.
8. Clear plastic propagator lid (with vent) to fit seed tray.
9. Plant labels.
10. Permanent marker pen.
11. Hormone rooting powder or solution (optional).
12. Dibber (see Note 3).

2.4. Propagation by Grafting

1. Healthy *C. roseus* plants approximately 6–8 weeks old.
2. Phytoplasma-infected plant for propagation.
3. Plant pot saucers.
4. Small plant canes.
5. Clear plastic bags (approximately 20 × 30 cm).

6. Elastic bands.
7. Sterile scalpel blades (e.g., Swann Morton No. 10A or 22).
8. Parafilm® M sealing film (at least 38 mm wide).
9. Sharps disposal vessel.
10. Permanent marker pen.

3. Methods

3.1. Growth of Healthy *C. roseus* from Seed

1. Mix between 25 and 50% perlite into seed and cutting compost (see Note 5). Put into a seed tray and tap the tray lightly on the bench to compact the compost.
2. Wet the compost in the seed tray by either sitting in a tray of water and allowing the compost to absorb water or watering gently from above with a rose on a watering can (see Note 6).
3. Thinly sow seeds of *C. roseus* (L.) G. Don and cover with a layer of compost. Label with the date and description.
4. Put tray into a glasshouse at approximately 20°C and normal daylight until germinated.
5. Once the seedlings have developed the first set of true leaves (these develop after the seed leaves) they are ready to be “pricked out.”
6. Prepare individual pots of the compost mix as in step 1. Do not fill pots to the very top but leave a gap of approximately 2 cm from the rim to allow easier watering of the plants. Use the dibber to make a hole in the center of the pot approximately 3 cm in diameter and 3 cm deep.
7. Remove each seedling into an individual pot. Holding the seedling only by the seed leaves (and not the stem or true leaves) use the dibber to loosen the compost around the roots, and then use the dibber to lift the roots and attached compost and support the weight.
8. Transfer the seedling into the hole made in the individual pot. Use the dibber to fill compost around the stem and gently firm in using your fingers. Maintain the compost level around the stem as it was in the seed tray.
9. Water the pots gently to settle the compost and top up the compost if required.
10. Place the plants in a glasshouse and grow on until well rooted.

3.2. Maintenance of Established *C. roseus* Plants

Both healthy and phytoplasma-infected plants (once established) should be maintained in this way. It is good practice to always maintain a stock of healthy parent plants which can then be used to



Fig. 3. Healthy *C. roseus* plants, *left*, an established parent plant and *right*, a young rooted cutting.

generate cuttings and/or seed as required. Young healthy plants raised from cuttings are used as rootstocks for maintaining and increasing stocks of phytoplasma-infected plants (Fig. 3). Generally it is wise to keep at least three established plants for each phytoplasma isolate because some phytoplasmas strains can cause plants to die very rapidly. If additional phytoplasma-infected plants are required, these can be created by either cuttings or grafting with equal success.

1. The glasshouse conditions are primarily determined for healthy growth of *C. roseus*. As most phytoplasmas are naturally found in temperate regions conditions suitable for *C. roseus* seem to also be appropriate for long-term maintenance of most phytoplasma strains; however, exceptionally some strains may need different conditions. The daytime temperature should be between 24 and 28°C, with a night time minimum of 16°C, which depending upon the country and time of year, may require artificial cooling and/or heating (see Note 7). The plants require 16 h of good intensity daylight, so supplementary lighting will be required for varying lengths of time depending upon the time of year and country. Humidity does not typically need controlling.
2. Plants should be watered with tap water close to ambient temperature. As a rule plants will likely need watering every 2 days, as *C. roseus* does not like to have water logged soil, however this will vary. Younger plants with less well established root

systems will require less water than established plants, and if overwatered can rot and die. To judge if a plant needs watering either touch the soil to feel how wet it is, or (with experience) the weight of a pot can be used. Depending upon the glass-house setup, plants can either be watered from the top (directly in the pot) or bottom using either plant saucers or capillary matting (see Note 8).

3. Established plants should be fed weekly with a commercially available broad range plant fertilizer following the manufacturer's recommendations (see Note 9).
4. Plants should be spaced out across the benches as much as possible so that they are not touching, or touching minimally. This helps to limit the spread of disease.
5. Established plants should be pruned as required to maintain the plant at a workable size and improve the structure. *C. roseus* can be pruned quite severely and will regrow from brown woody stems. Generally a scheme of pruning two to three times a year is adequate, with one pruning session before the onset of winter. When pruning either use secateurs (which are disinfected by wiping with 100% ethanol between each plant) or scalpel blades. Make clean cuts without tearing the stems. Always cut just above a bud or leaf joint. When pruning a plant the desired shape is an "open goblet," with a structure of stems around the sides and the center open. This helps air flow through the plant which reduces disease. Any diseased or dying stems should be removed as required throughout the year without waiting for a pruning session (see Note 10).
6. Established plants can remain in larger pots (approximately 20 cm diameter or larger) for prolonged periods of time, and do not need frequent potting-on. Generally they can be repotted once a year into a pot a few centimeters bigger. Younger plants will need potting on more frequently until they become established, perhaps two to three times a year, but only gradually increase the size of the pot. When potting on plants use commercially available multipurpose compost mixed with approximately 10% perlite (see Notes 5 and 11).
7. If any common diseases afflict the plants, then treat with an appropriate pesticide/insecticide following manufacturer's recommendations. Regular monitoring of the plants to enable early detection of disease yields the best results.
8. Plants within a collection should be routinely monitored for the presence of the phytoplasma by any molecular method of your choosing (see Chapters 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, and 26). This is especially important when creating new plants via grafting or cuttings to ensure the phytoplasma is successfully transmitted. Routine screening of the healthy plants should also be undertaken.

3.3. Propagation by Cuttings

Cuttings can be used to create new plants of both healthy and phytoplasma-infected *C. roseus*, with the process being completed in the same manner. However, if infected plants are being propagated, then this must be conducted within a quarantine facility.

1. Mix between 25 and 50% perlite into seed and cutting compost, fill individual pots, and tap lightly on the bench to compact the compost. Do not fill pots to the very top but leave a gap of approximately 2 cm from the rim to allow easier watering of the plants (see Note 5).
2. Select a stem from the phytoplasma-infected *C. roseus* or healthy *C. roseus* which requires propagating. If possible, select a young nonflowering shoot which shows some disease symptoms (if applicable) and is straight, 2–3 mm in diameter, and approximately 6–8 cm in length. Using a sterile scalpel blade gently cut the shoot to remove from the parent plant (see Note 12) (Fig. 4a left).
3. Prepare the shoot by carefully cutting away all the leaves (and flowers/buds if present) except the top three pairs, using a scalpel blade to leave clean cuts (Fig. 4a right).
4. (Optional) Dip the bottom 3 cm of the stem into hormone rooting compound (see Note 13).
5. Use a dibber to create a hole approximately 5 cm deep along the side of the plant pot.
6. Insert a prepared cutting into the hole until the top 3 cm are protruding above the compost and the leaves are close to (but slightly above) the compost level.



Fig. 4. Selected stems to act as cuttings or grafting scion; (a) suitable shoot removed from parent plant (left) and stripped of lower leaves (right); (b) pot of cuttings ready to grow.

7. Gently firm compost back around the shoot with your fingers.
8. Repeat steps 2–7 placing four or five cuttings around the edge of the pot. Ensure that the leaves are not touching each other (Fig. 4b).
9. Label the pot with the date and source of the cuttings (see Note 14).
10. Place the pot of cuttings in the seed tray and fit the propagator lid with the vent closed. Put into a glasshouse at approximately 20°C and normal day light.
11. Water gently as required (approximately twice per week) (see Note 15).
12. After 1 week open the vent on the propagator lid. If any cuttings start to rot or die, carefully remove these from the pot.
13. Once rooted (approximately 4 weeks) pot up individually (see Note 16). To do so, prepare individual pots as step 1.
14. Gently squeeze the sides of the pot of cuttings to loosen the compost. Place spread fingers over the top of the pot to support the stems, invert the pot and tap the bottom of the pot firmly. Tip onto its side, squeeze the side and the compost should slide out onto a bench.
15. Divide the compost so that each rooted cutting still has compost attached to its roots. Pot up individually following steps 7–10 in Subheading 3.1.
16. Once fully rooted in the new pot and the top growth is actively growing these can be used for grafting or as healthy material (see Note 17).

3.4. Propagation by Grafting

The method described below creates an “apical” graft union, which is the easiest manipulation and confers the best transmission rate due to two surfaces of the scion being exposed to the rootstock. The most common alternative is the “side wedge” or “top” graft where both the scion and rootstock stems are cut at a 45° angle and held together with film. This can be used to successfully transmit phytoplasmas; however, it is technically harder to achieve a good graft union site. Gather together all of the required equipment (Fig. 2) before commencing the grafting process (see Note 18).

3.4.1. Prepare the Rootstock

1. Firstly prepare the healthy plant which will be the rootstock (see Note 19).
2. Take a young (approximately 6–8 week old) well rooted healthy *C. roseus* plant (Fig. 5a) (see Note 20).
3. Using a sterile scalpel blade (see Note 21) carefully remove all of leaves from the plant at the stem. Use the blade to ensure a clean cut at each point rather than a tear which may introduce disease. Remove all stems apart from the main stem (see Note 22).

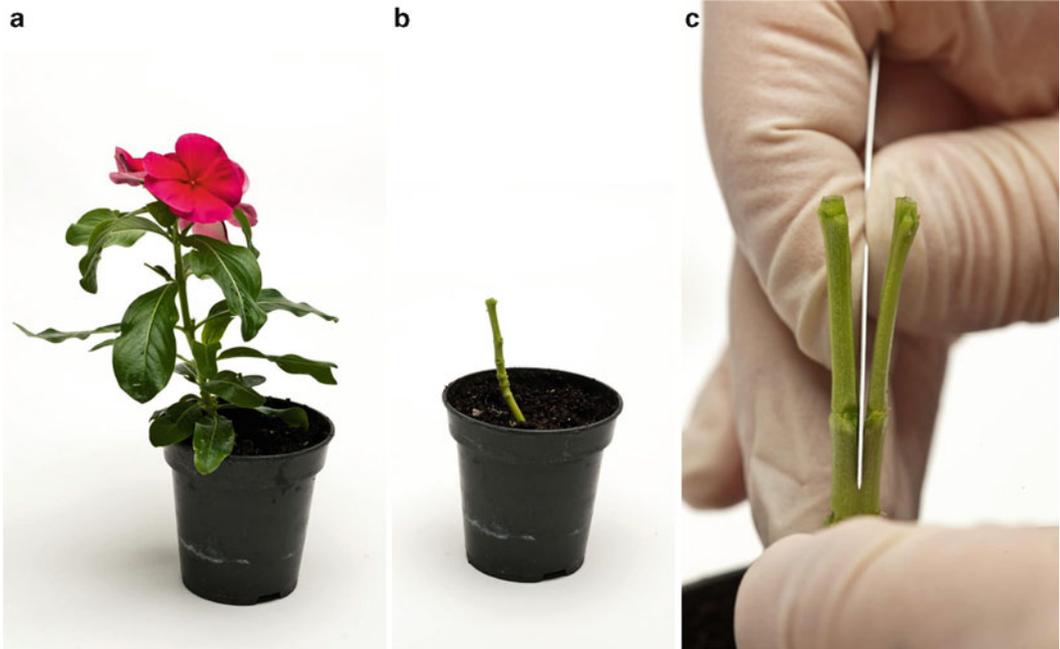


Fig. 5. Preparation of the healthy rootstock plant; (a) the rooted young healthy plant which will act as the rootstock; (b) the rootstock stripped of leaves; (c) the rootstock stem being cut in preparation for accepting the scion.

4. Remove the growing point of the main stem and then make a horizontal cut to reduce the height of the main stem to approximately 10 cm from the compost surface (Fig. 5b).
5. Cut vertically down the center of the stem for approximately 6 cm to open out the stem into a “V” shape (Fig. 5c) (see Note 23).

3.4.2. Prepare the Scion

1. Select a stem from the phytoplasma-infected *C. roseus* which requires grafting, this will be the scion.
2. If possible select a nonflowering shoot which shows some disease symptom and is straight, 2–3 mm in diameter, and approximately 6–8 cm in length. Using a sterile scalpel blade gently cut the shoot to remove from the parent plant (Fig. 4a left) (see Note 12).
3. Use the scalpel blade to remove the lower leaves from the stem (not leaving any material behind) leaving only the top 2–3 pairs of leaves (Fig. 4a right).
4. The lower 4–5 cm of the stem now needs cutting from two “sides” to expose the internal stem structure. Gently support the stem in one hand and then gradually cut into the stem to a depth of approximately 0.5–1 mm, tailoring the cut to a pointed shape at the nonleaf end of the stem. Repeat on the “opposite” side so that the shoot has two gradual cuts ending in a thin point with the phloem and xylem layers exposed along

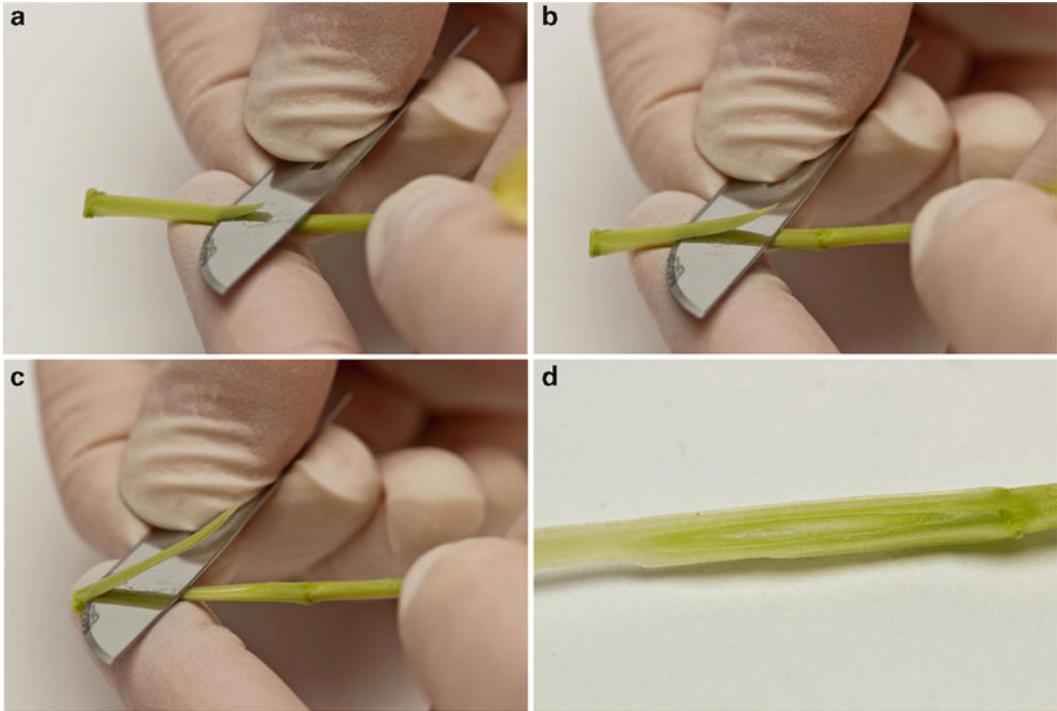


Fig. 6. The stages of preparing the scion for grafting showing; (a–c) cutting away of the scion outer stem layers; (d) the exposed internal structure of the scion stem.

the length of the cut. It may be necessary to make several passes to generate the cuts. The nonleaf end needs to be very thin to allow insertion into the rootstock (Fig. 6).

3.4.3. Create the Graft

1. Insert the cut scion into the cut main stem of the healthy plant; it may help to use the tip of the scalpel to open the cut in the rootstock stem. Ensure that the entire cut area of the scion is inside the cut rootstock stem. If necessary, increase the depth of the cut of the rootstock stem or trim the end of the scion (Fig. 7a).
2. Firmly hold and support the scion within the center of the stem and wrap the whole cut area with stretched parafilm[®], ensuring to start wrapping the parafilm[®] on the rootstock stem beneath the cut area. Care is needed to support the stem whilst stretching, pulling, and wrapping the parafilm[®]. Wrap the parafilm[®] over the whole length of the scion (Fig. 7b, c) (see Note 24).
3. Label plant with designation (of rootstock and scion origin).
4. Place three canes equidistant around edge of pot (see Note 25).
5. Place a clear plastic bag over the canes and secure around the plant pot with an elastic band ensuring the bag is completely sealed (see Note 26) (Fig. 8).

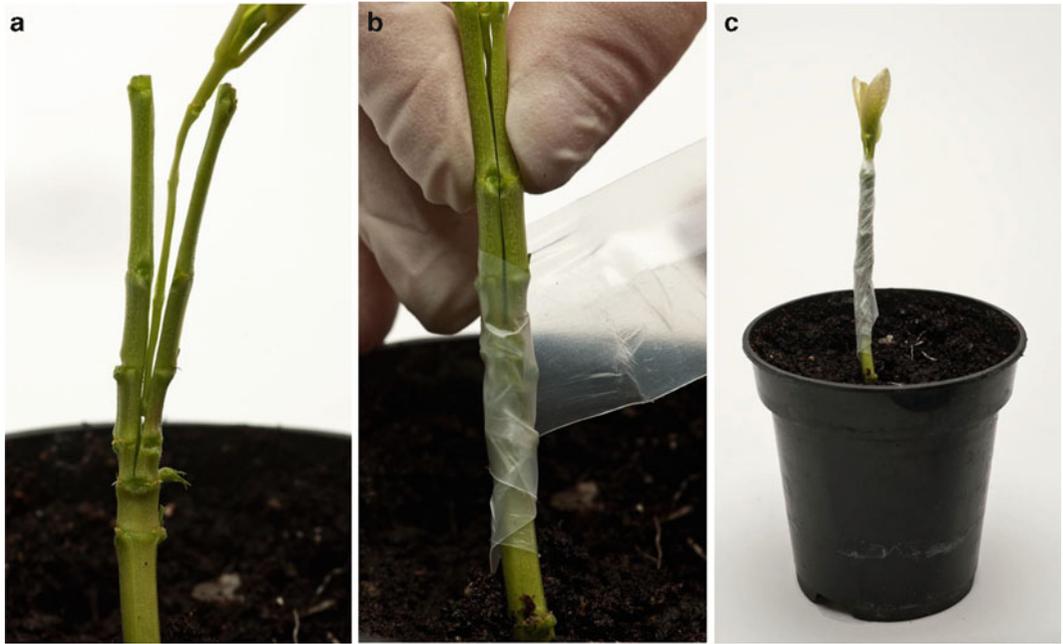


Fig. 7. Grafting of the scion into the rootstock showing; (a) how to insert the scion into the rootstock; (b) wrapping of the scion rootstock join with parafilm®; (c) the completed graft union.



Fig. 8. A completed grafted plant ready to grow on.

6. Place the pot in a saucer to enable watering from the bottom and place in a glasshouse at approximately 20°C and normal daylight (see Note 27).
7. After 3–4 days cut 3 small holes near to the top of bag and leave to grow (see Note 28).
8. Once the grafted scion is actively growing well (approximately 3–4 weeks), the plant is ready for removal from the bag (see Note 29).
9. Cut several larger holes into the plastic bag. Leave overnight for the plant to acclimatize and then remove from bag and canes the following day (see Note 30).
10. Grow on and pot up the plant as required following Subheading 3.2.

4. Notes

1. Whilst there are no reports of pesticide treatments (e.g., foliar sprays or soil drenches) being related to the elimination of phytoplasmas from *C. roseus*, it may be prudent to empirically determine this for treatments commonly or likely to be used in the given country. To do this, generate phytoplasma-infected plants surplus to culture collection and experimental requirements and then treat these with the chemical as per manufacturer's instructions. Test the plant for presence of phytoplasmas before and at intervals after treatment to ensure that the phytoplasma remains present.
2. Terracotta pots and saucers may be used although it is easier to disinfect and sterilize plastic pots (by autoclaving) so for this reason plastic is recommended. The main advantage of terracotta is that it absorbs and maintains some moisture meaning the plant root ball does not dry out as easily. For this reason (and especially in hot climates), it may be desirable to use terracotta pots for large established parent plants in a collection.
3. A dibber is a pointed stick commonly made of wood or plastic which is used to make a hole in compost or soil to allow seeds or plants to be planted without damaging the roots. Dibbers are commonly available from garden centers; however, if one is not available, a sturdy plant label or pen may be used in its place.
4. Multiple sources of seeds for *C. roseus* are available from commercial seed suppliers. Once a healthy line has been established it is possible to collect and store seeds from these plants. Flowers should be allowed to mature on the plant and once full and ripe the pods should be carefully removed from the plant, opened and the seeds removed. Separate any debris and store

the dry seeds in a small paper envelope in dry, cool conditions (ideally a fridge over silica). Seeds collected and stored in this manner will not remain viable for as long as commercially available seeds and should be used within 6 months. The germination success rate may also be lower.

5. Perlite is added to compost to aid and improve drainage. Sharp grit or vermiculate may also be used for this purpose. These are typically added at a rate of 25–50% for young plants and 10% for established plants.
6. The compost is watered prior to the addition of seeds as if watered from above once sown the seeds can easily be washed away or redistributed to the edges of the tray.
7. During the winter months, (if desired) bubble-wrap type insulation may be attached to the interior sides of the greenhouse glass to reduce heat loss and minimize heating costs, however ensure this does not touch the plants.
8. Water should be close to ambient temperature to avoid shocking the plants. It may be necessary to store water within the glasshouse (e.g., in a water butt or watering can) in some instances, for example in a cold winter if the water is piped from outside directly for watering. When watering, try to avoid splashing water onto the plants leaves as this may cause scorch damage. If possible, water plants in the morning to enable surplus water to drain away or evaporate during the course of the day. Excessive water will increase the humidity which, if very high, can promote disease within a glasshouse. If plants are housed on capillary matting (which can enable easier watering), then try to avoid moving the pots excessively. This breaks the root connections with the matting and limits water uptake by the plant until they become reestablished. Do not leave plants sitting in excessive water if plant saucers are used.
9. Do not be tempted to overdose the plants with fertilizers as this can be harmful. Plants potted up into fresh compost do not require feeding for 6 weeks due to the presence of fertilizers in the fresh compost. Some strains of phytoplasma in particular can cause yellowing or bleaching of the leaves which appears like nutrient deficiency; however, if the plants are being fertilized regularly, then this is not the case and does not require additional treatment.
10. Pruning before winter decreases the leaf load and removes any dead or diseased tissue. Plants can be prone to more disease in winter so this is an important preventative measure. Always prune to a bud or leaf so that sections of stem are not left to die back. Sometimes stems of an established plant can die back whilst the rest of the plant is healthy. If this occurs, remove the entire stem cleanly from as close to the plants base as possible. Plants usually recover and grown on well.

11. *C. roseus* does not like root disturbance so be very gentle and careful when repotting plants and never root prune specimens.
12. If a nonflowering shoot is not present on the plant, then select a shoot with the fewest flowers and buds and gently remove the flowers at the stem and cut the growing tip with the buds from the shoot. Ideally try and match the diameter of the scion stem with that of the rootstock; however, if this is not possible, the scion stem can be smaller than the rootstock, although not the other way round. Stems showing some phytoplasma symptoms generally give good transmission of the phytoplasma, however not all phytoplasma strains cause symptoms in *C. roseus*. In this case, select a healthy young shoot. Older shoots tend to propagate less well. Be careful and delicate when handling the stems to not crush them.
13. Hormone rooting compound can be used if desired. *C. roseus* does root well without this but it may increase the speed of rooting.
14. Each phytoplasma and plant within a collection should have a unique designation, and when propagating it is a good idea to record which plant is used as the source as you will likely have multiple plants of a given phytoplasma isolate.
15. Be very careful to not overwater cuttings as this will cause the stems to rot and die. Do not allow the pots to stand in water.
16. To tell if the cuttings are rooted, look at the bottom of the pot for fine white roots to begin emerging from the drainage holes. This will indicate the cuttings are well rooted and ready to be potted on.
17. If a young rooted cutting is intended to be used as a rootstock for grafting, then it is desirable for the plant to have one main stem. In this instance, leave the growing point of the plant intact, as this will enable one strong dominant stem to develop. However, if the plant is for a general collection or a parent plant, the growing point should be carefully removed once the plant is a reasonable size (roughly 10–12 cm in height) to promote the development of lateral stems, which will give the plant a better rounded shape and structure.
18. The primary advantage of using the “side wedge” or “top” graft technique is that in rootstock plants with two main stems it is easy to graft in two different scions. This may be to increase the “dose” of phytoplasma or to create a plant where two phytoplasmas are intentionally mixed together. If this is the desired outcome, then when generating rootstock plants remove the growing tip of the main stem when the plant is small (approximately 5 cm height) which will promote multiple lateral shoots to develop.

Once started the grafting process should be completed without any large gaps in time once the parent/rootstock plant

and scion have been cut. This is to limit any drying out of the cut surfaces, which if they became dry would mean the graft may not take as well.

19. Great care should be taken when grafting, especially when cutting down the rootstock plant and slicing the scion, to avoid injury.
20. The rootstock should be an established cutting from the healthy parent plants maintained, or grown from seed. These should have been routinely tested to confirm the absence of phytoplasmas; however, if desired, a sample of leaves from the rootstock plant can be collected as the plant is being prepared for grafting and used for confirmation that the plant is indeed free from phytoplasmas. The rootstock plant should be at most 12–14 weeks of age. Once the main stem has gone woody the grafts are much harder to create and take less well so the plant should not be used at this point.
21. Use one blade for each plant (healthy rootstock plant and each phytoplasma-infected plant to be grafted). This is to prevent the spread of any other disease (e.g., viruses) which may be in the plants. Either scalpel blades alone or a blade on a handle may be used for the grafting process with the choice a personal preference. Most people find that only a blade allows the greatest dexterity for making the maneuvers.
22. The extreme removal of all leaves and stems (other than the main stem) from the rootstock plant enables easier transmission and spread of the phytoplasma out from the scion and into the whole rootstock plant. If the rootstock plant remains larger at the time of grafting, then there is a greater area for the phytoplasma to spread into which can delay the spread of the phytoplasma throughout the entire plant.
23. Grafting as low down the rootstock as possible ensures that the grafted scion is structurally stable when the plant is fully grown.
24. The parafilm[®] prevents the graft union from drying out which would cause the graft to fail. This needs to be firmly applied with the film stretched so that it sticks to itself as it wraps around the stem.
25. The purpose of the canes is to prevent the plastic bag from touching the plant as this can cause scorch and damage the plant.
26. The size of the bag required will vary depending on the size of the plant, pot, and canes. Ensure that the elastic band completely seals the plastic bag against the pot. This is necessary to maintain high humidity for the first days after grafting; if this is not maintained, then the graft often fails to take.
27. This now needs to be in a quarantine glasshouse, which can be the same one that the main parent plants are kept in.

28. Occasionally the elastic band may perish before the plant is ready for removal from the bag. If this is the case, replace with a new band. The holes allow fresh air to enter the bag but still maintain an environment of high humidity. If the scion is excessively wilted, then leave the bag intact for up to 1 week. On occasion the scion may fail to take and will die, usually within the first 1–2 weeks. In this case, discard the plant as the phytoplasma is unlikely to have been transmitted. Once experienced, most grafters can achieve a 100% success rate of grafts.
29. To determine that the scion is actively growing, note that the scions shoot should be growing away from the growing tip increasing in stem length and producing new leaves. The rootstock may also have started reshooting from the base of the main stem at this point.
30. The grafted plant requires acclimatization to the environmental conditions before complete removal from the bag to prevent shock causing damage (or death) to the plant.

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