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DISEASE RESISTANCE IN TRANSGENIC COTTONS

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Abstract

Transgenic Upland cottons (*Gossypium hirsutum* L.) expressing the antifungal peptide D4E1 were evaluated for tolerance to Fusarium wilt caused by *Fusarium oxysporum* f.sp. *vasinfectum* (FOV) Atk. Sny & Hans race 1 in a small field with sandy soil, also infected with root-knot nematodes (*Meloidogyne incognita*). A transgenic line expressing the *uid*A (GUS) marker gene only and the original non-transgenic parent cultivar used for transformation were used as controls. The transgenic lines included in this study were shown to be effective against fungal pathogens in vitro and in planta in laboratory and greenhouse studies. Commercial Acala (*G. hirsutum*) and Pima (*G. barbadense*) cultivars were also planted alongside for comparison at the USDA-ARS, WICS, Cotton Enhancement Program, Shafter CA. Entries were planted in a Randomized Complete Block design with four replications on 10 m long plots and evaluated for plant survival, foliage symptoms, vascular root staining due to FOV in the presence of root-knot nematode, and agronomic traits. No significant differences were observed in all of the above evaluated traits between transgenic and control cottons, except for plant survival. Transgenic lines NO-373 and NO-358 showed a higher number of plant survival and stand during the season. It appears that the evaluation of *hirsutum* lines for FOV tolerance is much more complex possibly due to the following reasons - Upland cultivars may have higher levels of resistance, and exhibit a far less genetic variation for resistance among them rendering investigation of FOV symptoms difficult using the disease severity scales as used in this study. On the other hand, resistance for FOV in Pima cottons seems to be more complete at the host plant level. Further large scale evaluations of the transgenic cottons are needed to assess the full potential regarding to tolerance to plant pathogens including FOV.

Introduction

Fusarium wilt caused by *Fusarium oxysporum* f.sp. *vasinfectum* (FOV) Atk. Sny & Hans in cotton was first described by Atkinson (1892) in the USA. FOV is a soil-inhabiting organism and can survive for long periods in soils, even in the absence of cotton, making it nearly impossible to eradicate from the field. Eight genotypes of FOV, called races, have been described throughout the world. Until recently, only race 1 and race 2 were known to occur in the United States (DeVay, 1986; Smith et al., 1981). In 2005, four of the races were identified in California (Kim et al., 2005). These races were originally classified based on pathogenicity tests on different cotton species, *G. hirsutum*, *G. barbadense*, and *G. arboreum* L. (Armstrong and Armstrong, 1958, 1960, 1978; Ibrahim, 1966), and by their pathogenicity on alfalfa, soybean, and tobacco (Armstrong and Armstrong, 1978). Today, these races are categorized according to a number of genetic markers. Based on sequence differences in EF-1α, PHO, and BT genes and intergenic spacer (IGS) restriction enzyme digests, strains of FOV worldwide can be classified into five major lineages (Kim et al., 2005). Host-plant resistance is the most economic and effective strategy for *Fusarium* wilt control in cotton. In Pima cottons a single dominant major gene and one or more minor genes that may provide transgressive segregation have been reported for FOV resistance (Fahmy, 1927; Mohamed, 1963; Ulloa et al., 2005, 2006 in press) as well as two major dominant genes with inter locus additivity that provide a high degree of resistance in Sea Island cotton (Smith and Dick, 1960). However, conflicting results have been reported for Acala

and non-Acala Upland cottons regarding resistance to the *Fusarium* wilt disease (Smith and Dick, 1960; Kappelman, 1971). It has been difficult to identify and/or develop highly resistant Acala/Upland cottons to FOV, which suggests that resistance in *G. hirsutum* gene pool may be more complex than in Pima (*G. barbadense*) cottons.

The disease was first noted in California in 1959 (Gaber and Paxman, 1963) where the number of infested sites remained relatively few until the mid 1970s, after which the number of infested sites increased substantially (Hillocks, 1992). However, by 1974 cotton growers of the San Joaquin Valley were cheered by Dr. H.B. Cooper's announcement that a new wilt-tolerant variety was being released by the USDA-ARS Cotton Research Station, Shafter CA. (Turner, 1981). The *Fusarium* wilt evaluated in California and the western states during studies conducted in the 1960s and 1970s were caused by FOV races 1 or 2, and were typically found in sandy or sandy-loam soils with significant presence of root-knot nematode (RKN, *Meloidogyne incognita* [Kofoid and White] Chitwood) populations (Veech, 1984; Bell, 1984). It has been demonstrated that the susceptibility to *Fusarium* was substantially increased in the presence of the root-knot nematode (Garber et al., 1979). Cottons developed for resistance to FOV on soils infested with *M. incognita* usually maintained their resistance when simultaneously challenged by both organisms (Sasser, 1972; Heald and Orr, 1982).

The synthetic peptide D4E1 has been shown in our laboratory and greenhouse experiments to inhibit further development of pre-germinated conidia of *A. flavus, Fusarium* and other phytopathogens, including bacterial pathogens, at low concentrations (Rajasekaran et al. 2001). Moreover, we have demonstrated that crude protein extracts from leaf tissue of transgenic tobacco plants expressing the synthetic peptide D4E1 significantly reduced *in vitro* the number of fungal colonies arising from germinated conidia of *A. flavus* and *Verticillium dahliae* and showed greater levels of disease resistance *in planta* to the fungal pathogen, *Colletotrichum destructivum*, which causes anthracnose (Cary et al. 2000). Herein, we present results from a small scale field evaluation of four transgenic Upland cottons (*Gossypium hirsutum* L.) expressing the antifungal peptide D4E1 (Rajasekaran et al. 2005), a transgenic control entry with the GUS marker gene, the non-transgenic parent control, along with commercial Acala (*G. hirsutum*) and Pima (*G. barbadense*) cultivars for tolerance to *Fusarium* wilt race 1 in a typical sandy soil field also infected with root-knot nematode.

Materials and Methods

Transgenic cotton lines expressing the synthetic antifungal peptide D4E1 were produced as described (Rajasekaran, 2004). Ten cotton cultivars, which included four transgenic Upland cottons (*Gossypium hirsutum* L.) expressing the antifungal peptide D4E1 (NO-357, NO-358, NO-373, and NO-374), a transgenic control entry expressing the marker GUS gene only (GUS-C4) and the original non-transgenic parent cultivar used for transformation (AW4), along with the following commercial varieties - PHY-72, a commercial Acala (Phytogen Seed Co); SJ-CA_Suc_Check, a susceptible germplasm line from USDA-ARS; PHY-800, a Pima variety (Phytogen Seed Co); and DP744, a Pima variety, (Delta and Pine Land Seed Co), were planted at the USDA-ARS, WICS, Cotton Enhancement Program, Shafter CA. On 28 April 2005, 100 seeds each were planted from the above entries in a known infested field with FOV and RKN, and plants were grown in one-row plots 10 m long with 1 m row spacing in a Randomized Complete Block design with four replications. In order to determine the level of tolerance or resistance for each entry, five plants were assayed from each replication for foliar symptoms (F_Damage), vascular symptoms (Vascular_S), number of nodes (Node_C), plant height in cms (Plant_Ht), presence of and/or damage due to RKN (Root-knot), and plant population counts/survival (Stand). Data collection was performed for each plot-entry at three different stages of plant development: 1) plant establishment, 2) five weeks after establishment, and 3) before final evaluation at crop maturity in order to monitor and determine the level of tolerance/resistance to FOV race 1 in a typical sandy soil field with interaction due to presence of root-knot nematodes.

Evaluations of individual plants were rated for disease severity based on the following scale for A) foliar damage (F_Damage): 0 = no foliar symptoms, 1 = chlorosis and/or wilt restricted to cotyledons or first leaf and/or stunting, 2 = similar symptoms extended beyond the first leaf, 3 = moderate to severe foliar symptoms usually with loss of infected leaves, 4 = severe symptoms with the whole plant affected, 5 = plant dead; B) vascular damage

(Vascular_S): 0 = no vascular staining evident, 1 = light vascular staining evident as spotty areas, 2 = more continuous color staining - covering area equal to between one quarter and one half of stem cross-section, 3 = moderate vascular staining (intensity of the brown/black color) evident in a band most of the way around the stem cross section, 4 = vascular staining darker, and 5 = plant dead; and C) presence of RKN symptoms (Root-knot) 0 = no presence of RKN damage and 1 = presence of and/or damage due to RKN.

Results and Discussion

A small scale field experiment conducted in a sandy soil field infested with *F. oxysporum* (FOV) race 1 and root-knot nematodes (*M. incognita*) provided limited information for determining the level of tolerance/resistance in the transgenic cottons expressing the synthetic antifungal peptide D4E1 (Figs. 1 to 4). No significant differences were observed for foliar or vascular damage, number of nodes and plan height, except for plant survival or Stand (Fig 1) in transgenic cottons when compared with a transgenic or non-transgenic control entries, GUS-C4, or AW4. Significant differences were observed for all of the above traits when were compared with SJ-CA_Suc_Check (Figs. 1 to 4). It was difficult to determine seed germination with certainty under pressure from infestation with FOV and RKN in the field. However, based on plant survival (Stand), transgenic entries NO-373 and NO-358 maintained the large number of plants per plot during the growing season, suggesting the expression of the antifungal peptide D4E1 gene (Fig 1) during the early stages of plant germination and development. In addition, the transgenic line NO-373 showed less stunting when compared to all entries (Figs. 3 and 4).

Recently, Ulloa et al. (2006, in press) reported that contrary to Pima cottons, where resistance for FOV race 4 seems to be more complete at the host plant level, it was difficult to produce and/or identify highly tolerant Acala and non-Acala cotton plants in order to investigate further the resistance of Upland cottons to this pathogen. Our field trials demonstrated the lack of correlation between tolerance/resistant traits in Upland varieties to FOV symptoms (e.g., F_Damage or Vasculat_S) in four evaluation sites suggesting that Upland cultivars may have higher levels of resistance, and there is far less genetic variation for resistance among these Upland cultivars. In addition, the investigation of and/or differences in FOV symptoms in Upland cultivars were difficult to detect by the disease severity scales as used because of the presence of asymptomatic foliage and overall agronomic performance of plants in most cases (Ulloa et al. 2006, in press). The transgenic lines expressing the antifungal peptide need to be evaluated further for their efficacy against phytopathogens during plant growth and development as related to gene expression levels. To prevent possible cytoplasmic degradation of peptides and achieve high levels of gene expression for effective control of plant pathogens, it may be necessary to consider organelle expression of transgenes (DeGray et al. 2001). We have demonstrated previously in our laboratory and greenhouse studies that *Agrobacterium*-mediated transgenic cotton plants expressing the synthetic peptide D4E1 imparts antifungal traits. Crude protein extracts from leaves of transgenic cotton plants inhibited the colony development from germinated spores of *Fusarium verticillioides* and *Verticillium dahliae*, two of the most common fungi that cause seedling disease complex and wilt in cotton. *In situ* and *in planta* bioassays with *Aspergillus flavus,* the causal agent for aflatoxin contamination in cottonseeds, showed that the transgenic cotton plants expressing D4E1 are capable of controlling the growth and spread of the fungus in cotyledons of cottonseed in a consistent manner in both immature detached seeds and mature greenhouse-grown seeds (Rajasekaran et al. 2005). Transgenic cotton progeny seedlings also showed significant tolerance *in planta* to *Thielaviopsis basicola*, one of the fungal pathogens that causes seedling disease complex and black root rot (Rajasekaran et al. 2005).

In this study, the lack of significant differences among transgenic entries may also be the result of higher levels of resistance that is difficult to differentiate, presence of far less genetic variation for resistance among them and/or the technical difficulty to detect resistance or tolerance by the disease severity scales used. Additional evaluations are needed to assess the full potential of these transgenic cottons for tolerance to FOV and other phytopathogens.

Another important aspect for consideration is the conflicting results that have been reported for Acala and non-Acala Upland cottons regarding resistance to the *Fusarium* wilt disease (Smith and Dick, 1960; Kappelman,

1971). The inheritance of the resistance for Upland cottons to *Fusarium* wilt has been reported from dominant gene types with minor modifying genes (Smith and Dick, 1960) to quantitatively inherited and controlled by several major genes and minor modifying genes (Kappelman, 1971). After almost a century of breeding for wilt resistance in the U.S., it has not been possible to produce a commercially acceptable *G. hirsutum* wilt-immune cultivar (Hillocks, 1992). Even highly tolerant Acala cottons, which tend not to be affected in growth and yield, have been observed with high vascular brown staining in xylem vessels with no observable effect on the water requirements of the plant (Ulloa et al. 2006, in press). From the limited scale field trials reported in this study it appears that the expression of a single gene coding for the antifungal peptide D4E1 may not be sufficient to prevent infection by FOV. However, the results presented here show that the expression of the antifungal peptide D4E1 may prevent cotton seedling diseases caused by fungal pathogens such as FOV and other phytopathogens by providing a better rate of survival (Fig. 1). Further studies on the effect of the peptide and transgenic expression of the same in cotton plants on control of FOV and other phytopathogens are in progress in our laboratory.

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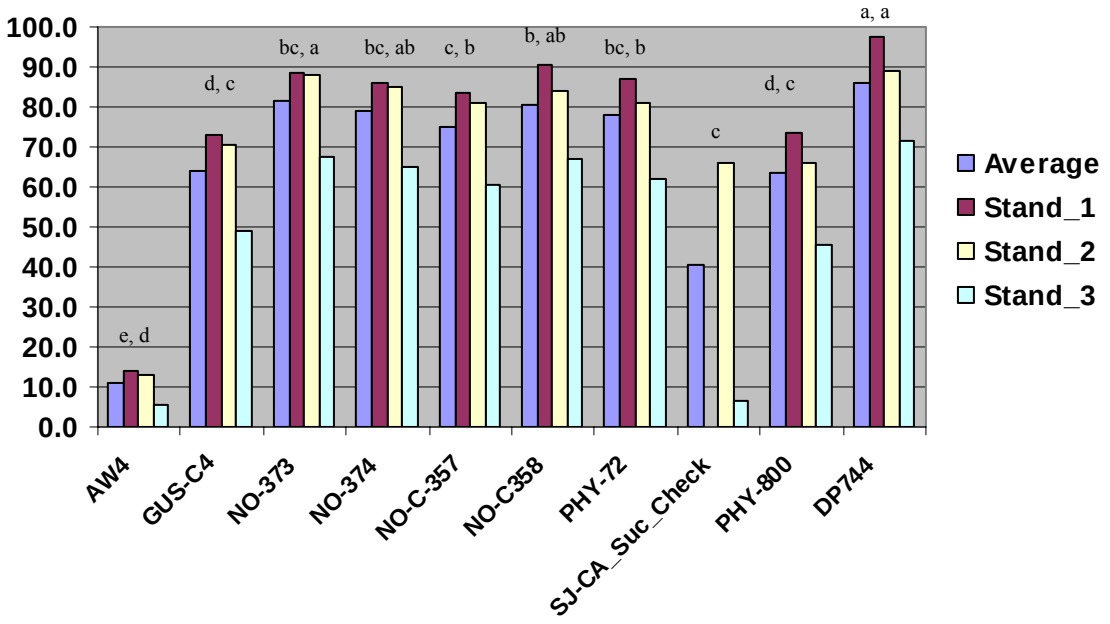

Figure 1. Plant survival during the growing season. The four bars indicated the plant count performed for each plot-entry at three different stages of plant development: 1) plant establishment (Stand_1), 2) five weeks after (Stand_2), 3) before final evaluation at crop maturity (Stand_3), and the average plant count (average) for four transgenic Upland cottons expressing the antifungal peptide D4E1 (NO-357, NO-358, NO-373, and NO-374), a transgenic control entry with the GUS marker gene (GUS-C4) and the original non-transgenic parent (AW4), commercial Acala (PHY-72), Pima (PHY-800 and DP744) cultivars.

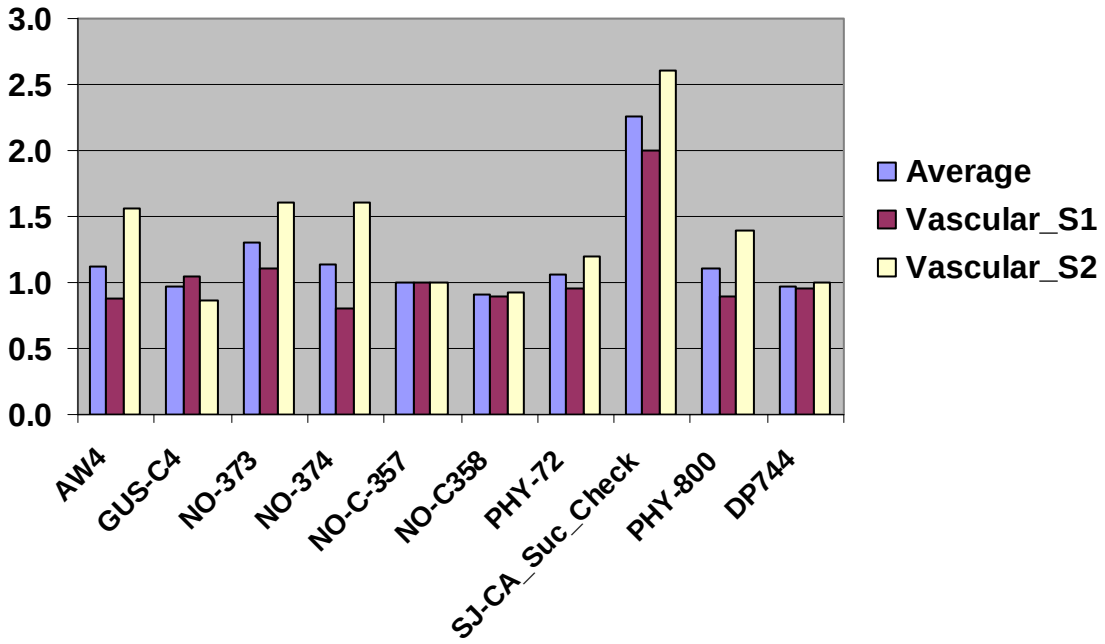

Figure 2. Root top vascular staining evaluation scores. The three bars indicated evaluation scores performed for each plot-entry from the average of five plants at two different stages of plant development: 1) plant establishment (Vascular_S1), 2), five weeks after (Vascular_S2), 3), and the average of the evaluation scores (Average) for four transgenic Upland cottons expressing the antifungal peptide D4E1 (NO-357, NO-358, NO-373, and NO-374), a transgenic control entry with the GUS marker gene (GUS-C4) and the original non-transgenic parent (AW4), commercial Acala (PHY-72) and Pima (PHY-800 and DP744) cultivars.

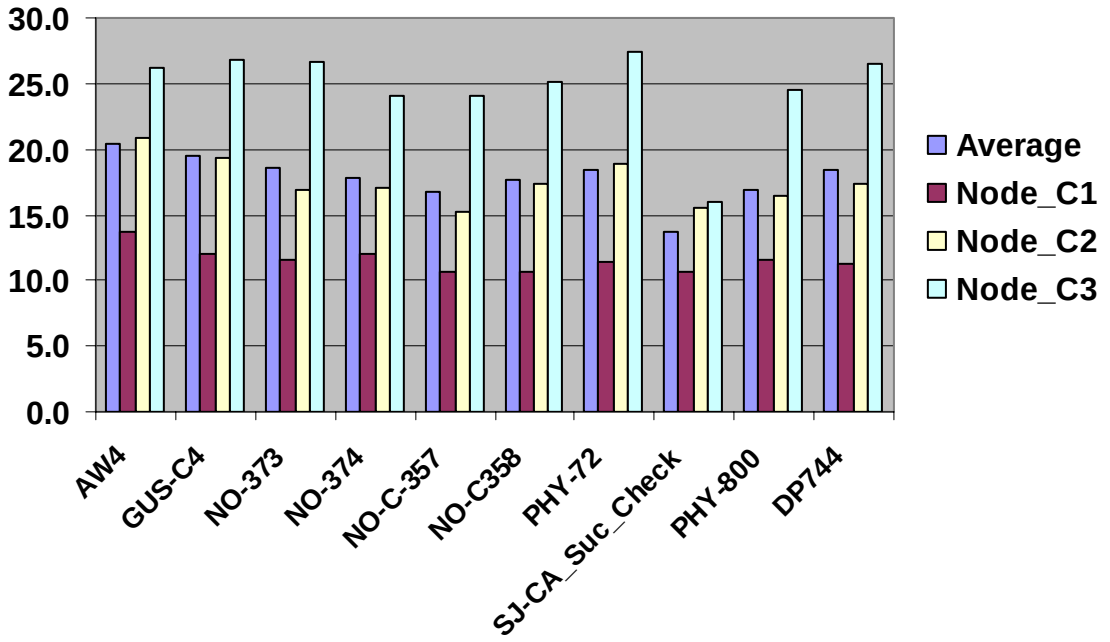

Figure 3. Number of node count during the growing season. The four bars indicated the number of nodes per plant for each plot-entry from the average of five plants at three different stages of plant development: 1) plant establishment (Node_C1), 2) five weeks after (Node_C2), 3) before final evaluation (Node_C3), and the average plant count (Average) for four transgenic Upland cottons expressing the antifungal peptide D4E1 (NO-357, NO-358, NO-373, and NO-374), a transgenic control entry with the GUS marker gene (GUS-C4) and the original non-transgenic parent (AW4), commercial Acala (PHY-72) and Pima (PHY-800 and DP744) cultivars.

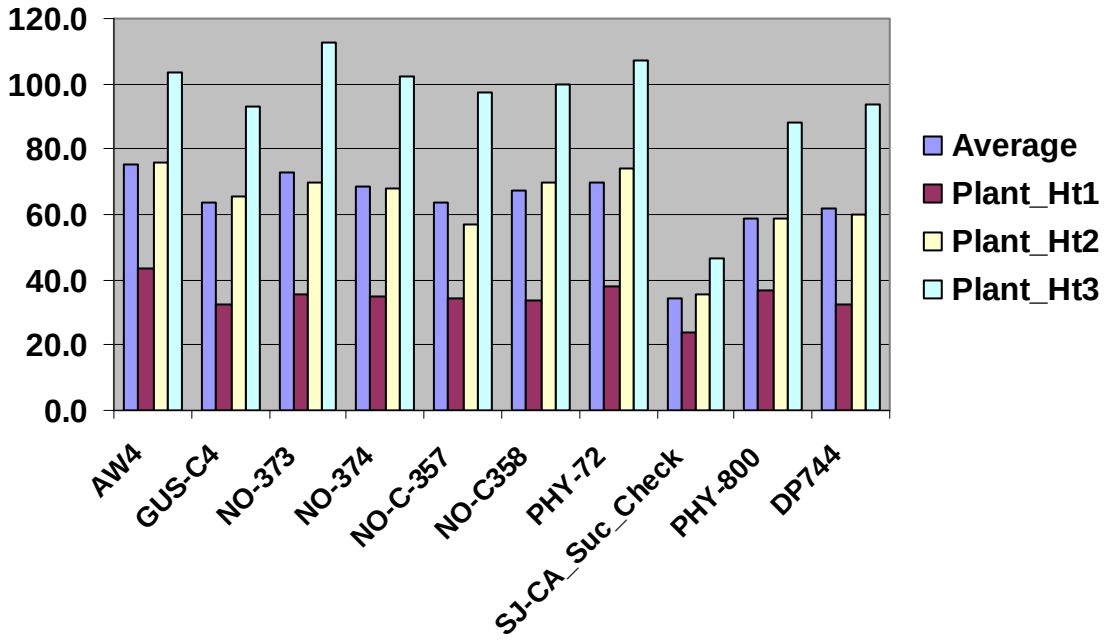

Figure 4. Plant height in centimeters during the growing season. The four bars indicated the plant height measurements performed for each plot-entry from the average of five plants at three different stages of plant development: 1) plant establishment (Plant_Ht1), 2) five weeks after (Plant_Ht2), 3) before final evaluation (Plant_Ht3), and the average plant count (Average) for four transgenic Upland cottons expressing the antifungal peptide D4E1 (NO-357, NO-358, NO-373, and NO-374), a transgenic control entry with the GUS marker gene (GUS-C4) and the original non-transgenic parent (AW4), and commercial Acala (PHY-72) and Pima (PHY-800 and DP744) cultivars.