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PLANT PATHOLOGY AND NEMATOLOGY

A Guide to Grafting for Cotton (*Gossypium Hirsutum* L.) Virus Transmission and the Successful Transmission of Cotton Leaf Roll Dwarf Virus

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ABSTRACT

A new virus in cotton (*Gossypium hirsutum* L.) required the need to graft plants to evaluate resistance. In searching the literature, several studies reported grafting, however the details surrounding the types of grafts, age, and acclimation environment are not described in detail. A graft is the union of rootstock and scion requiring good cambial tissue contact to be successful. Therefore, several different graft types, and the need for humidity was investigated. Initially, thirty plants were grown in the greenhouse. The first set of grafts were performed on fifteen plants between two graft types (T-graft and bottle shoot) and the need to be bagged for cambial humidity. The second set of fifteen were used to test the wedge, saddle-graft, whip-and-tongue, bottle shoot, and approach grafts on three plants each. The T-graft was chosen as the best for success as it provided the highest cambial contact. A set of twenty plants were grown to serve as rootstock for cotton leafroll dwarf virus (CLRDV) transmission. Two different infected CLRDV plants served as the scion for the virus which were grafted using the T-graft. Three leaves below the graft node were used to test for the virus using PCR. Fourteen of 20 grafts had successful transmission of CLRDV, regardless of graft success.

INTRODUCTION

As an emerging threat to the U.S. cotton industry, efforts are focused on finding host plant resistance to cotton leaf roll dwarf virus (CLRDV) in existing cotton populations. Large quantities of diverse breeding material need to be screened for a virus employing field screening, vector transmission, mechanical inoculation, grafting, or use of an infectious clone.

Field screening relies on the presence of a population of viruliferous insect vectors in the landscape but requires a full growing season and is not time effective. In addition, the vector may not be present or transmitting the virus, which is problematic as is the availability of the causal virus in plant reservoirs for vector acquisition. Vector transmission assays ensure specificity of the target virus but requires maintaining robust insect colonies. This type of screening can be completed in several months. Mechanical inoculation is a fast screening method but is ineffective for phloem-limited viruses, such as luteoviruses (Katis et al. 2007). An infectious cDNA clone is a good method for resistance screening of germplasm; however, clones can be costly and difficult to produce (Beckett and Miller, 2007). In the absence of an infectious clone, and with limited time and the uncertainty of insect availability; graft transmission is a suitable alternative screening method.

A graft is the union of two plant tissues referred to as the scion and the rootstock. The scion is the bud or stem being introduced that will grow to form the top portion of the plant and is grafted onto the rootstock, which will form the root system for the new composite plant (Hartmann et al. 2014a). Graft types can be divided into two groups based on the relative size of the scion to the rootstock. Grafts using the same size scion as rootstock include whip-and-tongue, wedge, saddle, and approach grafts. Grafts using dissimilar sized rootstock and scions include side-veneer, T-graft, chip budding, and cleft.

Grafting is primarily used in horticulture, and therefore little information is available concerning grafting in agronomic crops (Hartmann et al. 2014a). In cotton, grafting is employed primarily for virus transmission and is typically performed on 4-8 week old rootstock (Ahktar et al. 2002; Ahktar et al. 2013; Reddall et al. 2004; Rahman et al. 2005; Price et al. 2020). As a perennial, cotton has two stages of growth. Herbaceous tissue is produced first, followed

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by lignification of that same tissue. It is possible to graft woody cotton plant tissue; however, young herbaceous tissue is preferred due to its fast growth, bark slippage, and higher percentage of graft take. Grafts used successfully in cotton include wedge, modified bottle shoot, and T-graft. (Karaca et al. 2020; Reddall et al. 2004; Ahktar et al. 2002; Sharman, Personal Communication). The literature is limited on why particular grafts were chosen for cotton virus transmission work (Table 1.). Little information has been given on the factors necessary for successful grafts. Therefore, the primary objective of this work is to provide an overview of grafting types and techniques for success in cotton.

MATERIALS AND METHODS

Grafting Protocol Development

T-graft and Bottle shoot. Thirty Stoneville '6182' cotton seeds were sown in 11.36 L pots (Nursery Supplies®, Chambersburg, Pennsylvania), three plants per pot, in peatlite (PRO-MIX 'BX', Quakertown, PA) and placed on a greenhouse bench in full sun until the 4th true leaf stage at approximately 12 weeks. At this time, self-grafts were performed on 15 of the plants, 10 T-grafts and five bottle shoots. All grafts were made by cutting the terminal shoot off 0.5cm below the 3rd node. The terminal was trimmed to 15cm, all leaves below the two topmost expanded leaves were removed, and the terminal was placed in a jar of water while the rootstock was prepared.

For the T-grafts, two cuts were made in the shape of a "T" on the stem of the rootstock above the 2nd node, lightly cutting through to the cambial layer (Fig. 1). Using the bark lifting edge on the back of the grafting knife (Felco 3.90 20, Felco SA, Switzerland), the epidermal layer was lifted and water dripped into the cut. The terminal shoot was then prepared by trimming one side of the basal end into a wedge shape (Fig. 2). The basal end was placed inside the "T" shaped cut, cambial layers touching, and bound together with parafilm for stability and moisture retention (Fig. 1c).

Figure 1. T-graft: (A) The rootstock is prepared by cutting a "T" shape into the stem and lifting the epidermal layer free of the cambium. The scion, trimmed to a wedge, is then inserted into the flap made by the "T" cut, with the long cut side of the wedge placed against the cambium of the rootstock. (B) A healed T-graft with mases of callus tissue. (C) Progression series to completing a T-graft.

Figure 2. Basic wedge shapes of a scions used in wedge, saddle, whip-and-tongue, and T-grafts.

Table 1. Graft types examined for cotton grafting in the Southeastern U.S.

Type of Graft	Virus Studied	Study Examining Grafting in Cotton	Successful Graft Take (%)	Successful Virus Transmission (%)
T-graft	*Cotton leafroll dwarf virus*	*This study, virus transmission study only*	80	70
Wedge	*Cotton bunchy top virus*	Reddall et al. 2004	96	96
Saddle	-	Rea 1931	46.2	-
Approach graft	*Cotton leaf curl virus*	Nazeer et al. 2014	100	100
Bottle shoot (modified approach graft)	*Cotton leaf curl virus*	Akhtar et al. 2002	100	100
Whip-and-tongue	*Cotton leafroll dwarf virus*	*This study*	100	-

The bottle shoot graft is a modified approach graft (Fig. 3) in which the basal end of the scion is placed in a test tube of water that is bound to the rootstock (Fig. 4). The graft was performed by making a single long shaving cut to the rootstock that removed the epidermal layer, making an identical cut to the scion, then aligning the cuts together and binding them with parafilm (Fig. 3). The cut on the scion was made 10-15cm above the bottom of the stem. A test tube was then fitted over the basal end of the scion and taped to the rootstock for stability. The test tube of water was removed from the bottle shoot grafts after the scions had regained turgidity (approximately seven days).

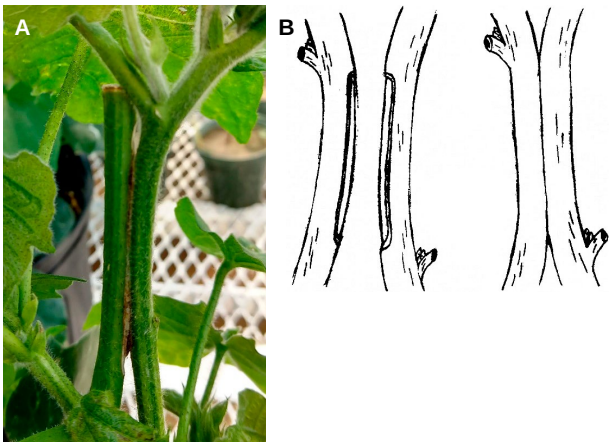

Figure 3. Approach graft: (A) Healed approach graft. (B) Two matching, shallow cuts are made in the stems, then the cuts are brought together and bound in parafilm.

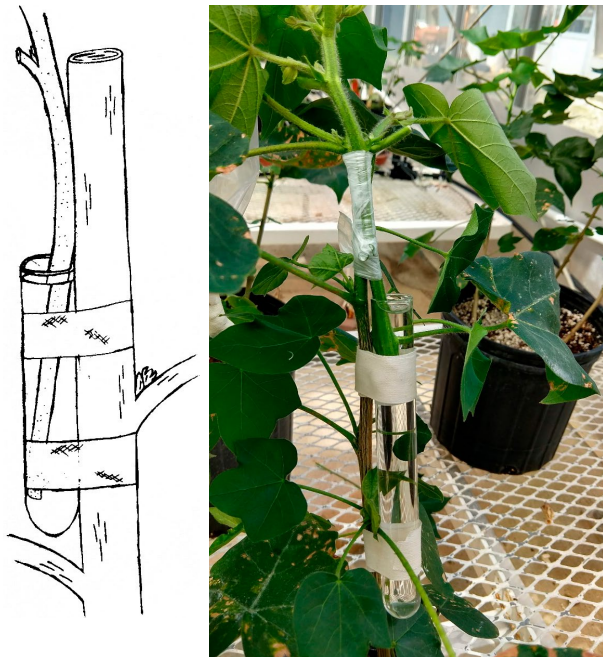

Figure 4. Bottle shoot graft. A standard approach graft fitted with a test-tube for water supply.

Five of the T-graft plants had a clear one-gallon plastic bag placed over them and taped shut below the union using grafting tape, creating a 100% humid environment (Fig. 5). The remaining five T-grafts were left uncovered. Bottle shoot grafts were not bagged since the water source is taped to the rootstock.

Figure 5. Bagged grafts in the acclimation phase of healing.

Types of Grafts

The wedge, saddle-graft, whip-and-tongue, bottle shoot, and T-graft were performed on the remaining 15 cotton plants with each graft type designated to a pot containing three plants. Wedge grafts were performed by preparing the terminal as stated above, except that both sides of the terminal were cut into a wedge shape. The rootstock was cut by centering the grafting blade on top of the cut stem and carefully rocking the blade to split the stem downward (Hartmann et al. 2014b) (Fig. 6). The scion was inserted into the split, ensuring cambial alignment between scion and rootstock along at least one side, then wrapped in parafilm.

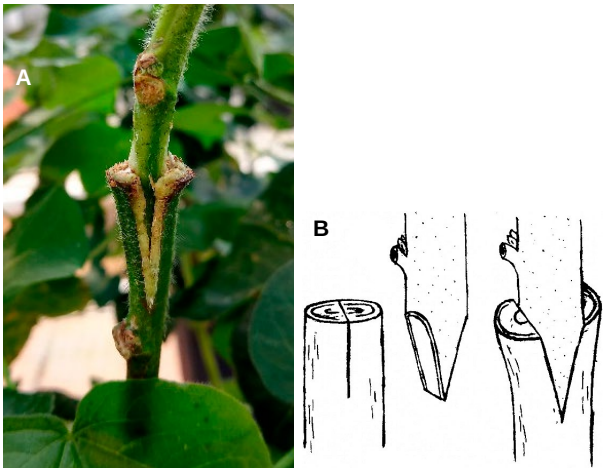

Figure 6. Wedge graft: (A) Healed wedge graft with callus tissue. (B) A single cut is made into the rootstock, parallel to the stem. The scion is cut into a wedge and inserted into the split.

A saddle-graft is an inverse wedge: the rootstock was cut into a pointed wedge shape and the scion was cut down the middle. The scion was then split over the apex of the wedge, cambiums aligned, and wrapped in parafilm (Fig. 7).

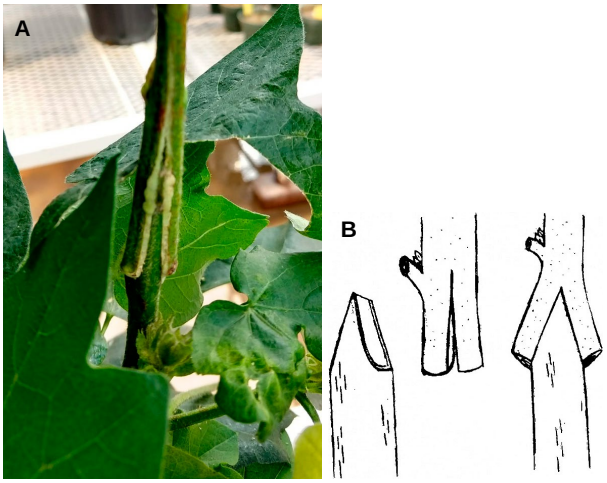

Figure 7. Saddle graft: (A) Healed saddle graft. (B) The rootstock is cut into a wedge shape and the scion is cut down the middle. The scion is then set over the apex of the wedge.

For the whip-and-tongue, identical, angled cuts are made to both the scion and the rootstock. A second angled cut is made under the first, creating the tongue. The tongues are inserted behind each other as the rootstock and scion are brought together, locking the graft together (Fig. 8). The graft was then wrapped in parafilm.

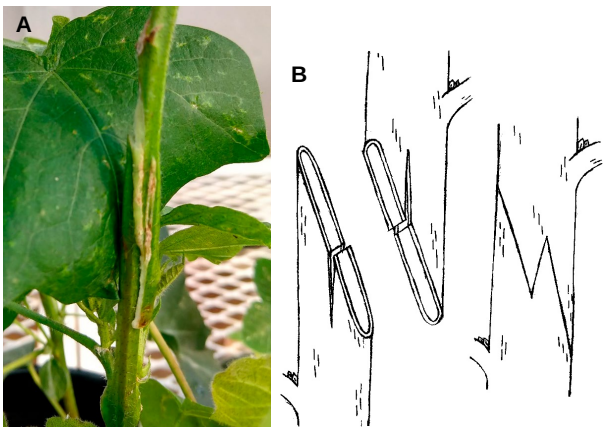

Figure 8. Whip-and-tongue graft: (A) healed whip-and-tongue graft. (B) Identical long, angled cuts are made to both rootstock and scion. A second set of identical cuts are made in the center of the first, creating the tongues. The tongues are inserted behind each other, locking the graft together.

T- and bottle shoot grafts were performed as above, with modification to the bottle shoot graft. Bottle shoot grafts were modified from the preliminary experiment by increasing the length of the scion to 20cm.

All grafts were bagged to prevent desiccation of the scion. After grafts had regained turgidity (approximately five days), the bags were clipped to allow airflow and humidity acclimation. Three days later, the bags were fully removed.

Graft Screening – CLRDV Transmission

Twenty 'DeltaPine 1646' plants were sown as previously described and grown for 8 weeks. Two sets of 10 T-grafts were performed to assess the graft transmission of CLRDV. The first 10 grafts were performed using scions from CLRDV-infected cotton plants that had been transplanted from a field in Elmore County, AL (source A) onto a greenhouse bench at the end of the 2018 growing season. The second set of 10 grafts were performed using scions from greenhouse grown cotton plants (source B) that were placed in a field to acquire the virus under natural conditions before being returned to the greenhouse.

Plants were prepared for grafting by removing the terminal shoot 0.5cm below the 3rd node. Scions were collected and kept basal end down in a jar of distilled water to prevent loss of turgidity while rootstocks were prepared. T-grafts were performed on the side of the stem using CLRDV-infected scions prepared as above. Scions from the first source were 10cm long due to a zig-zag growth pattern and short internodes, while scions from the second source were 20cm, and straighter. As cuts were made to the rootstock, water was dripped into the wounds to prevent them from drying out. Because of the injury to plants, grafts were placed under a bench top shade structure (Agribon®, Berry Global, Evansville, IN) to reduce light stress and transpiration until fully healed (Fig. 9). To test for presence of the virus, three leaves were collected from the first node below the graft for a composite sample. Virus presence was confirmed via PCR (Thiessen et al. 2020).

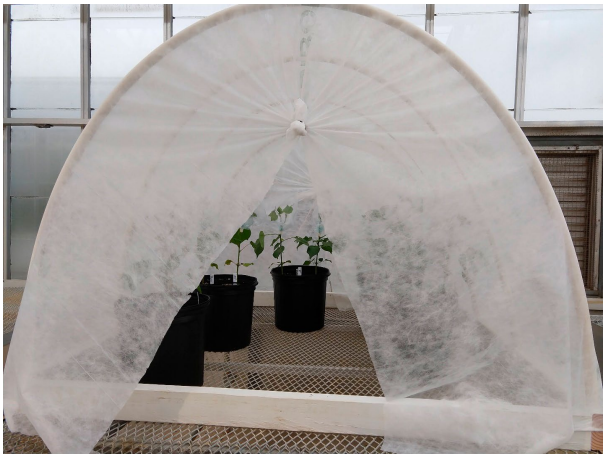

Figure 9. Benchtop shade structure for healing grafts.

RESULTS AND DISCUSSION

Grafting Protocol Development

Graft take is dependent on a myriad of factors, the most important to this study being cambial alignment and healing conditions. The first observable sign of a successful graft union is the formation of callus tissue between the scion and rootstock (Hartmann et al. 2014b). The callus tissue fills the gaps between the scion and rootstock tissues and links the vascular systems, allowing them to grow together.

The preliminary experiment proved that cotton grafts need high humidity conditions during healing because the scion can desiccate and die before the cambial tissue can link the vascular systems (Fig. 10). By protecting the scions with plastic bags some transpiration occurred, but created a high humidity environment that sustained the scion long enough for the callus to form which is consistent in other herbaceous grafts (Vu et al. 2013). For this study, humidity was 100% inside the bagged grafts. In the unbagged T-grafts, transpiration drew water away from the graft union and out of the leaves before the callus could form (Fig. 11). These scions wilted in less than two hours and were dead in three days. Therefore, all further experiments in this study were bagged.

Figure 10. Dead cotton T-graft.

Figure 11. Dead T-graft. Scion pulled away from dead T-graft to reveal the lack of callus in the rootstock.

Types of grafts

T-graft. The T-graft proved to be the best graft overall due to the high amount of cambial contact, required little time to perform, and was the easiest to bind with parafilm as the epidermal layer of the rootstock held the scion in place. The high amount of cambial contact was contributed by the rootstock as the entire cambium was exposed to the scion when the scion was inserted under the epidermal layer. High amount of cambial contact greatly increases the chances of both graft take and virus transfer (Mudge 2008). This graft is extremely easy to perform on young cotton tissue as the epidermal layer slips off the cambium.

Bottle shoot. Based on this study, bottle shoot grafts are not recommended for cotton grafting due to the difficulty in maintaining cambial alignment during binding, the need for 20 cm long, straight scions, and the added involvement of maintaining water levels in the test tubes. As described by Akhtar et al. (2002) and (2013), he removed the test tube from grafted cotton plants after 5 days and 7 days, respectfully, and was successful in both disease transmission and graft take. However, in this study, once the test tube of water was removed after the scions regained turgidity (approximately 7 days), the scions subsequently died within seven days. This was unexpected as the grafts had regained turgidity and mild callus had formed to sustain the scion. The vascular tissue formed between the scion and rootstock was insufficient to sustain the scion without an external water source. This method is time-intensive to assemble and maintain compared to the wedge and T-grafts. A symptom of CLRDV is zig-zag stems and these stems would be problematic to fit inside the test

tube. If a bottle or other large water source were to be used, support structures would have to be added to take the weight of the bottle making it impractical.

Wedge. Wedge grafts were effective and easy to cut, but difficult to maintain cambial alignment during binding due to the sap flow making conditions slippery. Care had to be taken with the cut downward into the stem as it was easy to injure oneself or break the stem if too much pressure was applied. This graft became more difficult when performed on practice plants older than 12 weeks but that had not yet lignified. Cotton forms pith in the center of the stem that is spongy and resists cutting (Janick 1986). The downward cut into the stem was hindered and the structural integrity of the scion wedge weakened by pith. This graft is best performed on young cotton or old cotton that is fully lignified.

Saddle. This graft was successful for all 3 grafts but had similar issues to the wedge graft regarding the pith but was less safe to perform. Cutting the wedge was more dangerous as the grafter now had to make long upward cuts on the rootstock in a less controlled fashion. When cutting a wedge on a scion, the scion is placed between the thumb and blade and pulled through without moving the thumb or blade. This maneuver is not possible on a potted rootstock and therefore the cuts were more erratic. Rea (1931) used the saddle graft in cotton with a success rate of 46.2-71.3%. His reduced success rate may be attributed to his use of mature wood as opposed to the faster growing herbaceous tissues.

Whip-and-tongue. Whip-and-tongue grafts were easy to perform on true herbaceous tissue, but not when pith was present. The benefit of the interlocking tongues is structural integrity and increased cambial contact (Hartmann et al. 2014b), both of which would aid novice grafters. While not reported in cotton, whip-and-tongue was included as it is useful for small diameter material (<1cm) and increased cambial contact, which is necessary for virus transmission.

CLRDV Graft Transmission

Six of 10 T-grafts using scion source A resulted in CLRDV transmission, two of which were dead scions. All grafts performed with scion source B survived, resulting in eight of 10 successful transmissions. The results indicate that union and scion survival are not necessary for CLRDV transmission. The only grafts that failed used scion wood from the field (source A plant). These grafts likely died because the source A plant was highly stressed from transplant shock, having been dug up from the field and moved into a greenhouse. The more robust state of the greenhouse grown (source B) plant likely contributed to the 100% survival rate.

Akhtar et al. (2002) and Reddall (2004) both indicated 100% transmission success of cotton leaf curl virus (bottle shoot) and cotton bunchy top virus (wedge), respectively, in their studies. Successful transmission of CLRDV by grafting has been reported by Price et al. (2020). Transmission in this study was likely affected by fluctuating viral titers within the source plants. CLRDV is a low-titer virus and can be present in a plant and undetectable by PCR (Conner, Personal Communication). Further, current molecular diagnostic protocols have not yet been developed that can characterize CLRDV titer levels in the host plant as qPCR protocols were not available at this time.

Graft Success

Of the 50 grafts performed across the grafting experiments, 36 were successful. The 5 unbagged T-grafts and the 5 bottle shoot grafts in the first experimentall failed due to lack of humidity. All of the graft types investigated in the second experiment (wedge, saddle, T-graft, bottle shoot, and whip-and-tongue), were bagged and completely successful. The grafts used for virus transmission had an 80% graft take success rate, with scion source A having 6/10 successful grafts and scion source B having 10/10. CLRDV transmission of those grafts was slightly lower at 70% successful transmittance, 6/10 and 8/10 for sources A and B, respectively.

CONCLUSION

The overall recommended graft with the highest cambial contact is the T-graft. All grafts investigated worked in cotton under greenhouse conditions, except for bottle-grafts. These studies have demonstrated the need to protect herbaceous cotton grafts from desiccation by providing humidity control. CLRDV can be transmitted via both living and dead scions, indicating that grafting is a viable method for resistance screening. Based on the percent success rate in this study, it is recommended to use five plants per line to determine and confirm a result. It is also recommended to use the most robust of the available diseased tissues to increase the chances of graft take and virus transmission.

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