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Antifungal properties of *Agapanthus africanus* L. extracts against plant pathogens

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Abstract

Crude extracts of various *Agapanthus africanus* plant parts were screened *in vitro* against eight economically important plant pathogenic fungi. Radial mycelial growth was inhibited significantly ($P<0.05$) in five test organisms, while *Pythium ultimum*, and to a lesser extent *Fusarium oxysporum* and *Alternaria alternata*, showed a degree of tolerance. Subsequently, these crude extracts were tested *in vivo* in the greenhouse against *Mycosphaerella pinodes*, the cause of black spot or *Ascochyta* blight in peas. Fourth internode leaves were removed from 4-week-old pea plants, placed on moist filter paper in Petri dishes and inoculated with a *M. pinodes* spore suspension 30 min before and after treatment with the extracts. The control of *Ascochyta* blight by different concentrations of the crude extracts was measured in terms of lesion size over a 6-d period at 20 °C in a growth cabinet. All crude extracts significantly reduced lesion development caused by *M. pinodes* when leaves were inoculated with spores both before and after treatment with the extracts. Neither of the extracts showed any phytotoxic reaction on the leaves, even at the highest concentration applied. A combined aerial part crude extract of *A. africanus* was additionally evaluated against sorghum covered (*Sporisorium sorghi*) and loose (*Sporisorium cruentum*) kernel smuts under field conditions over two seasons. The extract and a standard fungicide, Thiram that served as a positive control, were applied as seed treatments 1 h after artificially inoculating separate sets of sorghum seed with smut spores. Inoculated, but untreated seeds served as a negative control. The extract reduced the incidence of both loose and covered kernel smuts significantly ($P<0.05$) and compared favourably with the prescribed fungicide, Thiram. Both treatments resulted in significant yield increases compared to the untreated control. From these results it was concluded that the aerial part crude extract of *A. africanus* possesses sufficient *in vivo* antifungal activity to warrant a further investigation.

Keywords: *In vitro*; *In vivo*; Antifungal activity; *Agapanthus africanus*; Crude extracts; Plant pathogens

1. Introduction

In an attempt to reduce the use of synthetic pesticides, extensive investigations into the possible exploitation of plant compounds as natural commercial products, that are safe for humans and the environment (Duke, 1993; Daayf et al., 1995), have been undertaken over the past two decades. Although extracts from various wild plants have been reported in the past to be effective against a wide range of micro-organisms *in vitro* (Blaeser and Steiner, 1999; Sato et al., 2000; Pretorius et al., 2002a), these extracts failed to inhibit pathogen growth *in vivo* (Benner, 1993) and failed to control diseases under field conditions in most cases. Moreover, many of these extracts were also reported to have phytotoxic effects in crops following foliar applications (Benner, 1993). Although *in vitro* screening of plant extracts is an important first step in identifying plants with application potential in agriculture, *in vivo* confirmation of this potential is essential in the search for plant-derived preparations with the potential to be commercialized (Gorris and Smid, 1995). As an example, promising results were obtained by Tewari and Nayak (1991) who showed that leaf extracts of *Piper betle*, *Ocimum sanctum*,

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Nyctanthes arbortristis and *Citrus limon* were not only effective *in vitro*, but equally effective *in vivo* in controlling rice pathogens, while no phytotoxic effects were observed in the host plants.

A screening programme in our laboratory over the past 6 years, where crude extracts of more than 3000 South African plants have been tested for antifungal activity, *Agapanthus africanus* (L.) Hoffm. (Family Agapanthaceae; Subfamily Liliaceae) was identified as one of the most potent. *A. africanus* is an evergreen plant indigenous to South Africa (van der Una, 1971). The species is found growing wild from the south-western Cape eastwards into Kwazulu-Natal and further North into Mozambique (van Wyk et al., 1997) and is also adapted to conditions in Europe, Australia, New Zealand and North as well as South America. *Agapanthus* roots are traditionally used by local communities in South Africa as medicine for various disorders (van der Una, 1971). According to Kaido et al. (1997), infusions or decoctions of *A. africanus* are traditionally used by Xhosa women during pregnancy to induce labour. It is also frequently used to treat constipation in pregnancy, as antenatal or post-natal treatment of the mother and for high blood pressure (Duncan et al., 1999). However, its antifungal potential against plant pathogens has not scientifically been investigated to date.

In this study, the *in vitro* antifungal activity of crude extracts from different *A. africanus* plant parts was initially investigated against eight economically important plant pathogens in terms of mycelial growth inhibition. Subsequently, the *in vivo* control of *Mycosphaerella pinodes* (Berk & Blox) Vesterger (Ascomycetes) by these crude extracts was followed under greenhouse conditions using detached pea (*Pisum sativum* L.) leaves while a possible phytotoxic effect was quantified.

M. pinodes is a major constraint to field pea production and is the most destructive (Xue, 2000) and widespread (Lawyer, 1984; Baard and Los, 1989) disease throughout the field pea-growing areas of the world (Hagedorn, 1984). According to Allard et al. (1992) all aerial parts of the pea plant are susceptible to infection while growth, yield and seed quality are all adversely affected. The fungus infects pea seedlings as they emerge causing girdling stem lesions that reduce field pea populations and increase lodging. Later, it also causes necrotic lesions on leaflets and stipules and, in exceptional circumstances, abscission of the leaflets (Hagedorn, 1984). *M. pinodes* is spread via pycnidiospores throughout the season (Banniza and Vandenberg, 2003). After germination of spores, the fungus grows over the plant surface for some distance before forming an appresorium and penetrating the cuticle (Clulow et al., 1991). Symptoms may appear as early as 24 h after infection under optimal conditions and are characterized by brown to purplish, coalescing lesions on aerial tissue (Roger et al., 1999). Non-germinated spores remain viable for up to 21 d under dry conditions (Banniza and Vandenberg, 2003). Infection and disease development are highly dependent on temperature and leaf wetness (Roger et al., 1999). Management of *M. pinodes* is limited to fungicide seed treatment and crop rotation (Lawyer, 1984). However, there are indications that the long-term use of chemicals favours the development of plant pathogen resistance (Goldman et al., 1994). Currently, there is no potentially effective natural product to serve as an alternative in controlling the disease and no pea cultivars showing effective resistance against *Ascochyta* blight are currently available (Wroth, 1998; Warkentin et al., 2000; Xue and Warkentin, 2001).

Finally, the *in vivo* control of seed-borne sorghum covered (*Sporisorium sorghi* Link; Clinton) and loose kernel smuts (*Sporisorium cruenta* Kuhn; Potter) by a combined *A. africanus* aerial part crude extract was investigated under field conditions over two growing seasons. Sorghum (*Sorghum bicolor* L. Moench) is an important source of food in Ethiopia and serves as staple food for the majority of people (Abera et al., 1995). Both of these smut species are major factors (Abera et al., 1995) that account for sorghum yield reduction of as high as 25–30% in Ethiopia. As the use of synthetic seed treatment chemicals is beyond the reach of the majority of subsistence farmers in Ethiopia, farmers are dependent on other locally available control measures. Crude extracts of the root part of *Dolichos kilimandscharicus* (local name, Bosha) is used in a slurry form to treat sorghum seeds for the control of sorghum smuts under field conditions. However, this has been practised on a small scale and only had a limited impact in the past. Experimentally, treatment of sorghum seed with *D. kilimandscharicus*, *Phytolacacca dodecandra* (berries) and *Maerua subcordata* (root) material in a powder form appeared to be effective in controlling both pathogens to a certain degree, but not nearly as effective as the standard chemical, Thiram (Tegegne and Pretorius, 2007).

2. Materials and methods

2.1. Plant material

Whole *A. africanus* plants were initially collected in the Blyde River Canyon Nature Reserve (BRC), South Africa. The taxonomic identification of the species was performed by a taxonomist from the National Museum, Bloemfontein, South Africa. A voucher specimen (NMB6819) was processed according to standard procedures and deposited in the herbarium of the museum. Bulk samples of the species were later collected in Bloemfontein (29°07′S; 26°11′N) between January and March 2002 and 2003.

One hundred *P. sativum* cv. Mohanderfer seeds, obtained from a local seed merchant, were sown 2 cm from the surface in 20 pots at five seeds per pot using Bainsvlei soil and applying a standard NPK fertilizer mixture. The plants were allowed to grow for 4 weeks in a greenhouse while maintaining the soil at field capacity. After 4 weeks, two fully expanded leaflets of the same age were removed carefully from the fourth nodes of each plant

and used for monitoring the potential of the crude flower, root, leaf and combined aerial part extracts of *A. africanus* to control *Ascochyta* blight *in vivo*.

2.1.1. Other materials

Potato dextrose agar (PDA), plate count agar (PCA) and nutrient broth were purchased from Merck (Germany). Methanol was purchased from Sigma (Germany) and of the purest grade available. Thiram® was supplied by the Melkassa Agricultural Research Centre, Nazreth, Ethiopia.

2.2. Preparation of crude extracts

Dried plant material was powdered using a Retsch SM2000 cutting mill, soaked in 95% methanol (*v*/*g*) at a ratio of 2 ml dry weight on a roller mill overnight and the supernatant subsequently decanted. This was repeated five times. The combined suspensions were filtered twice, first under vacuum through a double layer of Whatman filter paper (Nos. 3 and 1) and then by gravity through a single sheet of Whatman No. 1 filter paper. The methanol was removed from the clear supernatant by means of vacuum distillation at 30–35 °C using a Büchi Rotary Evaporator. The remaining aqueous solution was referred to as the crude extract.

2.3. Screening of crude extracts for in vitro antifungal activity

Seven of the eight fungal pathogens used in this study, selected from different taxonomic groups, were provided by the Plant Protection Research Institute (PPRI) of the Agricultural Research Council (ARC) in South Africa including the following (strain numbers and taxonomic groups in brackets): *Botrytis cinerea* Pers.: Fr. (PPRI 5209; Hyphomycetes), *Fusarium oxysporum* Schlechtend.: Fr. (PPRI 7972; Hyphomycetes), *Sclerotium rolfsii* Sacc. (PPRI 7975; Agonomycetes), *Rhizoctonia solani* Kühn (PPRI 7973; Agonomycetes), *Botryosphaeria dothidea* (Moug.: Fr.) Ces&De Not. (PPRI 7974; Loculoascomycetes), *Pythium ultimum* Trow (PPRI 6821; Oömycetes) and *Alternaria alternata* (Fr.: Fr.) Keissler; Deuteromycetes (PPRI 6219). An eighth fungus, *M. pinodes* Berk. & Bloxam; Vestergr. (Ascomycetes; strain DPP 185) was isolated by the Department of Plant Pathology, University of the Free State, Bloemfontein, South Africa, from diseased leaves and stems of a winter pea cultivar collected in the central pea-growing area of Ethiopia at the time of senescence.

The agar dilution method described by Rios et al. (1988), with slight modification, was used for determining the inhibition of mycelial radial growth of the test organisms by the plant extracts. All plant pathogenic test fungi were cultured on 2% (*m*/*v*) malt agar, prepared according to the specifications of the manufacturers, and autoclaved for 20 min at 121 °C. On cooling to 45 °C in a water bath, 300 µl of a 33% (*m*/*v*) streptomycin solution was added to the basal medium for controlling bacterial growth. Dried material of each plant extract was dissolved in 100 ml sterile distilled water and amended in the agar to yield a final concentration of 1 g l^{-1}. Working in a laminar flow cabinet, the medium was poured into 90 mm sterile plastic Petri dishes, to a thickness of 2–3 mm, and allowed to set. The centre of each test plate was subsequently inoculated with a 5-mm size plug of 7–10 d-old cultures, for each of the pathogens separately. A plate containing only the basal medium served as control.

Additionally, a plate containing a specific standard fungicide registered for each fungus was used at concentrations recommended by the manufacturers to serve as a positive control against each test organism separately in order to determine the effectiveness of the extracts by comparison. These included Bravo 720 (chlorothalonil; registered against *Botrytis cinerea* and *Rhizoctonia solani*), CungFu 538SC (copper hydroxide; registered against *F. oxysporum* and *Sclerotium rolfsii*), Eria® (difenoconazole and carbendazim; registered against *Botryosphaeria dothidea* and *M. pinodes*) and Metazab 700WP (metalaxyl and mancozeb; registered against *P. ultimum* and *A. alternata*). Plates were incubated for 4 d at 25 ± 2 °C (March et al., 1991) in a growth cabinet. Each assay was replicated four times and the screening procedure repeated twice. Radial mycelial growth was determined after 4 d by calculating the mean of two perpendicular colony diameters for each replicate. The measurement included the diameter of the assay wells (March et al., 1991; Pfaller et al., 1992) and was calculated as percentage mycelial growth inhibition according to the formula of Pandey et al. (1982): $(dc-dt)/dc \times 100$, where dc = average diameter of the fungal colony of the negative control and dt = average diameter of the fungal colony treated with the extracts. The data from two screening experiments were pooled and averaged.

2.4. Isolation of Mycosphaerella pinodes

M. pinodes was isolated from diseased leaves and stems of various winter cultivars of field pea at the time of senescence. Collections of the infected plant material were made from the central and south-eastern pea-growing areas of Ethiopia. Pieces of the diseased tissues were surface sterilized for 1 min in 96% (v/v) ethanol, 3 min in a 3.5% (v/v) NaCl solution (Moussart et al., 1998) and 30 s in 96% (v/v) ethanol. The tissues were subsequently aseptically transferred to corn meal agar amended with streptomycin (0.3 ml l^{-1}) in 9 cm Petri dishes and incubated at 20 ± 1 °C in a growth chamber.

Isolates initially obtained from the plant material were then grown on Coon's medium (Ali et al., 1978) consisting of 4 g maltose, 2 g KNO_3, 1.2 g $MgSO_4$, 2.7 g KH_2PO_4 and 20 g agar. Cultures were incubated for 14 d to obtain pycnidiospores. To obtain an isolate derived from a single

uninucleate cell, a suspension of pycnidiospores was streaked on 15% water agar, incubated overnight at $20 \pm 1\,^{\circ}C$ and examined under a dissecting microscope ($80\times$ magnification). A germ tube arising from one cell of a pycnidiospore was severed and transferred to Coon's agar (Clulow and Lewis, 1992). Six isolates of *M. pinodes* were obtained. All isolates from a single-spore and cultures were maintained on Coon's agar slants and stored in the dark at $5\,^{\circ}C$.

2.5. Preparation of a M. pinodes spore suspension

Oatmeal agar was prepared by gently heating 30 g of oats in 1 l distilled water for 1 h, stirring frequently, and subsequently filtering through a fine sieve upon which the volume was readjusted to 1 l. Twenty g of technical agar and 0.1 g Keltane AP was added to the filtrate to yield a 2% (*m*/*v*) agar concentration. The agar was autoclaved for 15 min, poured into Petri dishes and allowed to cool before inoculation of three oatmeal plates with *M. pinodes* mycelia. Plates were incubated in a 12 h photoperiod incubator at $20\,^{\circ}C$ for 14 d, to ensure the production of pycnidiospores.

To prepare the inoculum (spore suspension), sterile distilled water was added to the 14-d-old cultures dislodging spores gently with a sterile glass rod. The suspension was subsequently filtered through four layers of cheese cloth in order to remove the mycelia and the concentration of pycnidiospores was determined by means of a haemocytometer. The pycnidiospore concentration was adjusted to 1×10^5 spores per ml (Nassir and Hoppe, 1997) with sterile distilled water prior to the inoculation of pea leaves.

2.6. In vivo assessment of crude extract phytotoxicity and antifungal activity against M. pinodes using detached pea leaves under greenhouse conditions

The methodology has been previously described in Pretorius et al. (2002b). All treatments were replicated four times and the *in vivo* assessment was repeated twice. Data of the two assessments were pooled and averaged.

2.7. In vivo assessment of crude extract antifungal activity against sorghum covered and loose kernel smuts under field conditions

Different lots of sorghum seeds were artificially inoculated with either covered (*S. sorghi*) or loose (*Sporisorium cruentum*) kernel smuts spores, 1 h before application of seed treatments, at the rate of 5% (w/w). An aerial crude extract of *A. africanus* was suspended in water at a rate of $2.0\,g\,l^{-1}$. Sorghum seed lots of 90 g each were treated with 15 ml of the crude extract by mixing thoroughly in a small plastic bag 24 h before planting. A standard synthetic seed dressing fungicide, Thiram, was applied in the same way at the rate of 0.25% (w/w) per kg of seed and served as a positive control. Sorghum seeds artificially inoculated with either loose or covered smut spores, but that were not treated with the extract or synthetic fungicide, served as a negative control.

Subsequently, irrigated field trials were conducted at Melkassa Research Centre, Ethiopia during the 2003 and 2004 growing seasons. Plots were arranged in a randomized complete block design and treatments were replicated three times. Treated and control sorghum seeds were planted by hand in five rows, leaving 0.75 cm between rows, in $18.75\,m^2$ plots. Standard fertilizer was applied and plots were kept at field capacity by means of furrow irrigation. Disease incidence was recorded as percentage infected plants. Grain yield was determined on the whole plot.

2.8. Statistical analysis of data

Analysis of variance (ANOVA) was performed on the data, using the SAS (SAS/IML software; Version 6; SAS Institute) program. Tukey's least significant difference (LSD) procedure ($P<0.05$; Steele & Torrie, 1980) was applied to separate means.

3. Results

3.1. In vitro antifungal activity of A. africanus crude extracts against plant fungal pathogens

Crude methanolic extracts of all different plant parts markedly inhibited the mycelial growth of all test fungi, *in vitro*, at a concentration of $1\,g\,l^{-1}$ (Fig. 1). Extracts of separated plant parts completely (100%) or almost compeletely (>97%) inhibited the mycelial growth of *B. cinerea, S. rolfsii, R. solani*, *B. dothidea* and *M. pinodes* and showed a relatively high degree of control against *F. oxysporum* (77%), *P. ultimum* (64%) and *A. alternata* (60–80%). In the case of the latter three fungal pathogens, the combined aerial part extract showed a higher degree of control (>80%) than did extracts from the separate plant parts.

Subsequently, the antifungal activity of only the combined aerial part crude extract was tested against all test fungi at a concentration range. Based on a therapeutic standard (100% mycelial growth inhibition), the minimum inhibitory concentration (MIC) was lowest for *B. dothidea* ($0.8\,g\,l^{-1}$) followed by *P. ultimum* and *M. pinodes* ($1\,g\,l^{-1}$) while the mycelial growth of *B. cinerea*, *S. rolfsii* and *R. solani* was completely inhibited at an MIC of $1.2\,g\,l^{-1}$ (Table 1). Based on a prophylactic standard (80%), mycelial growth was inhibited in six of the test organisms at a concentration lower ($0.4–0.8\,g\,l^{-1}$) than the original $1\,g\,l^{-1}$ concentration used in the screening process. Measured against both standards, *A. alternata* and *F. oxysporum* showed the highest degree of tolerance.

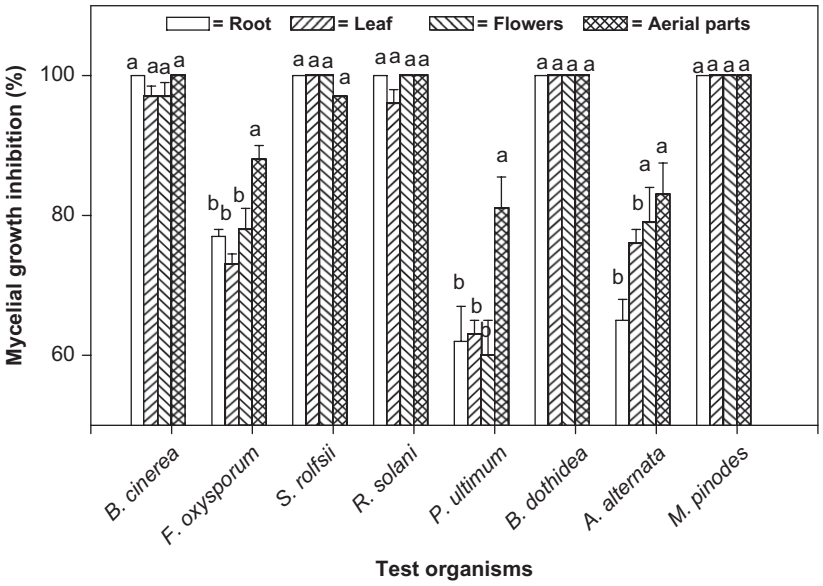

Fig. 1. *In vitro* screening for mycelial growth inhibition of eight plant pathogens by crude extracts of different *Agapanthus africanus* plant parts at a concentration of $1\,g\,l^{-1}$. Bars designated with different letters differed significantly ($P<0.05$) according to Tukey's least significant difference (LSD) statistical procedure.

Table 1
Comparison of the *in vitro* mycelial growth inhibition of eight plant pathogens by a combined aerial part crude extract of *Agapanthus africanus*, at minimum inhibitory concentrations (MICs) based on a therapeutic standard of 100% mycelial growth inhibition, to that of standard fungicides registered against the individual pathogens

Treatment	% Mycelial growth inhibition							
	B. cinerea	*F. oxysporum*	*S. rolfsii*	*R. solani*	*P. ultimum*	*B. dothidea*	*A. alternata*	*M. pinodes*
[a]Extract	98.5±2	87.8±1	97.5±2	99.5±1	83.5±2	100±0	86.0±1	100±0
[b]Standard fungicide	85.3±2	91.5±2	90.8±2	81.5±2	88.0±1	96.3±2	96.5±2	100±0
[c]$LSD_{(T)(0.05)}$	5.26	5.86	2.05	2.25	2.39	4.18	4.77	0
Correlation (+/−)	+	+	+	+	−	+	−	+

[a]In the case of the combined aerial part extract the calculated MICs based on a therapeutic standard of 100% mycelial growth inhibition was applied for each test organism separately.

[b]The registered standard Bravo 720 (chlorothalonil; $3.6\,mg\,l^{-1}$) was used for *B. cinerea* and *R. solani*, Cungfu 538SC (copper hydroxide ; $444\,m\,l^{-1}$) for *F. oxysporum* and *S. rolfsii*, Eria® (difenoconazole/carbendazim; $10\,mg\,l^{-1}$) for *B. dothidea* and *M. pinodes* and Metazab 700WP (metalaxyl and mancozeb; $1.89\,mg\,l^{-1}$) for *P. ultimum* and *A. alternata*.

[c]Statistical values according to Tukey's least significant difference (LSD) statistical procedure ($P<0.05$).

3.2. *In vivo* antifungal activity of *A. africanus* crude extracts against *M. pinodes* on detached pea leaves

Treatment of detached pea leaves with crude extracts from different *A. africanus* plant parts, both before and after inoculation with *M. pinodes* spores, resulted in significant differences among extracts in suppressing lesion development. Compared to both the untreated control (Fig. 2k; Table 2) and the standard fungicide (Fig. 2i; Table 2), treatment of leaves with extracts 30 min before inoculation was visibly more effective in suppressing lesion development than the post inoculation treatment, but at different MICs. The combined aerial part extract (Table 2; Fig. 2b–d) was most effective at the lowest MIC of $0.5\,g\,l^{-1}$ followed by the flower extract at $1\,g\,l^{-1}$. The root and leaf extracts were only effective at an MIC of $2\,g\,l^{-1}$.

When extracts were applied 30 min after spore inoculation, suppression of lesion development on pea leaves by the combined aerial part extract (Table 2; Fig. 2g and h) and the flower extract was again significantly better than that of the other extracts as well as the standard fungicide (Table 2; Fig. 2j). Complete suppression of lesion development was observed at a concentration of $1\,g\,l^{-1}$ for the aerial part and $2\,g\,l^{-1}$ for the flower extract.

In vivo phytotoxicity rating of the aerial plant parts, flower, root and leaf crude extracts of *A. africanus* in terms of its interaction with and potential to induce necrosis in pea leaves, revealed that neither of the crude extracts were

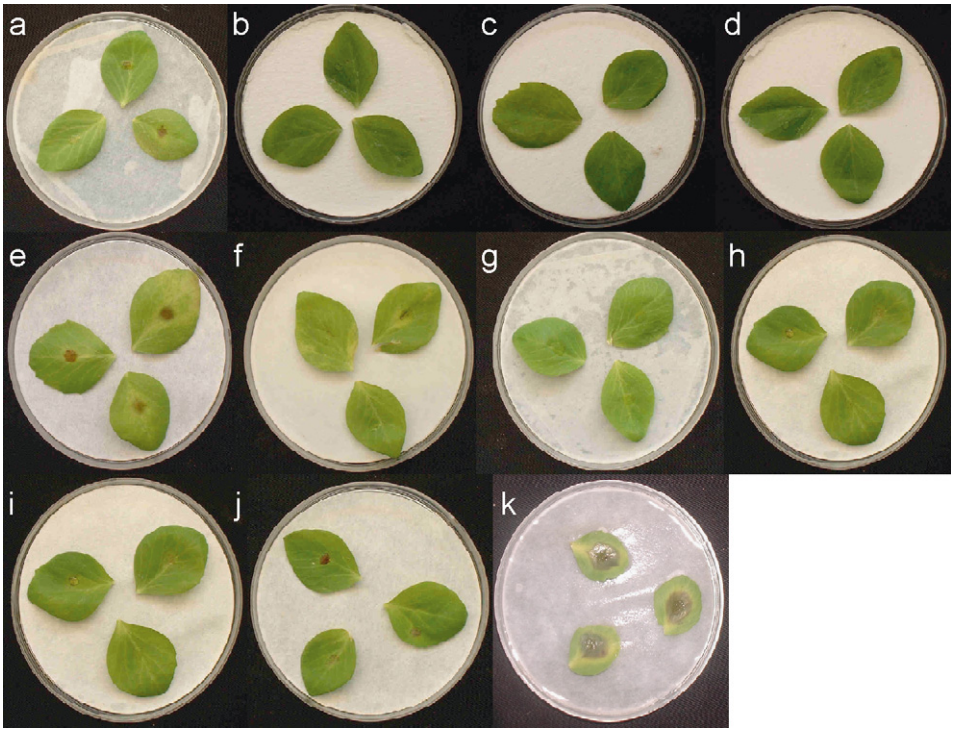

Fig. 2. *In vivo* control of spore infection by *M. pinodes* in pea (*Pisum sativum*) leaves by different concentrations of a combined aerial part crude extract of *A. africanus*. Pea leaves were treated 30 min *before inoculation* [(a) 0.25, (b) 0.5, (c) 1.0 and (d) 2.0 g l^{-1}] and 30 min *after inoculation* [(e) 0.25, (f) 0.5, (g) 1.0 and (h) 2.0 g l^{-1}]. (i) Standard fungicide applied 30 min *before* inoculation at 10 mg l^{-1}, (j) Standard fungicide applied 30 min *after* inoculation at 10 mg l^{-1} and (k) *M. pinodes* spore inoculation only.

Table 2
Mean lesion diameter (mm) following direct inoculation of detached fourth node pea (*Pisum sativum*) leaflets with a *Mycosphaerella pinodes* spore suspension 30 min before and 30 min after treatment with a concentration (m ml^{-1}) range of crude extracts from different *A. africanus* plant parts

Treatment	Crude extract concentration (g l^{-1} distilled water)							
	Mean lesion diameter (mm)							
	Before inoculation				After inoculation			
	0.25	0.50	1.00	2.00	0.25	0.50	1.00	2.00
Aerial part extract	3.9d	0d	0d	0a	5.3c	1.0d	0e	0b
Flower extract	9.5b	2.4c	0.5d	0a	9.3b	6.8b	0.2e	0b
Root extract	6.2c	5.9b	5.3c	0a	13.4a	10.8a	7.4c	1.5b
Leaf extract	10.9a	8.2a	8.9b	0.6a	14.2a	9.7a	8.9b	1.3b
Standard fungicide[1]	0e	0d	0d	0a	3.2c	3.2c	3.2d	3.2a
Spore suspension only	12.8a				12.8a			

Within columns, values designated with different letters differed significantly ($P<0.05$) according to Tukey's least significant difference procedure for comparison of means.
[1]Carbendazim/difenoconazole (10 mg l^{-1}).

phytotoxic even at the highest concentration tested (results not shown).

3.3. *In vivo* antifungal activity of an aerial part crude extract of *A. africanus* against sorghum covered and loose kernel smuts under field conditions

Compared to the corresponding untreated controls, treatment of sorghum seeds with an *A. africanus* aerial part crude extract after spore inoculation and before planting completely (100%) and significantly ($P<0.05$) reduced the incidence of both covered and loose kernel smuts during the 2003 and 2004 growing seasons (Table 3). In both cases control by the crude extract compared favourably with that of the synthetic fungicide, Thiram.

Inoculation of pre-planted sorghum seed with covered or loose smuts spores, without also treating the seeds with either Thiram or the crude *A. africanus* extract (untreated

Table 3
Effect of seed treatment with an *A. africanus* combined aerial part crude extract on the percentage covered and loose kernel smut incidence in sorghum under field conditions during the 2003 and 2004 growing seasons

Seed treatment	Mean plant population	Mean smut incidence at harvest (%)	Yield ($t\,ha^{-1}$)
2003			
Sorghum covered kernel smuts			
Aerial part extract [$2\,g\,l^{-1}$]	171a	0b	3.0a
Thiram [0.25% (w/w)]	175a	0b	2.6a
Spore inoculation only	173a	15a	1.6b
Sorghum loose kernel smuts			
Aerial part extract [$2\,g\,l^{-1}$]	175a	0b	2.9a
Thiram [0.25% (w/w)]	175a	0b	2.1a
Spore inoculation only	175a	28a	1.3b
2004			
Sorghum covered kernel smuts			
Aerial part extract [$2\,g\,l^{-1}$]	192a	0b	5.1a
Thiram [0.25% (w/w)]	190a	0b	4.8a
Spore inoculation only	198a	33a	3.7b
Sorghum loose kernel smuts			
Aerial part extract [$2\,g\,l^{-1}$]	192a	0b	4.0a
Thiram [0.25% (w/w)]	195a	0b	4.9a
Spore inoculation only	191a	45a	2.3b

Values designated with different letters, within columns, differed significantly ($P<0.05$) according to Tukey's least significant difference (LSD) statistical procedure for comparison of means.

control), significantly decreased the final yields in both seasons although the recorded smut incidence was much higher during the second season (Table 3). In comparison, treatment of inoculated sorghum seeds with the *A. africanus* aerial part crude extract resulted in yields almost doubling during both growing seasons. There was no significant difference in yield between plots treated with either Thiram or the *A. africanus* crude extract.

4. Discussion

Preliminary assessment of the *in vitro* antimicrobial potential of crude extracts from different *A. africanus* plant parts confirmed significant antifungal activity. *B. cinerea*, *S. rolfsii, R. solani, B. dothidea* and *M. pinodes* were most responsive to treatments with extracts from all plant parts. Except in the case of *S. rolfsii* (98%), both the root and combined aerial part extracts completely (100%) inhibited radial mycelial growth of the other four listed test organisms emphasizing the broad-spectrum fungicidal potential of the plant. This was especially significant in light of the experience that mycelial growth inhibition by fungicides is more difficult to accomplish than inhibition of spore germination. The Oomycete, *P. ultimum*, and to a lesser extent *F. oxysporum* and *A. alternata,* were more resistant to treatment with crude extracts from different plant parts. Generally, Oomycetes such as *P. ultimum* are regarded as being more tolerant to fungicides than fungi (Sancholle et al., 1984) and this is consistent with the results of this study. However, the combined aerial part extract was still capable of inhibiting mycelial growth of these two pathogens by >80%. This is especially promising, in the event that development of a natural product with *A. africanus* as donor plant might be considered in future, as non-destructive collection of aerial plant parts might pave the way for the sustainable establishment of *A. africanus* as a new or alternative agronomic crop.

Based on the initial *in vitro* results with *A. africanus* crude extracts, and in an attempt to test the latter potential, the *in vivo* antifungal properties of crude extracts from different plant parts were tested against *M. pinodes* using detached pea leaves. Crude extracts of all the different plant parts suppressed lesion development on detached pea leaves to variable degrees depending on the concentration as well as the time of inoculation. However, compared to the other plant part extracts, the combined aerial part crude extract was most effective at all concentration levels both when applied before and after inoculation of detached pea leaves with *M. pinodes* spores. The latter also showed the lowest MIC of $0.5\,g\,l^{-1}$. The flower extract performed second best in suppressing lesion development at a relative low concentration of $1\,g\,l^{-1}$.

As the combined aerial part extract contained compounds from flowers, flower stalks and leaves, the possibility of different active substances contained in the different plant parts acting synergistically in either inhibiting spore germination or mycelial growth or both is not excluded. The latter is supported by a report of Michael (1999) on the variability in bioactivity and the relative differential distribution of bioactive compounds in different plant parts. The ability of the combined aerial part extract as well as the flower extract to completely suppress lesion development, even when applied after inoculation of detached pea leaves, is especially significant considering that the standard fungicide failed to do so.

Compared to the combined aerial part and flower extracts, treatment of detached pea leaves with root and leaf extracts was less effective in preventing *M. pinodes* infection at lower concentrations when applied both before and after spore inoculation. The necrotic lesions measured on pea leaves treated with root and leaf extracts at concentrations lower than $1\,g\,l^{-1}$ were similar to that measured on control leaves inoculated with spores only. However, when applied to detached pea leaves before spore inoculation, both extracts still showed significant suppression of lesion development at the highest concentration of $2\,g\,l^{-1}$.

The findings of this study also corresponded with a previous report (Mekuria et al., 1999) on the *in vivo* antifungal effects of plant extracts applied before or after inoculation to either intact plants or detached leaves. The authors obtained similar results when testing crude extracts from various Bryophyte species against *Phytophthora infestans* on intact tomato plants when applied either

before or after inoculation. Extracts applied 2–5 d before inoculation provided the best results. Recently Amadioha (2000) reported the same tendency in an oil extract from *Azadirachta indica* (neem) seeds to reduce infection and spreading of *Pyricularia oryzae* in rice when applied 2 d before inoculation.

Experimental *in vivo* results on the potential of plant extracts to control fungal infections of crops under controlled conditions contribute to the assessment of the application potential of the extract under field conditions in organized agriculture. Further, according to Roger and Tivoli (1996), control measures based on the reduction of initial inoculum at an early stage, before spore infection establishes, is the most desirable method of *Ascochyta* blight control. *Ascochyta* blight can develop within days under favourable moisture and temperature conditions and currently available fungicides must be applied before the pathogen invades host tissue to ensure successful control. Interestingly, the present study confirmed that a crude extract of combined aerial *A. africanus* parts has the potential to be applied as both a preventative and corrective measure against infection of pea plants by *M. pinodes* spores at a concentration as low as $0.5\,g\,l^{-1}$. The latter is in concert with the findings of Dey and Singh (1994) as well as Moussart et al. (1998) that *M. pinodes* is non-systemic and can be treated on the plant surface.

Although more *in vivo* testing is necessary on other diseases, including foot rot caused by soil or seed-borne *M. pinodes* spores (Moussart et al., 1998) and other fungal pathogens, indications are that the combined *A. africanus* aerial part extract possesses the inherent potential to be applied as a broad spectrum natural fungicide. Further, its application potential in an integrated pest management (IPM) system (Rios et al., 1988; Lydon et al., 1989; Simmonds et al., 1992), especially at the resource-poor farmer level (Poswal et al., 1993), is not excluded. However, a decisive factor in evaluating plant extracts for their application potential as natural pesticides in agriculture is the possible phytotoxic effect on the crops that are sprayed (Benner, 1993). None of the different plant part extracts of *A. africanus* caused phytotoxic yellowing or necrosis on detached pea leaves even at the highest concentrations applied, supplying a rationale for additional tests under field conditions. Consequently, the potential of an *A. africanus* crude extract to control sorghum loose and covered smut was tested in Ethiopia. Pre-treatment of sorghum seeds with a combined aerial part crude extract of *A. africanus* at a rate of $2\,g\,l^{-1}$ completely prevented infection by both covered and loose kernel smuts under field conditions during both growing seasons. This compared favourably with the decrease in disease incidence by the standard synthetic fungicide, Thiram, and confirmed the efficacy of the crude extract. Although both covered and loose kernel smut incidence was much higher in the untreated controls during the 2004 than the 2003 field trial, all (100%) plants grown from inoculated seeds were totally protected against infection by the two smut diseases following seed treatment with the aerial part crude extract. The significant differences in grain yield obtained between plants grown from treated and untreated seeds during both growing seasons confirmed the impact that both diseases had on yield as well as the efficacy of the crude extract in controlling both diseases.

In light of previous reports on the contrasting inhibiting effect that natural plant extracts with antimicrobial properties can have on seed germination (Tran and Tsuzuki, 2002) and seedling establishment (Deena et al., 2003), this aspect was monitored during the field trial (results not shown). Concern with regard to these possible side effects as well as the reliability of natural plant extracts under field conditions was expressed almost a decade ago by Mathre et al. (1995). However, treatment of sorghum seeds with a combined aerial part crude extract of *A. africanus* had no apparent inhibitory effect on either seed germination or seedling growth. The latter, together with the significant *in vitro* and *in vivo* antifungal activities demonstrated in this study for *A. africanus* extracts against a broad spectrum of fungal pathogens, confirmed both its application potential and reliability.

Although the synthetic fungicide, Thiram, is very effective in controlling covered kernel and loose smuts infection in sorghum, the application of crude plant extracts seems a convenient, effective and economical alternative for the majority of Ethiopian subsistence farmers who cannot afford synthetic chemicals. Additionally, the risk associated with synthetic chemicals as well as consumer resistance towards its application in agriculture must be accepted as important issues when considering in depth studies on the application potential of natural products in agriculture. In light of the latter remark, utilization of *A. africanus* as donor plant for the antifungal active compounds in manufacturing a natural product may give the plant new crop status; an aspect that may even be considered by developed countries.

In conclusion, crude extracts of *A. africanus* consistently showed significant *in vitro* and *in vivo* antifungal activity against plant pathogens under laboratory and glasshouse as well as field conditions, respectively. This supplied a rationale for the isolation, purification and identification of antifungal active substances through nuclear magnetic resonance spectroscopy (NMR). A patent on these compounds has been registered and further research concerned with developing a natural fungicide with *A. africanus* as donor plant is ongoing. It is envisaged that, in the event of the extract passing all toxicology tests, the development of *A. africanus* as a new crop will be an added bonus especially to small-scale farmers that are not able to farm with conventional crops on a small piece of land.

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