

Endophytic fungi associated with shoots and leaves of *Vitis vinifera*, with specific reference to the *Phomopsis viticola* complex

Lizél Mostert¹, Pedro W. Crous¹ & Orlando Petrini²

¹Department of Plant Pathology, University of Stellenbosch, P. Bag X1, Matieland 7602, South Africa

²Tera d'Sott 5, CH-6949 Comano, Switzerland

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Phomopsis cane and leaf spot caused by *Phomopsis viticola* is an economically important disease in many of the vine-growing areas of the world. The aims of this study were to investigate whether *P. viticola* grows endophytically in various vine tissues, and to monitor its distribution over the growing season. Asymptomatic shoots were collected at eight different growth stages. Nodes, internodes, leaf petioles, leaves, tendrils and bunch rachides were surface sterilised, cut into smaller pieces and plated out on potato dextrose agar. After the fungal endophytes were identified, the relative importance (RI) values were calculated and a correspondence analysis performed on the data of taxa with RI larger than 5%. Of the 46 different fungal taxa found, 20 were present at relative importance values of more than 1%. An analysis of the relative importance of the different species showed that the most frequently isolated fungi were members of the *Alternaria alternata* complex (40%) and *Sphaeropsis* sp. (27%). The *Phomopsis viticola* complex had a relative importance of 9% and accounted for 3% of the isolations. *P. viticola* was mainly isolated from the nodes and internodes, the plant parts in which *P. viticola* usually causes disease symptoms. Two different *Phomopsis* spp. were isolated, with 94% of the isolates representing *P. viticola* taxon 2, and the rest taxon 1. Isolations made from diseased vine material during the last two years revealed taxon 2 to be dominant in vineyards in the Western Cape. Inoculations into healthy, young vine tissue also showed taxon 2 to be a primary pathogen, further suggesting that it is probably a latent pathogen rather than an endophyte. In contrast, taxon 1 appears to be a true endophyte, and does not seem to be an important pathogen on vines.

Keywords: *Phomopsis viticola*, *Vitis vinifera*, fungal endophytes.

Phomopsis viticola (Sacc.) Sacc. is a well-known pathogen of *Vitis vinifera* (Reddick, 1909; Pearson & Goheen, 1994), causing an economically important disease, Phomopsis cane and leaf spot, in many of the vine-growing areas of the world. Disease symptoms can be seen on the leaves as dark brown spots with yellow halos, followed by black lens-shaped lesions on the lower nodes of the green shoots during spring (Chairman & al., 1982). Yield loss can occur due

six leaves unfolded – stage 12 (29.09.97), beginning of flowering – stage 19 (29.10.97), berries pea-sized – stage 31 (25.11.97), beginning of berry ripening – stage 35 (19.01.98), berries ripe for harvest – stage 38 (11.02.98) and after harvest – stage 41 (26.02.98). The vineyard was also monitored for *Phomopsis* cane and leaf spot symptoms from Aug. 1997 to Feb. 1998.

The following plant tissues were investigated (Fig. 1): nodes (N), internodes (I), leaf petiole (Lp), leaf (L), tendril (T) and bunch peduncle (Bp). Berry rot due to *P. viticola* has never been observed in South Africa, and berries were therefore excluded from this study. Plant tissues were sampled from six sections across the length of the shoot. The first two sections were from the previous year's spur and the third to sixth sections from the new shoot.

Two samples (5 mm long) were respectively sampled over the length of the internodes, leaf petiole as well as tendril. The nodes were cut into four sections to distinguish between the ends and the sides of the sample. Five pieces ($5 \times 5 \text{ mm}^2$) were sampled from the leaves: at the leaf base and apex, on the main vein, side vein, and between a side vein and the leaf margin (Fig. 1).

After preliminary trials, tissue pieces were sterilised using the following protocol: 30 sec in 70% ethanol, 2 min in NaOCl (1%) and 15 sec in 70% ethanol. Tissue samples were plated on potato dextrose agar (PDA, Biolab) amended with Streptomycin (1 ml/L) and incubated at 25 °C. Two pieces from the internode, leaf petiole, tendril and rachis were respectively plated on each Petri dish. Each node (four pieces) and leaf (five pieces) were plated on separate dishes. Fungal growth from plated tissue pieces was monitored daily. Emerging fungal colonies were hyphal-tipped and transferred to PDA slants for further identification.

Data analyses

The relative frequencies were calculated for each species from every plant part and time interval. The relative importance values (RI) of endophyte species isolated were computed (Ludwig & Reynolds, 1988). After standardisation of the RI values within each sample by assigning the most frequent species the value of 100%, the other RI values were computed as percentages of it. To assess the distribution of the fungi in the plant and throughout the season, a correspondence analysis (CA) was used to visualize the patterns of correlation between fungal species, tissues, and time. Simple CA was performed on a reduced matrix that contained all fungal species with standardised RI values (over all samples) of at least 5%, using the package SimCA 2.1 (Greenacre, 1992).

to stunting of vines, loss of vigor, reduced bunch set (Pscheidt & Pearson, 1989) and in severe cases, death and breaking off of shoots (Berrysmith, 1962). It has been reported that *P. viticola* overwinters as mycelium within the woody parts on infected canes, spurs, pruned shoots and dormant buds (Nicholas & al., 1994; Pearson & Goheen, 1994). Other than mycelium, inoculum can also be carried over to the next season through pycnidia in the cortex of diseased, one-year-old vine canes (Pearson & Goheen, 1994). In these cases the symptoms of the previous season's disease, whitening of the spurs or the presence of pycnidial fruiting bodies would be visible indicators of inoculum that could cause disease under prolonged periods of rain in spring. *P. viticola*, however, has been isolated from healthy grape leaves after intensive surface sterilisation, suggesting that it is an endophyte of *V. vinifera* (Cardinali & al., 1994). Presently no information is available pertaining to its endophytic distribution within vines, and whether it can be a source of inoculum in healthy looking vines. Furthermore, in Australia up to four different *Phomopsis* taxa have been associated with *Phomopsis* cane and leaf spot disease (Merrin & al., 1995), some of which could possibly be endophytes.

Several well-known pathogens have frequently been reported from endophyte studies (Sieber & al., 1990; Johnson & al., 1992; Smith & al., 1996). These observations can be understood in the light of Petrini's (1991) and Wilson's (1995) definition that a fungus can be endophytic as well as pathogenic, depending on the phase of its life cycle.

The aims of this study were, therefore, to determine which *Phomopsis* taxa occurred endophytically in apparently healthy South African vines, to determine their distribution within the tissue, and thus establish potential sources of inoculum for new vine growth.

Materials and methods

Plant samples

Ten plants without any *Phomopsis* cane and leaf spot symptoms were collected for this study in a vineyard at Nietvoorbij, Stellenbosch, planted with the cultivar Riesling. The disease was present within the vineyard, however, and was noted on specific vines in the spring of 1997–1999. For the duration of this study the vineyard was treated for powdery mildew with penconazole (Topaz[®]) according to the recommendations of the manufacturer. Canes were sampled at eight different stages of shoot development coded according to the Eichhorn-Lorenz system (Pearson & Goheen, 1994): winter dormancy – stage 01 (08.09.97), bud burst – stage 05 (18.09.97), five to

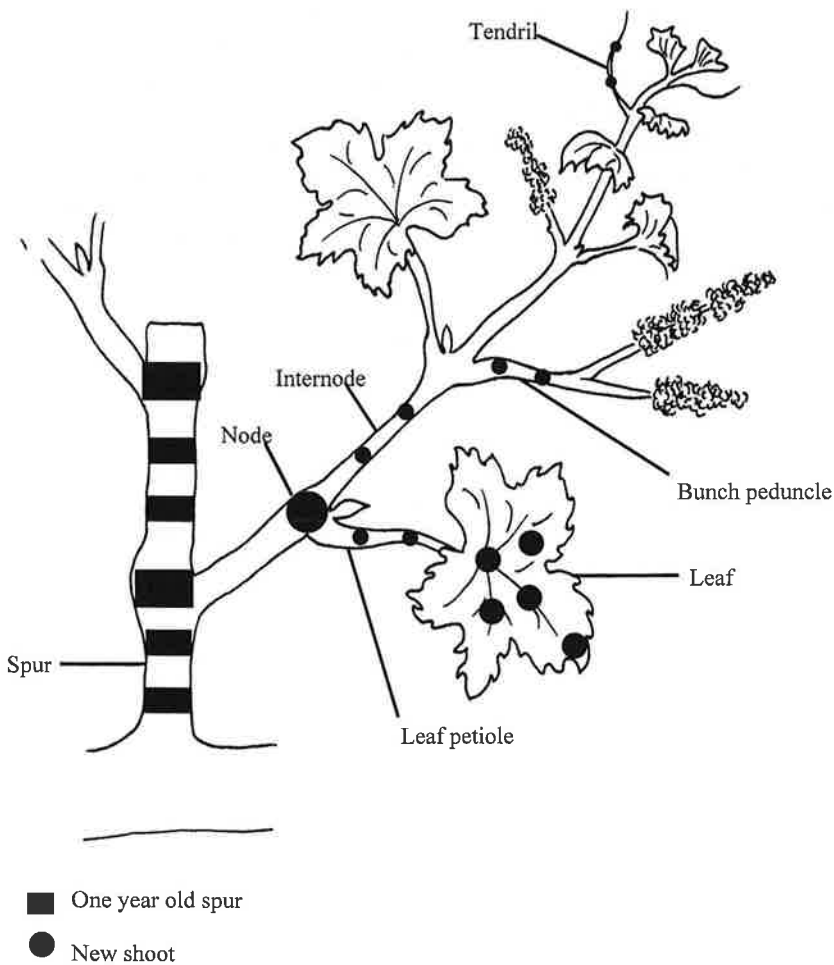


Fig. 1. – Diagrammatic illustration of the plant tissues analysed.

Pathogenicity

To distinguish between the 'true' endophyte and pathogenic *Phomopsis* spp. isolated in this present study, their disease reactions were also determined (Clay, 1991; Freeman & Rodriguez, 1993) by means of pathogenicity tests (Webber & Gibbs, 1984). The pathogenicity of eight isolates, representing the two distinct morphological groups, was tested on green shoots of the cultivar Riesling. Shoots were wounded using a 3 mm diam. cork borer. Colonised mycelium plugs from two-week-old cultures were used as inoculum and the wounds sealed with Parafilm. Uncolonised agar plugs were

used to inoculate control shoots. Each isolate including the control were replicated three times on separate plants. Plants were maintained in the laboratory at 25 °C under a 12 hr day/night light regime, and watered daily. After seven days the lesion length, width and shoot diameter were determined. A one-way analysis of variance (ANOVA) was carried out on the data to determine whether a difference in lesion size correlated with the two morphological groups identified in this study. The fungal isolates were re-isolated from the periphery of each respective lesion to prove Koch's postulates.

Results

Endophyte study

Two different morphological types of *P. viticola* were isolated, correlating with the Australian system, which distinguishes four taxa in the *P. viticola* complex (Merrin & al., 1995). Of all the *P. viticola* isolates obtained, 94% were morphologically similar to the Australian taxon 2, while 6% correlated with taxon 1.

The *P. viticola* complex occurred endophytically in vines. The low frequency of isolation suggests, however, that it was not a dominant endophyte. Out of 1705 isolates obtained, only 3% belonged to the *Phomopsis* species complex. When only the nodes (plant parts in which *P. viticola* usually causes disease) were taken into consideration, the relative frequency of isolation was 3.2% and the RI 10%. An analysis of the relative importance of the different species isolated showed that *P. viticola* taxon 2 accounted for a RI of 8%.

The most important endophytes isolated from vine are listed in descending order according to their RI values in Tab. 1. All the fungi isolated with their respective raw frequencies for time and plant tissue can be seen in Tab. 2.1 and 2.2. In general, *Alternaria* Nees

Tab. 1. – Relative importance (RI) of the most important endophyte species isolated from grapevines.

Taxon	RI (%) (overall)
<i>Alternaria alternata</i> complex	100
<i>Sphaeropsis</i> sp.	40
<i>Epicoccum nigrum</i>	27
Sterile black mycelium	26
<i>Pleospora herbarum</i>	18
<i>Alternaria tenuissima</i> complex	17
Sterile brown mycelium	17
<i>Phomopsis viticola</i> taxon 2	8

Tab. 2.1. - Fungi most frequently isolated from vines from dormancy (08.09.97) until flowering (29.10.97). The raw frequencies are given. A space represents no fungus isolated. Only those fungi with a relative importance of more than 1% have been considered^a.

	Stage 01 (08.09.97)			Stage 05 (18.09.97)			Stage 12 (29.09.97)			Stage 19 (29.10.97)			
	N ^b	I	Lp T Bp	N	I	Lp T Bp	N	I	Lp T Bp	N	I	Lp T Bp	
<i>Alternaria alternata</i> complex	46	13		14	13		19	2	2	17	4	13	1
<i>Alternaria tenuissima</i> (Kunze ex Pers.) Wiltshire complex	4	1					4	1		2	4	5	
<i>Alternaria infectoria</i> E. G. Simmons complex	2												
<i>Alternaria</i> sp.				1			1	1					
<i>Chaetomium</i> Kunze sp.	1						1	1	2	9	1	2	1
<i>Cladosporium cladosporioides</i> (Fresen.) G. A. de Vries													
<i>Epicoccum nigrum</i>	13			9	1		15	6		3	1		
<i>Gliocladium roseum</i> Bainier	1			2	1		2	3	1	3			
<i>Nigrospora oryzae</i>	1						1			1	1	1	1
<i>Phoma</i> Sacc. sp.						1							2
<i>Phomopsis viticola</i> taxon 2	7	2		3			4	3		6	2		3
<i>Pleospora herbarum</i>										2			
<i>Sphaeropsis</i> sp.				22	5		14	5		26	5		
<i>Sporormiella minimoides</i> (Cain) S. I. Ahmad & Cain													3
Sterile black mycelium	45	8		31	11		12	6		2			
Sterile brown mycelium	14	6		7			14	1		8	3		
Sterile pink mycelium										1		2	
Sterile white mycelium	1						1	1		1			
Sterile yellow mycelium							1	1		1			
<i>Verticillium</i> Nees sp.	3			2			4						

^a Isolates with standardised relative importance less than 1% include: *Acremonium* sp., *Ascochyta* sp., *Ascotricha* sp.1, *Ascotricha* sp.2, *Bipolaris cynodontis* (Marignoni) Shoemaker, *Coniochaeta* (Sacc.) Cooke sp., *Coniothyrium* Corda sp., *Curvularia clavata* B. L. Jain, *Drechslera* S. Ito sp., *Fusarium acuminatum* Ellis, *Fusarium chlamydosporum* Wollenw. & Reinking, *Fusarium oxysporum* Schltdl. emend. Snyder & H. N. Hansen, *Fusarium poae* (Peck) Wollenw., *Fusarium Link* sp., *Fusicoccum parvum* Pennycook & Samuels, *Gelasiospora* Dowding sp., *Geniculosporium* Chesters & Greenh. anamorph of *Hypoxylon serpens* (Pers.: Fr.) J. Kickx f., *Microsphaeropsis olivacea* (Bonord.) Höhn., *Nigrospora sphaerica* (Sacc.) E. W. Mason, *Phomopsis viticola* taxon 1, *Seimatosporium* Corda sp., *Sordaria lappae* Potebnia, *Trichoderma* Pers. sp., *Ulocladium botrytis* Preuss, unidentified coelomycete and *Veronaea* Cif. & Montemart sp..

^b Codes for the plant tissues: N = nodes, I = internodes, L = leaf, Lp = leaf petiole, T = tendril and Bp = bunch peduncle.

Tab. 2.2. – Fungi most frequently isolated from vines from pea-size stage (25.11.97) until after harvest (26.02.98). The raw frequencies are given. A space represents no fungus isolated. Only those fungi with a relative importance of more than 1% have been considered^a.

	Stage 31 (25.11.97)			Stage 35 (19.01.98)			Stage 38 (11.02.98)			Stage 41 (26.02.98)		
	Nb	I	Lp T Bp	N	I	Lp T Bp	N	I	Lp T Bp	N	I	Lp T Bp
<i>Alternaria alternata</i> complex	46	6	37 2 3 1	50	9	38 5 2 4	6	4	28 3 1 2	6	6	50 4 2 7
<i>Alternaria tenuissima</i> complex	6	2	5 1	10	14	2 1 1 1	5	2	8	1	4	2
<i>Alternaria infectoria</i> complex		1		1	1	1	2	1	2		1	1
<i>Alternaria</i> sp.	2	1		1			2	4			1	1
<i>Chaetomium</i> sp.	2	2	4 1	1								
<i>Cladosporium cladosporioides</i>	1	1							1		2	1 1
<i>Epicoccum nigrum</i>	33	1	3	23	1	5 2 1	1	2	2	2	1	1
<i>Gliocladium roseum</i>							4				1	
<i>Nigrospora oryzae</i>	2	9	1	4	1	8				1		
<i>Phoma</i> sp.				1		1		1		1		1
<i>Phomopsis viticola</i> taxon 2	2	3	1	4	1		6	4				1
<i>Pleospora herbarum</i>	6	4	11 2	9	4	12 2 1 1	3	1	10 1 2 2	1	5	11 1 2
<i>Sphaeropsis</i> sp.	16	1	1	51	25	1	2	13		1	7	2
<i>Sporormiella minimoides</i>		1				1	5			6		
Sterile black mycelium	8	2	1	5	1		3	2	2	2	1	6
Sterile brown mycelium	9	5	8 1	3	2	6 1	4	2	2	7	7	
Sterile pink mycelium						1	2	1	2	1	4	1
Sterile white mycelium	1	1	1	1		1			1			
Sterile yellow mycelium		1	1	1	2						1	1
<i>Verticillium</i> sp.		1	1	3								

^a Isolates with standardised relative importance less than 1% include: *Acremonium* sp., *Ascochyta* sp., *Ascotricha* sp.#1, *Ascotricha* sp.#2, *Bipolaris cynodontis*, *Coniochaeta* sp., *Coniothyrium* sp., *Curvularia clavata*, *Drechslera* sp., *Fusarium acuminatum*, *Fusarium chlamydosporum*, *Fusarium oxysporum*, *Fusarium poae*, *Fusarium* sp., *Fusicoccum parvum*, *Gelasinospora* sp., *Gelatinospora* sp., *Geniculosporium* anamorph of *Hypoxylon serpens*, *Microsphaeropsis olivacea*, *Nigrospora sphaerica*, *Phomopsis viticola* taxon 1, *Seimatosporium* sp., *Sordaria lappae*, *Trichoderma* sp., *Ulocladium botrytis*, unidentified coelomycete and *Veronaea* sp.

^b Codes for the plant tissues: N = nodes, I = internodes, L = leaf, Lp = leaf petiole, T = tendril and Bp = bunch peduncle.

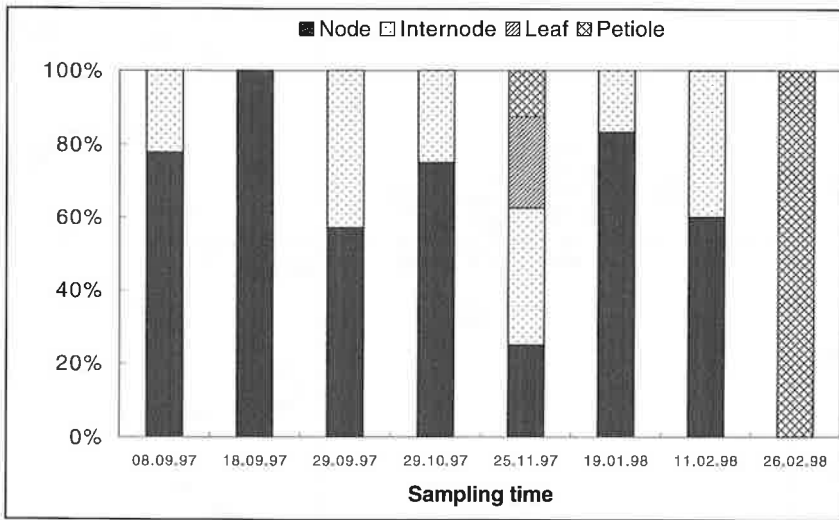


Fig. 2. – Isolation of *Phomopsis viticola* taxa 1 and 2 from the different plant parts.

spp. and a *Sphaeropsis* Sacc. sp. appeared to be the dominant endophytes occurring in grapevine plants in the present study.

The distribution of the *P. viticola* complex was scattered. A statistical analysis of the frequency data using the Chi-square test was therefore not advisable as it did not yield any reliable statistics, and for this reason no confirmatory statistical analysis was performed. Inspection of Figs. 2 and 3, on the other hand, reveals that *P. viticola*

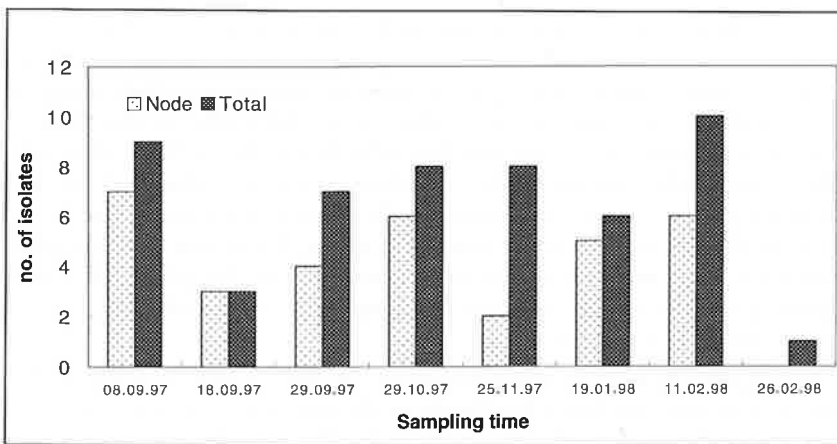


Fig. 3. – Frequency of isolation of *P. viticola* taxon 2 from the nodes as compared with the total number of isolations.

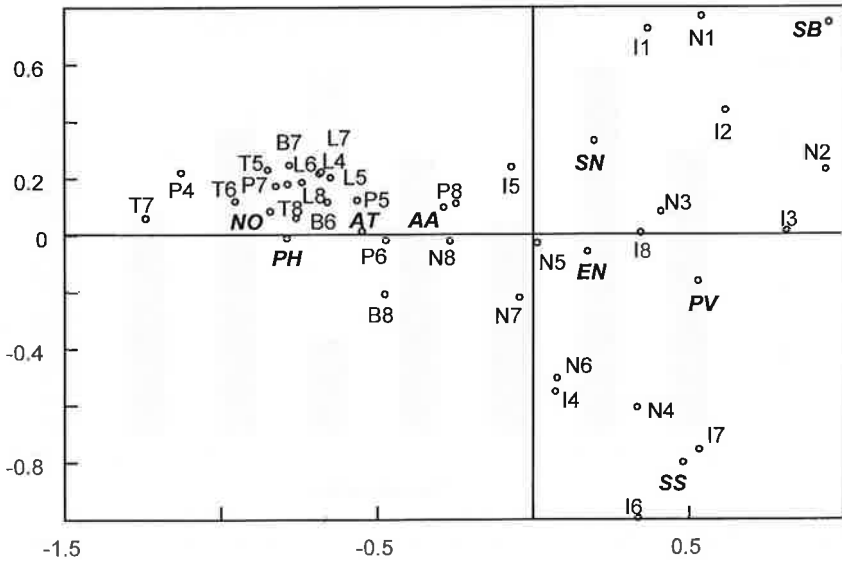


Fig. 4. – Results of the simple correspondence analysis showing the distribution of endophytes over the growing season in the host plant. It was performed on the matrix containing the most important fungi (RI $\geq 10.5\%$) isolated from vine plants at different sampling times. – The abbreviations used in the graphical display of the analysis are as follows: *Alternaria alternata* complex = AA, *Sphaeropsis* sp. = SS, *Epicoccum nigrum* = EN, Sterile black = SB, *Pleospora herbarum* = PH, *Alternaria tenuissima* complex = AT, Sterile brown mycelium = SN, *Phomopsis viticola* taxon 2 = PV and *Nigrospora khuskia-oryzae* = NO. Samples have been abbreviated as follows: N = Node; I = Internode; L = Leaf; P = Petiole; T = Tendril and B = Bunch peduncle. – The sampling times have been numbered from 1 to 8, thus, e.g.: N1 = Node, at sampling time 1.

was predominantly found in the nodes and internodes. Of the 51 *P. viticola* isolates obtained, 48 were isolated from the nodes and internodes, two from the leaf petiole, and one from a leaf. Only three of the isolates were obtained from above the fifth nodes of the shoots. Eighteen isolates were obtained from the bud sides of the nodes, and fifteen from the opposite side. No clear trend was observed for the frequency of *P. viticola* isolations with regard to tissue age (Fig. 3). This study, however, showed that 53% of the *P. viticola* isolates were obtained from the two nodes and internodes of the previous year's spurs, and 39% of the isolates from the first two nodes and internodes of the new shoots.

The distribution of endophytes in the host plant over the growing season was analysed by simple correspondence analysis (Fig. 4). The percentage of inertia explained by the first three components was approximately 72%, which indicated a good fit of the model to the data. The samples taken at an advanced stage of development

(times 5–8) were mostly colonized by the two *Alternaria* spp. and by fungi known to be saprobic inhabitants of plant parts [*Pleospora herbarum* (Pers. ex Fr.) Rabenh. and *Nigrospora oryzae* (Berk. & Broome) Petch]. On the other hand, the first axis separated most of the node and internode samples from all the others with both tissue types preferentially inhabited by *P. viticola*, *Epicoccum nigrum* and the two sterile mycelia. This confirmed the observations that *P. viticola* preferentially colonised the node and internode tissues. N7 and N8 were located close to the other samples, indicating that *Alternaria* spp. may actually have some competitive advantage during and after harvest over species such as *P. viticola*, the two sterile mycelia and *E. nigrum*. Further research, however, would be required to support this observation.

Pathogenicity tests

Pathogenicity tests were carried out on eight *Phomopsis* isolates to verify whether these isolates were true endophytes, or pathogens obtained from latent infections. The two taxa could not be distinguished on the basis of lesion length alone ($P = 0.2716$). Shoot diameter did not influence these measurements ($P = 0.1105$). Isolates of taxon 1, however, caused light brown, small lesions, while those caused by taxon 2 were generally larger and black in colour. Although the black discolouring of lesions was a prominent feature of taxon 2, obvious differences in virulence were also detected between isolates. These data correlated well with that observed in the field, where over the last two years no isolates of taxon 1 were obtained from diseased vines. In all cases the *Phomopsis* isolates could be re-isolated from lesions, while the control inoculations remained healthy.

Discussion

Phomopsis viticola was isolated endophytically from *Vitis vinifera*, but at very low frequency. Two taxa in the *P. viticola* complex, namely taxa 1 and 2, were isolated thereby confirming that more than one *Phomopsis* sp. occurs on grapevines in South Africa. Taxon 1 had a low isolation rate in this study and has yet to be associated with the disease in the field. This indicates that taxon 1 could be seen as a true endophyte. Data from isolations made from diseased vine material during the last two years showed taxon 2 to be dominant in vineyards in the Western Cape. Preliminary inoculations into healthy, young vine tissue showed taxon 2 to be a primary pathogen, and thus suggested that it was a latent pathogen rather than an endophyte. As both *Phomopsis* spp. occurred together in vines, it

should be established whether the presence of taxon 1 could make vines more resistant to infections by taxon 2, as reported by Matta (1971), where non-pathogenic strains of fungi conferred plants more resistant to pathogenic fungi.

These two taxa differ morphologically and studies are now underway to establish the genetic basis of the difference between the non-pathogenic endophytic taxon 1 and the pathogenic taxon 2. In other endophytic fungi with pathogenic and non-pathogenic isolates within the same species it was reported that a simple mutation at one genetic locus could switch endophytes to pathogens (Freeman & Rodriguez, 1993). In this study, however, isolates of taxon 1 and 2 were morphologically distinguishable, and therefore we believe them to be different species.

In general, *Alternaria* spp. and *Sphaeropsis* sp. appeared to be the most dominant endophytes occurring in grapevine plants in this study. It has already been reported that epiphytes are able to live endophytically within plant tissues (Petrini, 1986). *Alternaria alternata* (Fr.) Keissler was found to be the cause of decay of cold-stored table grapes (Swart & Holz, 1991). Furthermore, Swart & De Kock (1994) found that *Alternaria* fruit rot was caused by opportunistic forms of this fungus, which were isolated endophytically from asymptomatic bunches. Although isolations were not made from berries in the present study, the endophytic *A. alternata* found on the vine shoots and bunch peduncle might be a source of inoculum for infections of grape berries, and should be considered further. *Botryosphaeria* Ces. & De Not. spp., which have anamorphs in *Fusicoccum* Corda, *Diplodia* Fr., *Sphaeropsis* and *Lasiodiplodia* Ellis & Everh., are well-known pathogens of grapevines in South Africa (Pearson & Goheen, 1994). Whether the dominant *Sphaeropsis* sp. isolated here is a pathogen of vines has yet to be determined.

Positive correlation between the age of the plant material and the number of endophytic fungi colonizing the tissues has been reported (Petrini & Carroll, 1981; Fisher & Anson, 1986). In this study, however, the distribution of *P. viticola* over the growing season seemed to be independent from tissue age, although the total number of endophytes increased with time. No trends could be established due to the small sample size.

Phomopsis viticola was found to be tissue specific to the lower nodes and internodes, correlating with the basal three to six internodes, where the disease symptoms are usually observed (Pearson & Goheen, 1994). These results suggest, therefore, that this area had to be targeted for disease control. No clear difference was observed between the number of *P. viticola* isolates obtained from the bud side and the non-bud side of the node. This indicates that *P. viticola* not only resides asymptotically in the bud, but in the whole node.

This study showed that the majority of *P. viticola* isolates were obtained from the two nodes and internodes of the previous year's spurs. It therefore indicated that viable *P. viticola* isolates resided in apparently healthy pruned spurs, which could act as an inoculum source for new growth. To control disease inoculum in apparently healthy pruned spurs, eradicator fungicides can be applied during dormancy. This, however, is not common practice because of the toxicity of these fungicides (Chairman & al., 1982). Alternative, safer fungicides have shown potential as winter treatments of dormant canes (Castillo-Pando & al., 1997).

It is evident from this study that *Phomopsis viticola* taxon 2 could occur latently within healthy vine tissue. Further research now needs to be focused on latent infections, and the influence of contact and systemic fungicides thereon.

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