



Sweet orange scab with a new scab disease “syndrome” of citrus in the USA associated with *Elsinoë australis*

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ABSTRACT

Elsinoë fawcettii causes citrus scab and *E. australis* causes sweet orange scab and there are different pathotypes of each species. Citrus scab is widely distributed, whereas sweet orange scab is limited mostly to southern South America. In 2010, *E. australis* was detected by PCR in Texas for the first time in the USA and subsequently found in commercial areas of Texas. In this study, cultures were obtained in Texas mostly from diseased fruit with symptoms similar to “late-season windscar” and identified as the Natsudaikai pathotype of *E. australis* by sequencing of the PCR amplification products and sequencing of the translation elongation factor and ITS regions. Inoculations of detached leaves and fruit of grapefruit, sweet orange, and tangerine produced scab-like symptoms on all organs and species. The fungus was re-isolated from the inoculated organs and PCR tests on re-isolated fungal colonies and lesions confirmed the presence of *E. australis*. The disease represents sweet orange scab of citrus with a new scab disease symptomatology, but the host range, ecology and epidemiology of this pathotype are still not completely understood. Thus far, Natsudaikai pathotype has been reported only from South Korea.

Key words: *Citrus paradisi*, *Citrus reticulata*, *Citrus sinensis*, *Elsinoë australis*, *Elsinoë fawcettii*, ITS region.

INTRODUCTION

Two scab diseases on citrus are common commercial problems in many humid citrus areas: citrus scab, caused by *Elsinoë fawcettii*, and sweet orange scab (SOS), caused by *E. australis* (Chung, 2010; Whiteside, 1975). Citrus scab (syn. Sour orange scab) was recorded in the Gulf Coast region of East Texas before 1923, and was confirmed to be present in the Lower Rio Grande Valley (LRGV) in 1973 (Timmer, 1974). *Elsinoë fawcettii* has many pathotypes (Timmer et al., 1996) that vary considerably in the hosts they attack, but fruit and leaves of many tangerines (*Citrus reticulata*) and their hybrids, grapefruit (*C. paradisi*), lemons (*C. limon*) and other citrus species are affected. This species only rarely affects sweet orange fruit, but never attacks leaves of sweet orange (*C. sinensis*). Only the Florida broad host range (FBHR) pathotype of *E. fawcettii* is occasionally found on sweet orange (Timmer, 1974; Whiteside, 1975). Citrus scab is cosmopolitan in its distribution. Sweet orange scab is less well-studied, but generally affects only fruit of sweet orange

and mandarins (*C. reticulata*) and their hybrids (Jenkins, 1933). It is generally confined to southern South America and has never been reported in the USA (Timmer, 1974). Hyun et al. (2001) recovered another pathotype of *E. australis* from a single tree of *C. natsudaikai* in South Korea and termed the South American strains as the Sweet Orange pathotype and the Korean strain as the Natsudaikai pathotype. None of the known pathotypes of sweet orange scab affects leaves. Both pathotypes of *E. australis* and some pathotypes or groups of pathotypes of *E. fawcettii* can be distinguished using specific DNA primers (Hyun et al., 2007).

Immature young fruit up to 6 to 8 weeks after petal fall are highly vulnerable to citrus scab (Whiteside, 1975). The fungus survives and spreads through the production of conidia on the fruit surface (Whiteside, 1975). Conidia are fragile and short-lived, but need only 2 to 3 h of continuous moisture to germinate and infect citrus tissues (Agostini et al., 2003; CABI, 2010).

In this paper, we report the first detection of the fungus in the USA, isolation and culture of the pathogen, inoculation of detached citrus leaves and fruit, re-isolation of the fungus, and confirmation of the identity through symptoms, fungal morphology, and PCR. We sequenced the partial translation elongation factor (TEF) and the internal transcribed spacer (ITS) and compared the sequences with those of isolates of *E. australis* and *E. fawcettii*.

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MATERIALS AND METHODS

Fungal morphology and pathogenicity tests

Fungal isolation

Symptomatic fruit and leaves were collected from citrus orchards in the Lower Rio Grande Valley in Texas in 2010. The fruit was then washed under running water for 10 min, soaked in sterile distilled water (SDW) containing a few drops of Dawn dishwashing detergent (Procter & Gamble) in a beaker for 20 min, rubbed clean with a sponge under running water, air dried for 30 min, soaked in SDW containing 2-5% Chlorox (5.95% sodium hypochlorite) for 5-10 min, rinsed with SDW several times, and left on paper towels placed on a workbench for 2-3 days. The fruit were swabbed with 95% ethanol, dried in a laminar flow hood, and scab pustules were scraped using a sharp scalpel to deposit tiny flakes onto the surface of the Whiteside's semi-selective medium (Whiteside, 1975). The plates were kept on a workbench in the laboratory at 25°C and observed daily for fungal growth. After 7-10 days, the slow-growing colonies were transferred to antibiotic-amended potato dextrose agar (PDA) (39 g PDA, 0.1 g tetracycline, and 0.1 g streptomycin per liter of water). Once pure cultures of the fungus were successfully established on antibiotic-amended PDA, stock cultures were established on PDA and maintained by transferring to new plates every 30-45 days to maintain viability of the fungus.

Conidial production

For inoculation assays, conidia were produced using procedures described by Timmer (1974) with minor modifications. Briefly, colonies from isolates SOS-035 and SOS-200 (Table 1) were grown on PDA with no antibiotics for 7 to 10 days. Small pieces of actively growing mycelium without agar were removed from the PDA and placed on the bottom of a petri dish, crushed with a spatula into minute fragments, and then 5 to 6 mL of liquid Fries medium was poured into the plate and stirred to dislodge the fragments from the petri dish. The plates were incubated for 48 h at 25-27°C and observed under a stereomicroscope to check for the growth of microcolonies on the bottom of the plates. The microcolonies were washed three times by pouring SDW into the plates, leaving for 10-20 min, swirling the plates to make sure all the nutrients were dissolved in the water, and then carefully pouring water from the plate. Autoclaved pond water was then added to the plates until the microcolonies were barely covered. The petri dishes were incubated overnight in the dark at 25-27°C. The pond water was poured from the plate, the conidia were dislodged using a camelhair brush, SDW was added to the plate and the plate was shaken vigorously. For inoculations, the conidial concentration was adjusted to 1×10^5 mL⁻¹ using a haemocytometer.

Inoculation of detached immature young leaves and fruit

Three young leaves ($\frac{1}{3}$ - $\frac{1}{2}$ expanded) per cultivar were collected from grapefruit, sweet orange, lemon and tangerine. In two separate leaf inoculation experiments for each isolate, two leaves were inoculated with spore suspensions from SOS 035 or SOS 200 (Table 1) and one leaf with SDW remained as a control. The inoculation experiments were repeated three times. The leaves were disinfected by placing them for 2 min in 0.5% Chlorox, dipping in 50% ethanol for 2 min each, rinsing in SDW several times. The leaves were blot-dried on sterile paper towels and placed either on water agar (1.5%) or sterile moist filter paper in petri plates. Five μ L of the conidial suspension was placed on the abaxial side of the leaf; the plates were sealed with parafilm, and incubated in a humid chamber at 25 to 27°C in the dark.

Three small, immature (3- to 8-week-old) citrus fruit for each species with the pedicel attached were collected from grapefruit, tangerine, and sweet orange trees. A small leaf was kept on the surface of the fruit, and wrapped with moist cotton. From the stylar end, 10 μ L of spore suspension derived from SOS 200 (Table 1) was poured between the leaf and fruit; the fruit was placed in a closed humid chamber and observed for the symptom development. Control fruit were treated similarly with SDW. The inoculation experiments were repeated twice.

Molecular diagnosis and characterization

DNA extraction

Fruit and leaf material showing typical scab symptoms (Figure 1) were collected from several places in Texas including citrus orchards, nurseries, dooryard trees, and retail grocery stores (Table 1) as part of an ongoing citrus commodity survey. The scab pustules were scraped from fruit (avoiding flavedo tissue) and from leaf samples using a sharp razor blade, collected in a 2 mL Lysing Matrix A tube (MP Biomedicals, LLC., Solon, OH) and the tissue was disrupted in a Mini-Beadbeater-96 (Biospec Products, Bartlesville, OK). The total genomic DNA was isolated using Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following manufacturer's instructions and the DNA was stored at -20°C until used.

Diagnostic PCR

Two μ L of DNA was used as a template in the diagnostic PCR tests consisting of 25- μ L reaction mixture with 2.5 μ L 10X PCR buffer (Invitrogen, Carlsbad, CA), 2.5 mM MgCl₂, 0.2 mM each dNTPs, 0.2 μ M each primers, and 1 Unit platinum Taq DNA polymerase (Invitrogen). The primer pairs Efaw-1 and Efaw-2, and Eaut-1 and Eaut-5 were used to specifically detect *E. fawcettii* and *E. australis*, respectively, and the PCR conditions were the same as described by Hyun et al. (2007). All further confirmative PCR tests to detect *E. australis* were done using the Eaut-5 primer pair. NS1/NS2 primers (Dams

TABLE 1 - The *Elsinoë* isolates used in the production of Neighbor-joining consensus tree based on internal transcribed spacer region and translation elongation factor 1 α gene. The isolates marked with (*) were isolated by the authors and the remainder are from the GenBank database that showed nucleotide sequence similarity to sequences from several *E. australis* isolates in this study

Geographical origin	Host plant	Isolate No.	GenBank accession numbers
Weslaco, TX, USA*	Marrs sweet orange	SOS 002	KC211034
Weslaco, TX*	Marrs sweet orange	SOS 012	KC211035
Mission, TX*	Sweet orange	SOS 019	KC211036
Mission, TX*	Sweet orange	SOS 031	KC211037
Mission, TX*	Sweet orange	SOS 035	-
Mission, TX*	Rio Red grapefruit	SOS 040	KC211038, KC211048
Mission, TX*	Rio Red grapefruit	SOS 047	KC211039, KC211050
Mission, TX*	Sweet orange	SOS 054	KC211040, KC211051
Mission, TX*	Sweet orange	SOS L54	KC211041, KC211049
Mission, TX*	Tangerine	SOS 061	KC211033, KC211047
Weslaco, TX*	Sweet orange	SOS 138	-
Mission, TX*	Sweet orange	SOS 200	-
Austin, TX*	Rio Red grapefruit	SOS 225	-
South Korea (Jeju Island)	Yuzu (<i>C. junos</i>)	Yuzu 1-3	FJ010285
São Paulo, Brazil	Murcott tangor	VRG-7L-5	FJ010283
Florida	Temple tangor	Smoak-1	FJ010280
South Korea (Jeju Island)	Satsuma mandarin	SM24-4	FJ010279
New Zealand	Yen Ben lemon	70298	FJ010216
Florida	Murcott	MUR-3	FJ010240
São Paulo	Sweet orange	VRG-BRT	FJ010284
São Paulo	Sweet orange	FZ-STM-CL	FJ010242
Argentina	Satsuma mandarin	Ea-4	FJ010239
Argentina	Satsuma mandarin	Ea-3	FJ010238
South Korea (Jeju Island)	Natsudaïdai	KNa-1	FJ010250
South Korea (Jeju Island)	Natsudaïdai	KNa-2	FJ010251
South Korea (Jeju Island)	Natsudaïdai	KNa-5	FJ010252
South Korea (Jeju Island)	Natsudaïdai	KNa-9	FJ010253
South Korea (Jeju Island)	Natsudaïdai	Na-1	FJ010259
Hidalgo County, TX*	Sweet orange	S5174A	KC211042, KC211053
McAllen, TX*	Sweet orange	S5174B	KC211052
McAllen, TX*	Grapefruit	S5077B	KC211043, KC211054
McAllen, TX*	Grapefruit	S5074A3	KC211044, KC211055
McAllen, TX*	Grapefruit	S5074A1	KC211045, KC211056
Spring, TX*	Citrus spp.	S3829C	KC211046
USA	Unknown	AFTOL-ID.1360	QQ677902
USA	Unknown	AFTOL-ID.1853	DQ767641
South Korea (Jeju Island)	Satsuma mandarin	SM3-1	FJ010360
South Korea (Jeju Island)	Yuzu	YuZu2-2	FJ010357
South Korea (Jeju Island)	Yuzu	Yuzu2-1	FJ010356
South Korea (Jeju Island)	Kinkoji (<i>C. obovoidea</i>)	Maru-2	FJ010331
South Korea (Jeju Island)	Satsuma mandarin	SM16-4	FJ010346
South Korea (Jeju Island)	Natsudaïdai	Na-2	FJ010336
Australia	Jojoba (<i>Simmondsia chinensis</i>)	Ea-J02	GU126378
Australia	Jojoba	DAR77387	GU126376
Argentina	Satsuma mandarin	Ea-1	FJ010312
Argentina	Valencia sweet orange	70041	FJ010289
Argentina	Satsuma mandarin	70212	FJ010291

et al., 1988; Glass & Donaldson, 1995) were used as internal controls to confirm presence of amplifiable DNA in the extract. Furthermore, EaNat-1 primers were used to test for the Natsudaïdai pathotype. PCR amplification products were separated by electrophoresis on 1% agarose gels, ethidium-bromide stained, visualized under UV light,

and photographed using Biospectrum imaging system (UVP, Upland, CA).

PCR for sequence characterization of TEF and ITS regions

DNA was isolated from fungal cultures (Table 1) using a Qiagen kit as previously described. PCR reactions

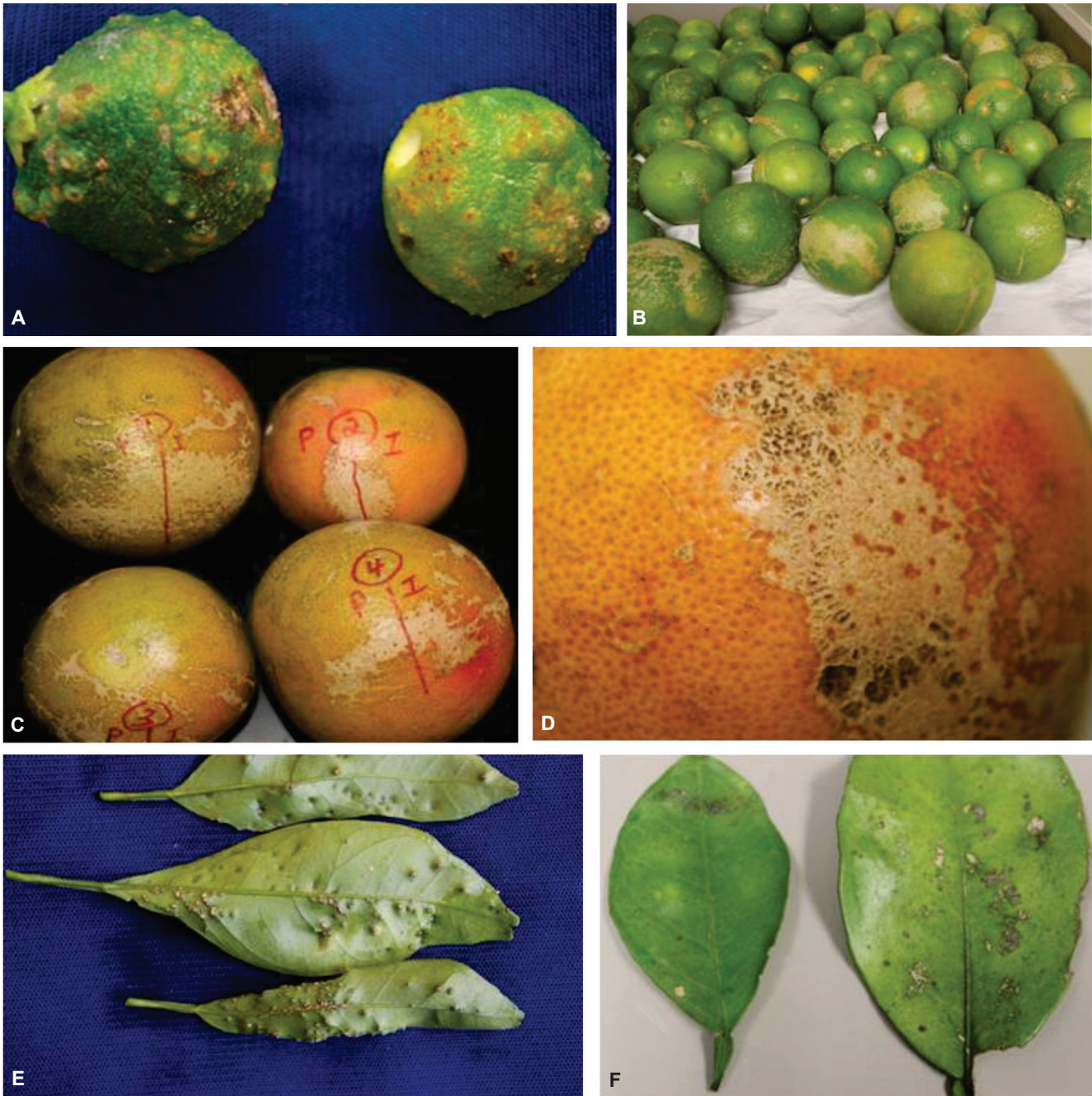


FIGURE 1 - Field symptoms of sweet orange scab with a new scab disease symptomatology associated with *Elsinoë australis* on citrus fruit on **A.** satsuma mandarin; **B.** sweet orange; **C.** grapefruit; **D.** a close-up of grapefruit “late season windscar” symptom and on leaves of **E.** satsuma mandarin; **F.** sweet orange.

were performed using 12.5 µL Takara Ex Taq master mix (Clontech Laboratories Inc., Mountain View, CA), 0.5 µL each primer (10 µM), 9.5 µL water, and 2 µL DNA. TEF runs using primers elongation-1-F and elongation-1-R (Hyun et al., 2009) consisted of 95°C for 120 sec, 35 cycles of 94°C for 30 sec, 60°C for 60 sec, 72°C for 120 sec, followed by 10-min extension at 72°C. A longer portion of the region spanning ITS1-ITS2 was reproduced from culture samples using the primer pair Elsi.24F/ITS4. PCR products were sequenced directly at MCLAB (MCLAB,

San Francisco, CA) and analyzed using Geneious Pro 5.4.4 software (Drummond et al., 2011). Forward and reverse sequences were aligned and edited and multiple alignments were carried out with Geneious Alignment. Neighbor-joining phylogenetic trees were estimated with Geneious Tree Builder using the Tamura-Nei genetic distance model, and replicated with bootstrapping (1,000 replications).

The PCR amplification products were excised from the gels and extracted using the Qiaquick gel extraction kit (Qiagen). Purified DNA fragments were ligated into

pCR4-TOPO cloning vector and transformed into DH5 α -T1R one-shot chemically competent *Escherichia coli* cells using the TOPO TA cloning kit for sequencing (Invitrogen). Further plasmid DNA extractions were carried out using QIAprep spin miniprep kit (Qiagen) and the purified DNA was sequenced at the Iowa State University DNA sequencing facility. Sequences were edited and analyzed using Geneious Pro 5.4.4 and homology searches were conducted using BLASTn program in GenBank (www.ncbi.nlm.nih.gov).

RESULTS

Symptoms observed, disease occurrence and background

In July 2010, a lemon fruit collected with scab-like symptoms from a residential area in Spring, TX (a Houston suburb) as part of an ongoing citrus commodity survey gave a positive PCR result for *E. australis*. The fruit had small, flat lesions similar to scab on lemons caused by species of *Elsinoë*. Later, Satsuma mandarin (*C. unshiu*) fruit collected from East Texas also produced a PCR-positive test result for *E. australis*. These fruit had more prominent and extensive scab-like lesions that were not typical of SOS symptoms described from other countries. Additional samples collected in East Texas by the United States Department of Agriculture - Animal and Plant Health Inspection Service - Plant Protection and Quarantine (USDA-APHIS-PPQ) were found to be positive for *E. australis* using specific DNA primers and sequence analysis of the PCR products (Hyun et al., 2007). *Elsinoë australis* was isolated in pure culture from samples received by the PPQ. On August 23, 2010, USDA-APHIS announced the presence of *E. australis* in residential properties in Harris and Orange counties in Texas and Orleans parish in Louisiana.

Later, several grapefruit and sweet orange samples collected in the LRGV of Texas were also positive for *E. australis* in molecular diagnostic assays and it was detected on samples from Mississippi and Florida in October and December of 2010, respectively. The fungus was identified by PCR in several counties in Florida and was detected in Arizona in January, 2011. Many of the fruit and leaves assayed had lesions similar to those expected of a scab disease. However, many positive PCR reactions were from a wide range of symptoms including those associated with windscar, thorn punctures, bird damage and various types of damage to leaves and fruit.

Symptoms in the field were variable. In the Lower Rio Grande Valley of Texas, PCR positive results were obtained primarily from a symptom usually referred to by growers as “late-season windscar” (J. W. Sauls, personal communication). However, symptoms on fruit ranged from small, flattened off-white pustules to large coalesced lesions (Figure 1 A, B, C, D). The leaf lesions were relatively large and coalesced on the margins of the upper side of the leaf (Figure 1E, F). The aforementioned symptoms are in contrast to those reported by Bitancourt

& Jenkins (1937) and Jenkins (1933) in South America as small lesions on the lower side of the sweet orange leaf, mostly on midrib. The PCR positive samples for *E. australis* that were sent to PPQ from various locations varied in size, shape and color. On some leaves, small raised rust-to-brown, rounded scabs were seen on the lower side of the leaves. On tangerine, lemon, and grapefruit, small, elongated rust-colored lesions on underside and upper side of leaves were observed. On several citrus species including tangerines, grapefruit, other non-specified citrus and *Fortunella* sp., foliar lesions were large, broad, coalesced and somewhat crust-like, rust-colored and darker brown or black lesions extending along the margins on the underside of leaves. Sweet orange and other unidentified citrus showed small lesions dispersed through the leaf blade on the lower and upper side of the leaves. On fruit of grapefruit, satsumas and tangerines, circular or elongated, depressed areas of the peel were surrounded by scabby cream- to rust-colored tissue with puckering of the green part of the peel around the lesion, alternatively the entire lesion was covered in scabby tissue. Scabby cream-to-brown lesions were seen surrounding the petiole of some fruit, particularly grapefruit. On other fruit, scabby lesions were dispersed, as if spread by water splash or scratches, and were in some cases crusty and brown to dark brown and slight raised. Microscopic examination, isolations on selective media (Whiteside, 1975) and PCR amplifications indicated that on some samples, there were several fungi growing on some lesions on fruit and leaves, resulting in a certain variation in symptoms making it difficult to relate the symptoms exclusively to the presence of *E. australis*.

The USDA-APHIS established a technical working group with researchers and authorities from universities and regulatory agencies to develop survey and disease mitigation strategies. In December, 2010, SOS quarantine areas were established for Texas, Mississippi and Louisiana and conditions were outlined that must be met to allow interstate movement of fruit to protect citrus-producing states and trading partners. Equivalent measures were also imposed on fresh fruit imports, as several shipments of Tahiti lime (*C. latifolia*) were positive for *E. australis* by PCR. Later, in March, 2011, APHIS revised the federal order to include Florida and Arizona in the quarantine areas. APHIS has also established guidelines for use of approved fungicides in plant nurseries and packinghouse procedures to be followed to allow interstate movement of fruit.

Fungal colony and conidial characteristics

Colonies resembling *Elsinoë* spp. were obtained in pure culture from more than 25 fruit samples. Colonies were slow-growing, beige, pink, orange, orange-beige, to purplish beige, about 17 mm in diameter in 30 days, very irregularly shaped with a wrinkled, cerebriform surface which resembled callus tissue (Figure 2). The conidia

produced were hyaline, ovoid and typical of those described for *E. australis* (CABI, 2010; Timmer, 1974). The fungal cultures are available at TAMUK Citrus Center, Weslaco, TX and USDA-APHIS, Edinburg, TX.

Detached leaf and fruit inoculation

Detached leaf assays showed scab symptoms 5 to 7 days after inoculation and sweet orange, grapefruit and lemon were highly susceptible (Figure 3). Fruit of grapefruit, tangerine, and sweet orange inoculated with *E. australis* isolates developed scab symptoms 8 to 10 days after inoculation (Figure 4). The fungus was successfully re-isolated from the inoculated leaf and fruit tissue by the methods described above. Furthermore, the PCR tests on DNA isolated directly from the lesion on the inoculated tissue and also on DNA

isolated from the re-isolated fungal colonies confirmed the presence of *E. australis*.

Molecular characterization

PCR amplifications using DNA isolated from citrus scab lesions as a template and *E. fawcettii*-specific primers, Efaw-1 and Efaw-2, produced PCR amplicons of two sizes: 717 bp and 334 bp (not shown). Homology searches in GenBank indicated 100% identity with an E-value of 1e-173 to previously deposited *E. fawcettii* sequences. PCR amplifications using the two primer sets Eaut-1 and Eaut-5 on new scab disease lesions including “late season wind scar” resulted in two amplification products of 548 bp (not shown) and 363 bp (Figure 5), respectively, with 100% identity to *E. australis* in GenBank homology searches. Amplifications using

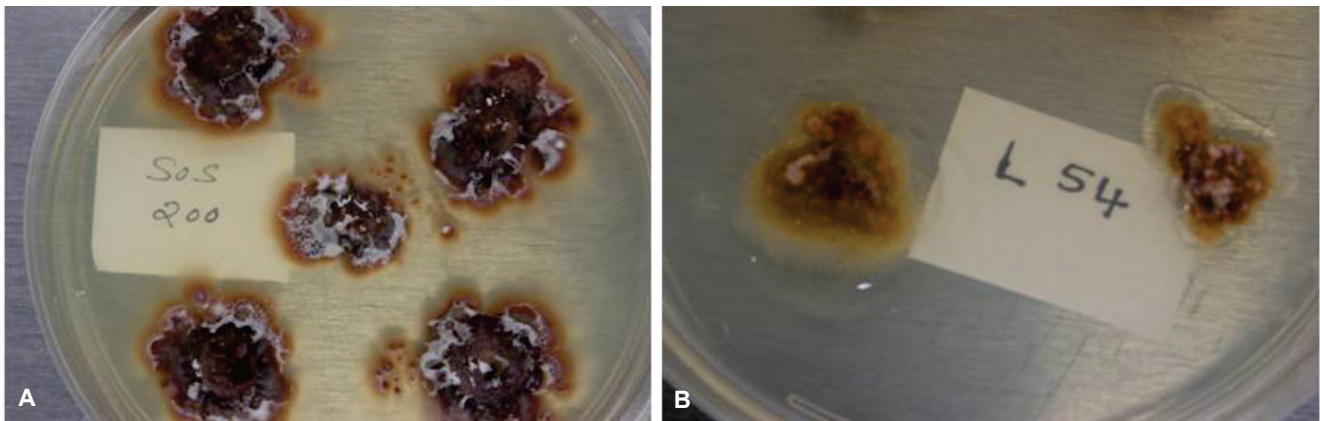


FIGURE 2 - *Elsinoë australis* isolates obtained in pure culture showing very irregular shape with a wrinkled, cerebriform surface resembling callus tissue. *a*

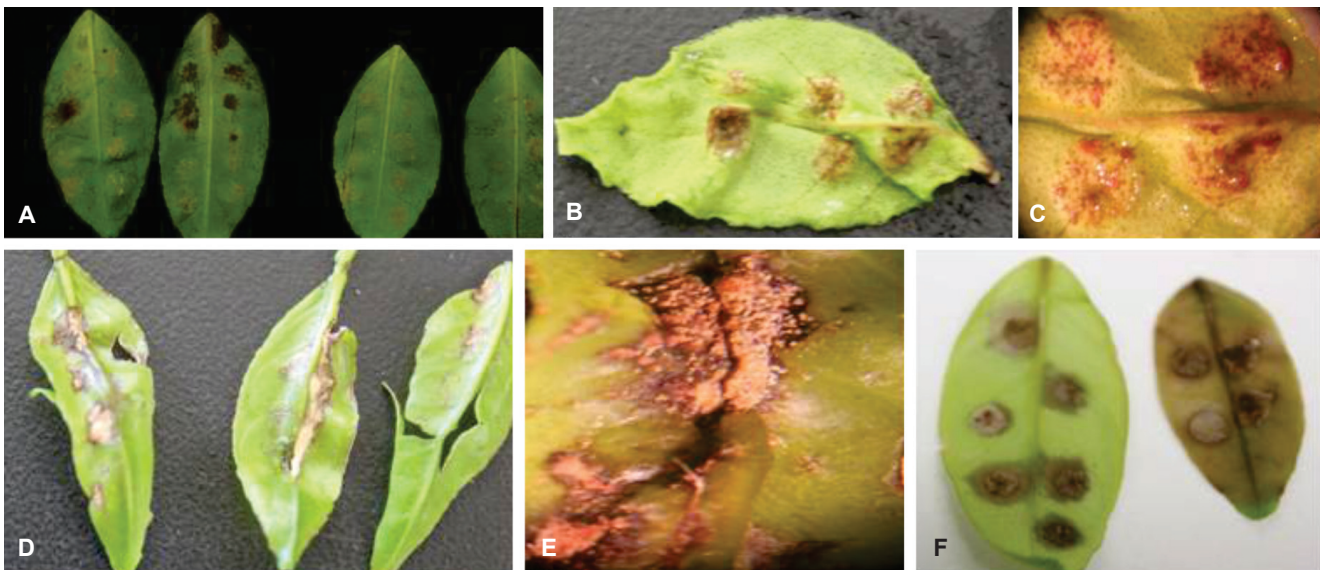


FIGURE 3 - Young citrus leaves developed scabby lesions 7 days after inoculation with conidial suspensions. **A.** Rio Red grapefruit; **B.** mandarin; **C.** a close-up of mandarin lesion; **D.** Pineapple sweet orange; **E.** a close-up of sweet orange lesion and **F.** lemon.

EaNat1 primers targeting the Natsudaikai pathotype resulted in 578-bp PCR amplicons as expected (Figure 5). The Satsuma mandarin leaf sample collected from East Texas and sweet orange leaf collected from Mission, Texas (Figure 1) gave a positive PCR test result using Eaut-5 primers for *E. australis*. These are the only leaf samples in the Lower Rio Grande Valley from which we ever detected the fungus and no cultures have been obtained from symptomatic leaves. Some of the initial test results in detecting *E. australis* were confirmed by USDA-APHIS-PPQ molecular diagnostic laboratory in Beltsville, MD.

The sequence analysis further confirmed the identity of these isolates as *E. australis*. Neighbor-joining (NJ) analysis of the TEF resulted in three clades, each

corresponding with one of three *Elsinoë* species studied (Figure 6). All domestic *E. australis* suspects fell within the *E. australis* clade. Furthermore, these suspects are all members of an *E. australis* subclade comprised entirely of the Natsudaikai pathotype. A similar NJ analysis of the ITS region, minus *E. veneta*, resulted in two clades, each with compositions corresponding to *E. australis* and *E. fawcettii* (Figure 7). All *E. australis* domestic suspects are members of the *E. australis* clade. Within this *E. australis* clade, three domestic suspects are members of a subclade that contain all samples of the sweet orange pathotype, whereas the remaining occupy unresolved positions in the *E. australis* clade along with all Natsudaikai pathotype samples.

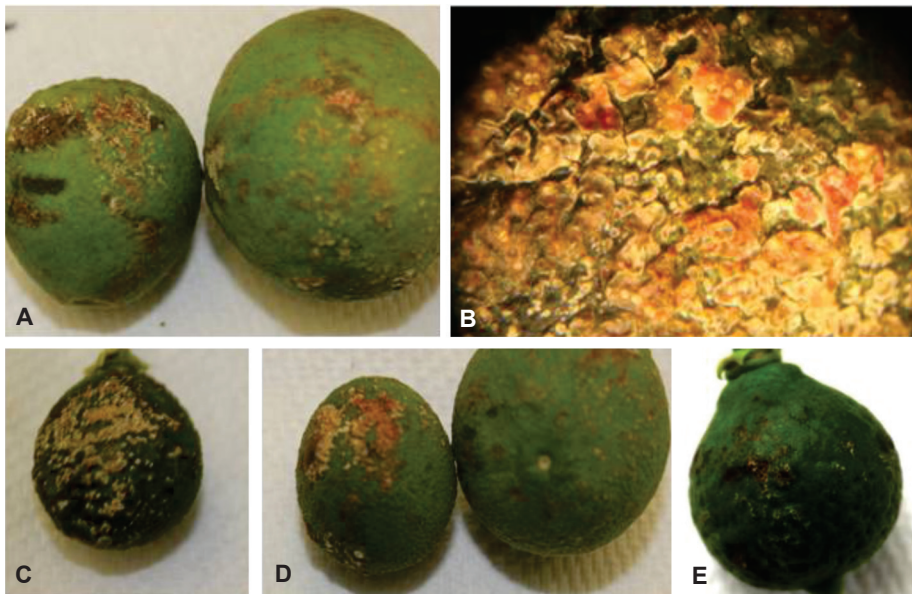


FIGURE 4 - Small immature citrus fruits developed scabby lesions 10 days after inoculation with conidial suspensions. **A.** grapefruit; **B.** a close-up of the grapefruit lesion; **C.** sweet orange and **D.** satsuma mandarin; **E.** satsuma mandarin.

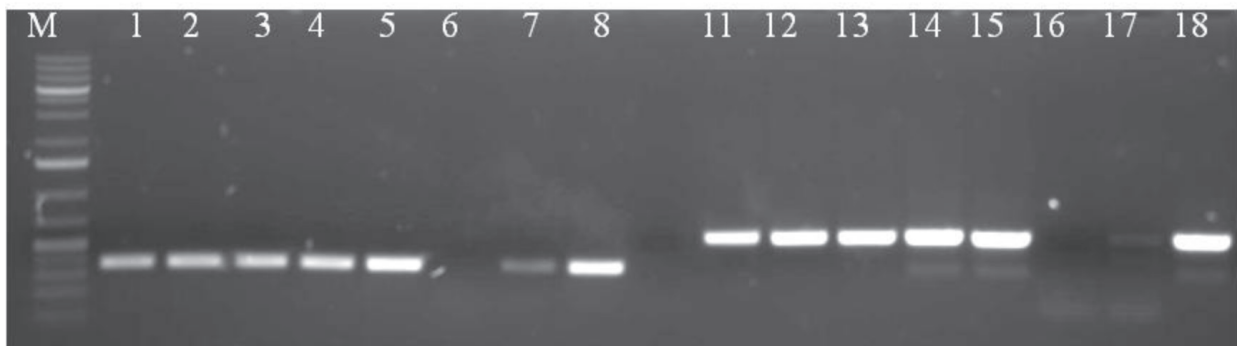


FIGURE 5 - PCR amplification products obtained from the sweet orange scab disease lesions associated with *Elsinoë australis* of the grapefruit and sweet orange collected in Lower Rio Grande Valley orchards using *E. australis* species-specific primers Eaut-5 and Natsudaikai pathotype-specific primer set Ea Nat 1. Lanes 1-4 (grapefruit) & 5-8 (sweet orange): 363-bp PCR amplicons obtained using Eaut-5 and lanes 11-14 (grapefruit) & 15-18 (sweet orange): 578-bp fragments obtained using Ea Nat 1 primer set. M = 1Kb DNA ladder (Fermentas).

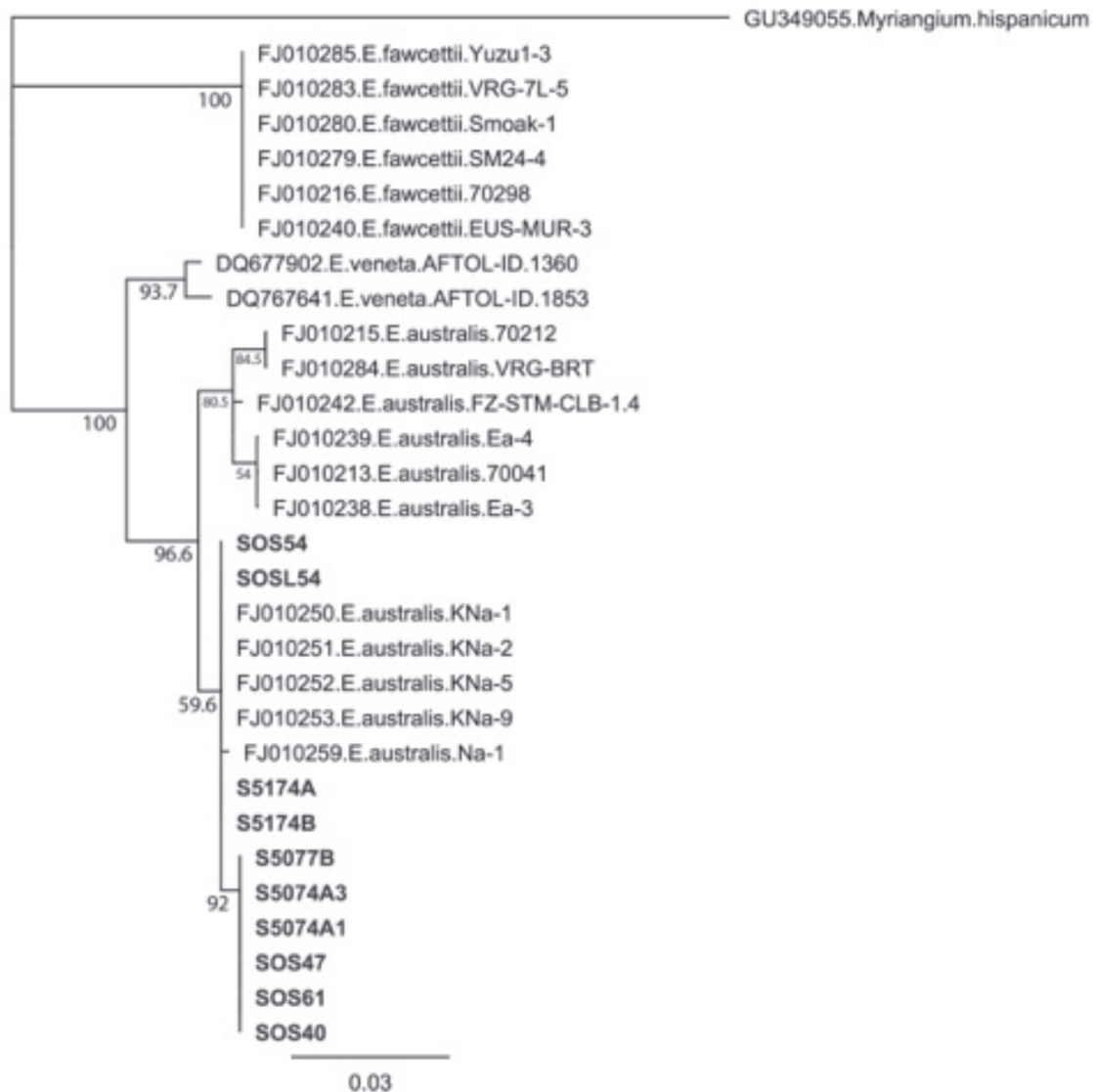


FIGURE 6 - Neighbor-joining consensus tree (1000 bootstrap replications) based on the alignment of partial elongation factor 1 α (TEF) gene nucleotide sequences obtained for the *Elsinoë australis* isolates recovered by the authors (bold) and several other sequences from GenBank database that showed high similarity.

DISCUSSION

In this study, many fruit samples from different citrus species and a few leaf samples were positive for *E. australis* by PCR. We have successfully isolated *E. australis* from grapefruit, sweet orange, and mandarin fruit collected in Texas and confirmed the isolate identity by colony characteristics, conidial morphology, and PCR. Moreover, we were able to inoculate detached leaves of grapefruit, sweet orange, and lemon and detached fruit of grapefruit, lemon, and sweet orange under laboratory conditions and inoculated fruits and leaves showed scab symptoms. PCR assays using tissue collected from the infected lesions indicated presence of the fungus, and the identity of *E. australis* was further confirmed by re-isolation of the fungus from inoculated detached leaf and fruit on

Whiteside's semi-selective medium. Furthermore, PCR on the re-isolated fungal colonies confirmed the pathogen to be *E. australis*.

This study is the first report of *E. australis* in the USA. We found that in Texas it affects many species of citrus including lemon and grapefruit in addition to sweet orange and mandarins. The symptoms associated with the presence of the fungus vary widely; many are typical scab symptoms, but many would not have been diagnosed as scab even by professionals with considerable experience with the disease. The lack of a specific symptom associated with samples positive for PCR for *E. australis* has made diagnosis of the disease for regulatory purposes very difficult and determination of the presence of the pathogen has depended primarily on PCR using specific primers. We have determined that the *E. australis* isolates used in

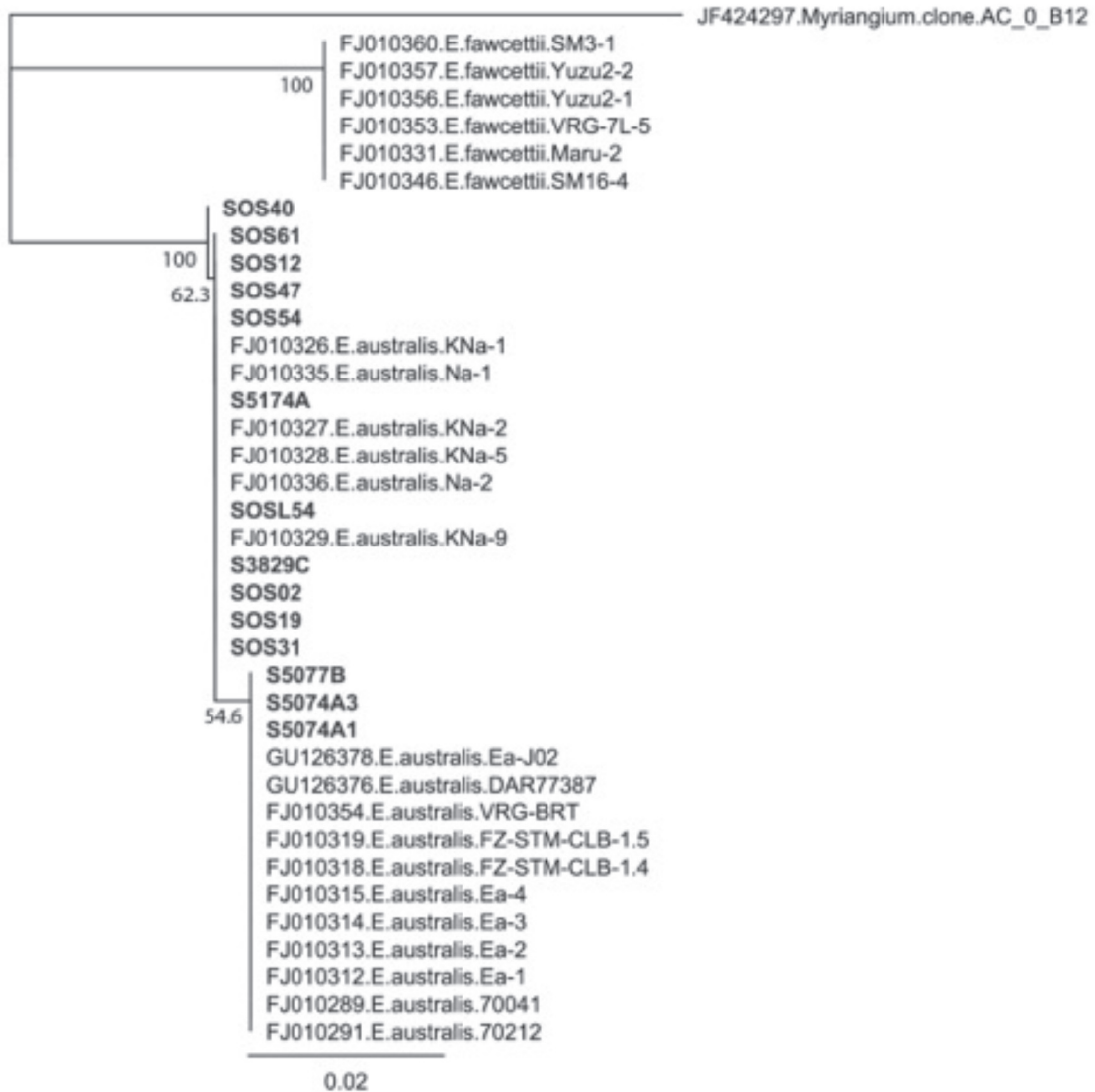


FIGURE 7 - Neighbor-joining consensus tree (1000 bootstrap replications) based on the alignment of internal transcribed spacer region (ITS) gene nucleotide sequences obtained for the *Elsinoë australis* isolates recovered by the authors (bold) and several other sequences from GenBank database that showed high similarity.

this study are pathogenic to fruit and leaves of many citrus species in laboratory tests; however, symptom expression under field conditions is unknown. It differs from sweet orange scab in South America in producing symptoms on leaves as well as on fruit although few symptomatic leaves were positive by PCR.

Molecular analysis indicates that all of the isolates recovered from the USA correspond closely with the Natsudaikai pathotype of *E. australis* from South Korea. There is no indication that the fungus was introduced from South America. Interestingly, the Natsudaikai pathotype of *E. australis* was also recently isolated and proven to cause a scab disease of jojoba in Australia (Ash et al., 2012). The isolates of *E. australis* from jojoba are apparently not pathogenic to citrus, as demonstrated by leaf and fruit

inoculations. The origin of *E. australis* in the USA remains unknown, but this species may be much more widely distributed on other plant species and in other areas than was previously thought and may have been present for some time.

PCR-positive samples and some cultures have been obtained from semi-arid to arid citrus areas in Texas and Arizona. This is surprising since scab is primarily a disease problem on citrus in humid areas (Whiteside, 1975) and normally would not be expected to occur, much less, cause significant disease on citrus in those areas. Citrus scab in Florida does not spread readily from tree-to-tree (Timmer, personal observations). Most of the spread occurs on nursery trees which are grown in high densities with overhead irrigation where the opportunity for spread

is very high. Conidia of *Elsinoe* spp. are very fragile and desiccate quickly. However, they do germinate and infect plants in 2-3 h (Agostini et al., 2003; Whiteside, 1975). It is possible that these fungi are really adapted to areas of high dew and little rain. They may have some means of survival on mature leaves and other organs which allows them to withstand long periods in the absence of moisture. This may explain a conundrum with sweet orange scab in South America. There, the disease affects only fruit with no apparent infection on leaves. It has always been a puzzle as to where the inoculum comes from for the next crop if the previous crop has already been harvested. The pathogen may survive as an epiphyte or endophyte in wounded citrus leaves or other organs.

As pointed out above, several symptoms are associated with the presence of *E. australis* on citrus fruit and leaves. The most common site for the fungus are wounds resulting from "late-season windscar". That symptom may be produced by the fungus or it may be residing there as an epiphyte or endophyte. PCR of normal healthy looking fruit rind or fruit with rust mite (*Phyllocoptruta oleivora*) damage or other smooth, superficial blemishes are rarely positive. Thus all of the symptoms associated with the presence of the fungus are deep wounds in fruit or leaves. Such tissues are sufficiently rough in texture to perhaps help retain the moisture on those surfaces longer than on smooth surfaces and this may favor growth of the pathogen.

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