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Molecular characterization of some *Elsinoë* isolates from leguminous hosts

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Pathogenicity of *Elsinoë* isolates from *Phaseolus coccineus*, *P. lunatus*, *P. vulgaris* and *Vigna unguiculata* was studied and the relatedness of the isolates examined at the molecular level using eight random 10-mer primers to amplify total DNA (RAPDs). Isolates differed markedly in their pathogenicity to the various hosts and there was some evidence that those from *P. coccineus and P. vulgaris* were different pathotypes. The isolate from *V. unguiculata* was the only one that was virulent on this host and was nonvirulent on *P. vulgaris*. A RAPD-based dendrogram with a cophenetic correlation coefficient of 0·975 was generated using the NTSYS-pc computer program. A UPGMA cluster analysis based on the RAPD banding pattern of the eight primers ranked *Elsinoë phaseoli* isolates from *Phaseolus* spp. into two major subgroups. One subgroup was composed of isolates from *P. lunatus* and *P. vulgaris*, and the other from *P. coccineus*, suggesting some specialization within the species. The isolate from *Vigna unguiculata* clustered separately from the *Phaseolus* isolates, as did the *Elsinoë* outgroups from *Citrus*, *Protea* and *Leucospermum*. These results suggest that the isolate from *Vigna unguiculata* could be an undescribed species of *Elsinoë*.

Introduction

Several leguminous crops of agricultural importance are susceptible to scab disease caused by *Elsinoë* spp. (Sivanesan & Holliday, 1971; Allen, 1983, 1991; Kannaiyan & Haciwa, 1993; Phillips, 1994a). Although bean scab is generally regarded as being of local and minor importance (Bates, 1957; Peregrine & Siddiqui, 1972; Mutitu & Mukunya, 1979; Allen, 1983), Schwartz (1991) states that yield losses of up to 70% can occur. Phillips (1994a) reported yield losses in the order of 40% in South Africa. In recent years, bean scab has become one of the most destructive diseases of beans in N.E. Zambia (J.C. Musanya, Ministry of Agriculture, Food and Fisheries, Kasama, 1994, personal communication) and cowpea scab is regarded as the most important disease of cowpeas in eastern Africa (Allen, 1983; Kannaiyan & Haciwa, 1993) where total destruction of the crop can occur.

Bean scab, as well as scab of several other food legumes, is commonly attributed to *E. phaseoli*, which is known throughout tropical America, Africa and India (Jenkins, 1931; Bruner & Jenkins, 1933; Sivanesan & Holliday, 1971; Emechebe, 1980; Phillips, 1994a). Little, however, is known about the genetic variability of *E. phaseoli* (Phillips, 1996). Pathogenicity experiments conducted by Bruner & Jenkins (1933) revealed evidence of host specificity among isolates of *E. phaseoli*. Although five cultivars of *Phaseolus lunatus* were susceptible to *E. phaseoli*, no symptoms could be induced on *P. vulgaris* or five other legume species. Similarly, Phillips (1996) found that isolates from *P. coccineus* were avirulent to *P. lunatus*. However, it is not realistic to attempt to delimit species among these *Elsinoë* isolates solely on the basis of their pathogenicity and virulence, because of their inherent instability and plasticity. This is still further complicated by the fact that their *Elsinoë* states are rarely if ever observed and their *Sphaceloma* anamorphs are generally morphologically conserved. In contrast, genomic fingerprinting techniques based on the polymerase chain reaction (PCR), using arbitrary primers and low annealing temperatures (RAPDs) (Welsh & McClelland, 1990; Williams *et al.*, 1990) have proven to be a relatively unbiased and informative approach in revealing genetic relationships within and between species in many organisms including fungi. For example, RAPDs has been used to distinguish genotypes in natural populations of the ectomycorrhizal fungus *Suillus granulatus* (Jacobson *et al.*, 1993), to differentiate pathotypes among isolates of plant pathogens such as *Leptosphaeria maculans*. (Goodwin & Annis, 1991), *Pseudocercosporella herpotrichoides* (Nicholson & Rezanoor, 1994), *Phaeoisariopsis griseola* (Guzmán *et al.*, 1995) and to distinguish among species of *Colletotrichum* (Freeman & Rodriguez, 1995) and *Cylindrocladium* (Victor *et al.*, 1996). In using this technique, Tan *et al.* (1996) were also able

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Table 1 Accession number and geographic origin of isolates of *Elsinoë* spp. used in this study

Isolate	Species	Host	Region	Date isolated
SP5	*E. phaseoli*	*Phaseolus coccineus*	Natal, S. Africa	1993
SP6		*Phaseolus coccineus*	Lothair, S. Africa	1993
SP7		*Phaseolus vulgaris*	Greytown, S. Africa	1993
SP8		*Phaseolus vulgaris*	Cedara, S. Africa	1993
SP9, IMI 056102		*Phaseolus vulgaris*	Zimbabwe	1954
SP10, IMI 302107		*Phaseolus vulgaris*	Chipata, Zambia	1986
SP11	*Elsinoë* sp. 1	*Vigna unguiculata*	Ricatla, Mozambique	1994
SP13	*E. phaseoli*	*Phaseolus vulgaris*	Dedza, Malawi	1994
SP14		*Phaseolus vulgaris*	Bunda, Malawi	1994
SP15		*Phaseolus vulgaris*	Bunda, Malawi	1994
SP17		*Phaseolus coccineus*	Mooi River, S. Africa	1994
SP18		*Phaseolus vulgaris*	Greytown, S. Africa	1994
SP19		*Phaseolus vulgaris*	Dedza, Malawi	1994
SP22		*Phaseolus coccineus*	Ogies, S. Africa	1994
SP26, CBS 156·31		*Phaseolus lunatus*	Cuba	1964
LC1	*Elsinoë* sp. 2	*Leucospermum* sp.	Betties Bay, S. Africa	1995
PR1	*Elsinoë* sp. 3	*Protea cynaroides*	Betties Bay, S. Africa	1995
CT1	*E. fawcettii*	*Citrus limon*	Tzaneen, S. Africa	1995

to distinguish between species and pathotypes of *Elsinoë* isolates associated with scab of *Citrus*.

The aim of the present study therefore was to use RAPDs to analyse the genetic differences in isolates previously characterized as *E. phaseoli* on leguminous hosts collected from southern Africa and to relate this to their pathogenicity. An *Elsinoë* isolate causing scab of cowpeas (*Vigna unguiculata*), originally referred to as *E. phaseoli*, was also included, as well as outgroup isolates of *Elsinoë* spp. from *Protea*, *Leucospermum* and *Citrus*.

Materials and methods

Isolates

Isolates of *Elsinoë phaseoli* were obtained from leaves and pods with symptoms, using the techniques of Phillips (1994b), and stored on potato dextrose agar (Biolab (Pty) Ltd, Midrand, Johannesburg, South Africa) (PDA) slants at room temperature. Subcultures were deposited in the National Collection of Fungi, Pretoria (PPRI). Accession numbers and origins of the isolates used are given in Table 1.

Pathogenicity

Seeds of the various legume hosts were sown in a soil/compost mixture in 10 cm diameter clay pots with one seed per pot. Plants were grown and inoculated in a greenhouse without supplementary lighting and where the air temperature ranged from 18°C at night to 26°C during the day. Relative humidity was not controlled but typically ranged from 40 to 70%.

Inoculum was prepared by the method of Phillips (1994b). Both primary leaves on each plant were inoculated within 24 h of unfolding. A spore suspension (20 μL of 10^4 conidia per ml) was spread over the surface of each leaf with a sterile glass rod. The plants were enclosed in aluminium foil packets secured around the pot with a rubber band. The packets were removed 18 h later. The plants were spaced out on the greenhouse bench in such a way that the leaves could not touch one another. Whenever necessary, water was applied to the surface of the soil, taking care that no water was applied to the leaves. Controls consisted of plants treated similarly, except that water was applied in place of the spore suspension. Because of the restricted amount of space available, the various hosts were inoculated on separate dates. Each experiment was arranged as a completely randomized design.

Disease was assessed 10 days after inoculation by counting the number of lesions on each plant. Lesion counts were log transformed before applying analysis of variance. In addition, disease was scored on a scale of 0–3 where 0 = no visible symptoms; 1 = very small pinprick type lesions; 2 = small dark brown lesions with no chlorosis; and 3 = pale brown/buff lesions surrounded by a chlorotic halo and with some distortion of the lamina.

DNA extraction

Total DNA of single conidial isolates was extracted from 1-month-old colonies grown on PDA at 25°C in the dark. Fungal colonies were scraped clean of agar, frozen in liquid nitrogen and ground to a fine powder. DNA was subsequently extracted by the method of Dellaporta *et al.* (1983), and quantified by ethidium bromide fluorescence on a UV transilluminator with known quantities of lambda DNA (Sambrook *et al.*, 1989).

Reaction conditions for RAPD-PCR

Reactions were carried out in 25 μL volumes in thin-walled Eppendorf tubes in an Idaho Technology Air

Table 2 Numbers of lesions per plant (log transformed data in parentheses) on four species of legume inoculated with 15 isolates of *Elsinoë* from legumes

						P. vulgaris					
						cv. Bonus		cv. Enseleni			
Isolate	Source host	*P. coccineus*		*P. lunatus*						*V. unguiculata*	
SP5	*P. coccineus*	34·3	(3·51)	19·0	(2·86)	5·5	(1·81)	66·0	(4·13)	2·9	(1·25)
SP6	*P. coccineus*	40·6	(3·67)	14·6	(2·63)	2·8	(1·25)	53·8	(3·94)	3·8	(1·45)
SP17	*P. coccineus*	37·6	(3·60)	14·6	(2·74)	3·4	(1·36)	37·4	(3·57)	0·9	(0·52)
SP22	*P. coccineus*	45·8	(3·80)	10·6	(2·29)	9·5	(2·25)	43·9	(3·72)	0·8	(0·43)
SP7	*P. vulgaris*	14·4	(2·57)	11·4	(2·43)	5·4	(1·72)	92·4	(4·44)	6·1	(1·79)
SP8	*P. vulgaris*	29·1	(3·34)	17·0	(2·82)	3·4	(1·46)	61·3	(4·03)	0·9	(0·48)
SP9	*P. vulgaris*	9·1	(2·01)	2·1	(1·00)	5·9	(1·80)	62·0	(4·09)	0·3	(0·17)
SP10	*P. vulgaris*	7·6	(1·94)	31·8	(3·41)	1·8	(0·80)	60·8	(4·08)	5·4	(1·54)
SP13	*P. vulgaris*	9·5	(2·26)	20·1	(3·03)	40·5	(3·63)	49·9	(3·88)	5·4	(1·77)
SP14	*P. vulgaris*	38·5	(3·63)	35·9	(3·55)	34·0	(3·47)	58·6	(4·04)	0·6	(0·40)
SP15	*P. vulgaris*	43·8	(3·75)	37·3	(3·50)	47·1	(3·81)	46·8	(3·84)	1·0	(0·57)
SP18	*P. vulgaris*	12·8	(2·39)	25·5	(3·17)	2·3	(1·09)	44·0	(3·78)	3·8	(1·49)
SP19	*P. vulgaris*	11·0	(2·33)	32·8	(3·40)	16·1	(2·66)	54·0	(3·99)	0·4	(0·22)
SP26	*P. lunatus*	0·0	–[a]	0·0	–	0·0	–	0·0	–	0·0	–
SP11	*V. unguiculata*	0·8	(0·45)	2·3	(0·98)	0·4	(0·22)	0·5	(0·31)	34·6	(3·40)
SED (98 d.f.)			(0·273)		(0·249)		(0·254)		(0·189)		(0·270)

[a]Isolate SP26 did not cause any symptoms and these data were omitted from the analysis of variance.

Thermo Cycler, model 1605 (Idaho Technology, Idaho Falls, Idaho, USA). Reaction mixtures contained 1–5 ng template DNA, 100 μM each of dATP, dCTP, dGTP and dTTP, 2·5 μL 10× ammonium sulphate buffer (670 mM Tris-HCl, pH 8·8, 40 mM $MgCl_2$, 160 mM $(NH_4)_2SO_4$, 0·01% Tween 20), 0·2 μM oligonucleotide primers, and 0·5 units of Taq DNA polymerase (Boehringer Mannheim Ltd, Mannheim, Germany). Mixtures were subjected to 40 cycles of denaturation at 94°C for 15 s, annealing at 38°C for 30 s, and elongation at 72°C for 35 s. These 40 cycles were followed by a final extension step of 4 min at 72°C. Eight 10-mer primers from Kit OPM of Operon Technologies (Alameda, California, USA): OPM-2, OPM-3, OPM-4, OPM-5, OPM-6, OPM-7, OPM-13 and OPM-14 were used. Products generated by PCR amplification were separated according to size in 1·5% agarose gels, stained with ethidium bromide and visualized under an UV transilluminator (312 nm). All analyses included a negative control.

Statistical analysis of RAPD patterns

The PCR bands that appeared consistently with each primer were numbered and their presence in each lane recorded. All RAPD amplifications were repeated twice, and both faint and intense bands shown to be reproducible in separate runs were scored.

The presence or absence of bands was compared using a computer software program NTSYS-pc (Rohlf, 1990) to generate a data matrix. This program analyses data by average linkage cluster analysis using shared fragments and simple matching similarity coefficients. The results obtained with all eight RAPD primers were combined in a single analysis. Each band in each pattern was given a number to distinguish it from all other bands in all eight patterns, and all the bands were included in the analysis. An Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sneath & Sokal, 1973) analysis was performed, based on the matching coefficient using the SAHN program of NTSYS. A dendrogram showing the relationship among the RAPD patterns was generated from these coefficients using the TREE program of NTSYS.

Results

Pathogenicity

Analysis of variance of the numbers of lesions per plant (Table 2) indicated significant differences between isolates. The isolate from cowpea (SP11) caused significantly fewer lesions on *P. coccineus*, *P. lunatus*, and *P. vulgaris* than any of the other isolates. Moreover, the number of lesions caused by the same isolate on *V. unguiculata* was significantly greater than that caused by any of the other isolates.

Disease severity on the basis of lesion types yielded further information on the variation in virulence amongst the isolates. In each combination of host and cultivar, lesion types were consistent on all plants in each replicate. Phillips (1996) showed that lesions in class 1 failed to produce conidia and those in class 2 produced significantly fewer conidia than those in class 3. Furthermore, class 1 and 2 lesions were atypical and damage to the host tissues was far less extensive than in class 3 lesions. Consequently, lesions in classes 0, 1 and 2 were scored as incompatible and assigned a '–'; lesions in class 3 were scored as compatible and assigned a '+'. These scores are presented in Table 3. All the isolates from *P. coccineus* were virulent on *P. coccineus*

Table 3 Pathogenic interactions between 15 isolates of *Elsinoë* and four legume hosts. The data represent compatible (+) or incompatible (−) interactions

Isolate	Source host	*P. coccineus*	*P. lunatus*	*P.vulgaris* cv. Bonus	*P.vulgaris* cv. Enseleni	*V. unguiculata*
SP5	*P. coccineus*	+	−	−	+	−
SP6	*P. coccineus*	+	−	−	+	−
SP17	*P. coccineus*	+	−	−	+	−
SP22	*P. coccineus*	+	−	−	+	−
SP7	*P. vulgaris*	−	−	−	+	−
SP8	*P. vulgaris*	+	−	−	+	−
SP9	*P. vulgaris*	−	−	−	+	−
SP10	*P. vulgaris*	−	+	−	+	−
SP13	*P. vulgaris*	−	−	+	+	−
SP14	*P. vulgaris*	+	+	+	+	−
SP15	*P. vulgaris*	+	+	+	+	−
SP18	*P. vulgaris*	−	+	−	+	−
SP19	*P. vulgaris*	−	+	+	+	−
SP26	*P. lunatus*	−	−	−	−	−
SP11	*V. unguiculata*	−	−	−	−	+

and *P. vulgaris* cv. Enseleni but nonvirulent on *P. lunatus*, *V. unguiculata* and *P. vulgaris* cv. Bonus. The isolate from *V. unguiculata* was virulent only on this host. The isolates from *P. vulgaris* displayed a range of virulence patterns on the various hosts tested, but none of them showed the same pattern as the isolates from *P. coccineus*. None of the *P. vulgaris* or *P. coccineus* isolates was virulent on *V. unguiculata*.

RAPD-PCR analysis

Twenty different random 10-mer primers from Operon Technologies Kit OPM were tested, and eight that produced reproducible RAPD patterns were used for a comparative analysis. The characteristic RAPD products generated by the eight randomly selected primers are summarized in Table 4. A total of 114 amplification products was generated. The number of amplification products (4–22), and their size (100–3500 bp) differed for each primer. Of 78 polymorphic products generated among the isolates from leguminous hosts, five products were monomorphic among *P. coccineus*, *P. lunatus* and *P. vulgaris* isolates. RAPD patterns obtained with primers OPM-2, OPM-5 and OPM-13 are shown in Fig. 1. A similarity matrix was generated from the RAPD markers (Table 4) using UPGMA. A dendrogram (Fig. 2) indicating the relationships among the *Elsinoë* isolates studied was constructed from a cluster analysis of the similarity matrix.

Isolates obtained from legumes grouped in three clusters. The first cluster represented isolates from *Phaseolus vulgaris* and *P. lunatus*, and the second isolates from *Phaseolus coccineus*. The third cluster represented the single isolate from *Vigna* and the three *Elsinoë* species used as outgroups. This third cluster can be more correctly regarded as four outgroups of very different species. The most polymorphic isolates were from *P. vulgaris*. In every other case primers always distinguished isolates from *Vigna*, *Protea* and *Leucospermum* from one another and from the *Phaseolus* isolates. Although there was some intraspecific variation with most primers, especially among the isolates from different *Phaseolus* species (Fig. 1), the single isolates from *Citrus*, *Vigna*, *Protea* and *Leucospermum* showed little similarity with those from *Phaseolus* which were more similar to one another.

Table 4 RAPD products generated by the eight primers used in this study

Primer	Nucleotide sequence (5′ to 3′)	Number of amplified products	Number of polymorphic products
OPM-2	ACAACGCCTC	15	9
OPM-3	GGGGGATGAG	14	11
OPM-4	GGCGGTTGTC	11	8
OPM-5	GGGAACGTGT	18	13
OPM-6	CTGGGCAACT	13	7
OPM-7	CCGTGACTCA	4	3
OPM-13	GGTGGTCAAG	17	12
OPM-14	AGGGTCGTTC	22	15

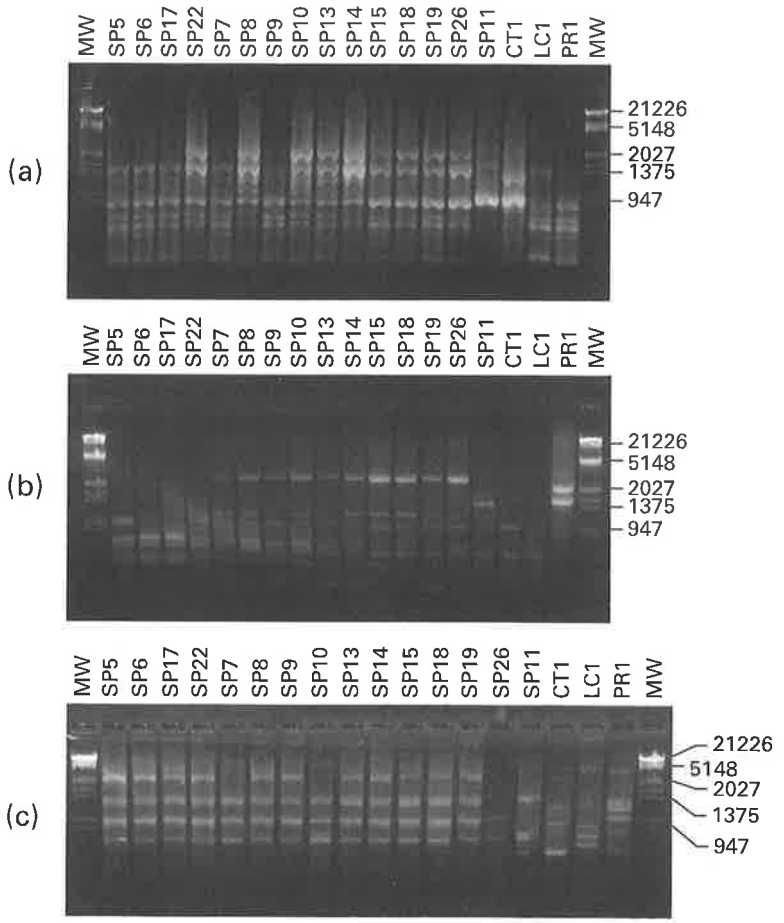

Figure 1 RAPD banding patterns of *Elsinoë* spp. generated using the primers (a) OPM-2, (b) OPM-5 and (c) OPM-13. Lanes 1 and 20, lambda marker digested with *Eco*RI and *Hin*dIII. Lanes 2–15 are *E. phaseoli*: 2, SP5; 3, SP6; 4, SP17; 5, SP22; 6, SP7; 7, SP8; 8, SP9; 9, SP10; 10, SP13; 11, SP14 12, SP15; 13, SP18; 14, SP19; 15, SP26. Lanes 16–19 are *Elsinoë* outgroups: 16, SP11 from *Vigna*; 17, CT1 from *Citrus*; 18, LC1 from *Leucospermum*; 19, PR1 from *Protea*. Additional information on isolates is given in Table 1.

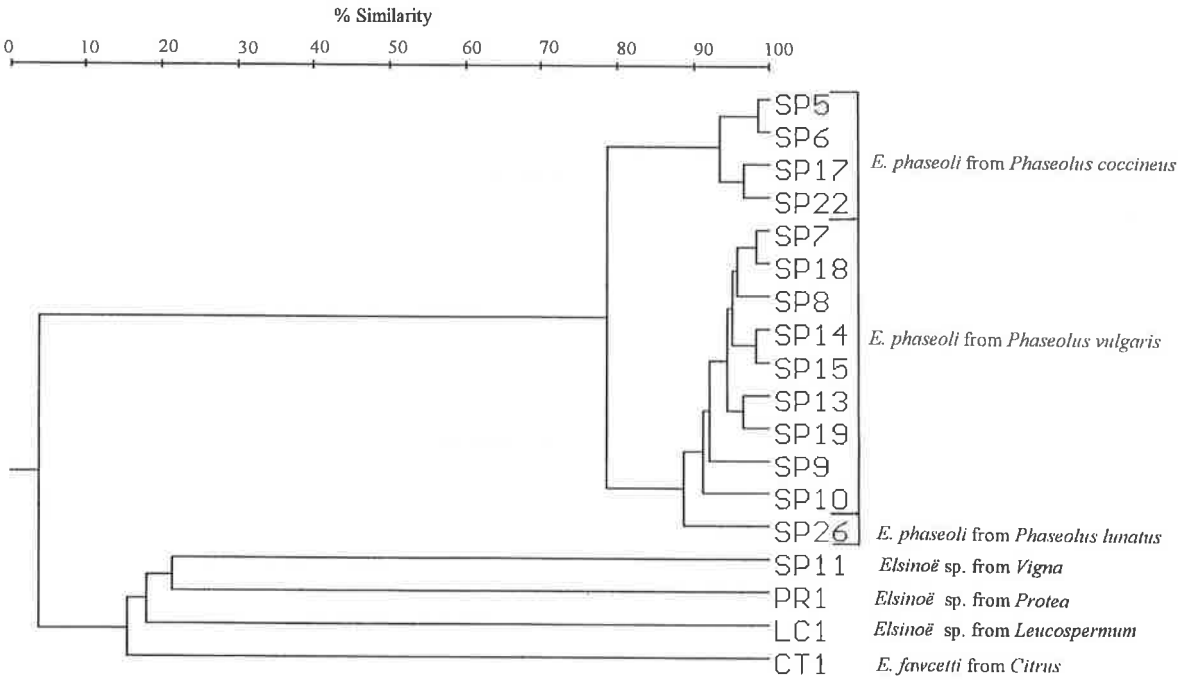

Figure 2 UPGMA dendrogram of RAPD-PCR similarities between *Elsinoë* spp. Isolates are labelled with accession numbers shown in Table 1.

Discussion

RAPD profiles were previously used to identify pathotypes and to detect genetic variation in *Pseudocercosporella herpotrichoides* (Nicholson & Rezanoor 1994; Campbell *et al.*, 1996) as well as to demonstrate that isolates of *Colletotrichum graminicola* occurring on maize and sorghum are genetically distinct (Vaillancourt & Hanau, 1992). They were used in the identification of mating populations in *Fusarium* section *Liseola*, and have also indicated variation among fungal isolates depending on their geographic origin (Guthrie *et al.*, 1992; Amoah *et al.*, 1996; Victor *et al.*, 1996). In spite of this, the technique has only recently been employed to assist in distinguishing between species and pathotypes of the genus *Elsinoë* (Tan *et al.*, 1996).

In the present study, using RAPDs to characterize *Elsinoë* isolates obtained from *Phaseolus* spp., two major subgroups were observed, one composed of isolates from *P. vulgaris* and the other from *P. coccineus*. This information reinforces the pathogenicity data, and suggests that the isolates from *P. coccineus* and *P. vulgaris* could be different pathotypes of *E. phaseoli*. On the other hand there is some evidence of geographical grouping in the dendrogram. Bruner & Jenkins (1933) reported a high degree of host specificity in *E. phaseoli* isolates from *P. lunatus*. In a more recent study, Phillips (1996) found that *P. coccineus* was resistant to most of the *P. vulgaris* isolates, but that *P. vulgaris* was susceptible to all the *P. coccineus* isolates. Bruner & Jenkins (1933) considered that *P. vulgaris* is not a host for *E. phaseoli*. For this reason, Staples (1958) referred to the *Elsinoë* on *Phaseolus* beans in Zimbabwe as *E. phaseoli* f.sp. *vulgare*. Phillips (1996) also demonstrated that isolates of *E. phaseoli* could be separated into four pathotype groups on the basis of their virulence on different bean genotypes. It is possible that differential reactions occur on different genotypes of other legume species and that this may be the reason for the differences in host range reported by various researchers (e.g. Bruner & Jenkins, 1933; Staples, 1958; Phillips, 1996). However, the molecular data presented here, together with the host range data, suggest that *E. phaseoli* may be composed of a number of *formae speciales*.

The isolate from *Vigna unguiculata* was as far removed from the two groups of isolates from *Phaseolus* as those from *Citrus*, *Leucospermum* and *Protea*. Several researchers have reported that isolates from *Vigna unguiculata* do not cause disease on *P. vulgaris*, and that *V. unguiculata* is resistant to isolates from *Phaseolus* spp. (e.g. Van Hoof, 1963; Emechebe, 1980; Phillips, 1996). This was confirmed in the present study. On the basis of the pathogenicity experiments reported here and elsewhere, it is not clear whether these observations are a result of host specificity, or whether cowpea isolates represent a different *Elsinoë* species. Host specificity is well known in other species of *Elsinoë*. Thus, Whiteside (1978) found that out of 24 isolates of *Elsinoë fawcettii* pathogenic on rough lemon and grapefruit, only 13 caused scab on sour orange and sweet orange. Allen *et al.* (1981) reported on the marked differences in susceptibility of cowpea isolates to scab. However, the RAPD data generated in the present study clearly show a high level of genetic distance between the *Vigna* isolate and those from *Phaseolus* spp. These results, together with pathogenicity and host range data, suggest that the isolate from *Vigna unguiculata* may be a different species from the one on *Phaseolus* spp. As the *Elsinoë* state is seldom collected for these fungi, further molecular studies are required to determine the amount of acceptable genetic variation within isolates from *Phaseolus*, and whether those from *Vigna* remain distinct as shown in the present study. As only one isolate from cowpea was available, confirmation of this aspect will have to wait until more isolates can be examined. However, there appears to be a high degree of specialization between isolates from *P. vulgaris* and *P. coccineus*. It seems likely that as more isolates from legumes are studied, additional pathotypes and species will be more clearly defined in this complex.

Most African countries regulate importation of crops from elsewhere to avoid the introduction of exotic pathogens. The ability to distinguish scab isolates by means of molecular analysis will greatly facilitate the regulation of movement of infected material into areas free from these species or pathotypes. It will also assist in the discovery of new misidentified species. The RAPD technique is sensitive, reliable and much quicker than host range differentiation studies, thereby enabling more rapid decisions regarding the movement of produce. Moreover, without a complete knowledge of the genetic variation and distribution of the *Elsinoë* spp. involved in scab disease of legumes in Africa, development of a disease management or breeding programme would not be possible.

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