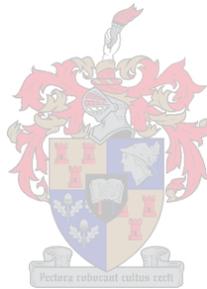


**THE BIOLOGY OF *ENDOPHYLLUM OSTEOSPERMI*, AND  
ITS USE FOR THE BIOLOGICAL CONTROL OF  
*CHRYSANTHEMOIDES MONILIFERA* SSP. *MONILIFERA***

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## **DECLARATION**

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work, and that I have not previously in its entirety or in part submitted it at any university for a degree.

## SUMMARY

*Chrysanthemoides monilifera* ssp. *monilifera* is a shrub indigenous to South Africa, which has become a serious weed of native vegetation in Australia. *Endophyllum osteospermi* is a microcyclic, autoecious, rust fungus that induces witches' brooms on *C. monilifera* ssp. *monilifera*. This rust is considered as a candidate biocontrol agent for use against *C. monilifera* ssp. *monilifera* in Australia.

The vegetative growth and reproductive output of healthy branches on bushes with different levels of *E. osteospermi* infections were measured at three sites. The growth of healthy branches on infected bushes was 26–81% less than that of healthy branches on uninfected bushes. The number of buds, flowering capitulae, fruiting capitulae, and cypselas on healthy branches of infected bushes was 35–75%, 45–90%, 15–99%, and 15–90% less, respectively, than those on uninfected bushes.

At five sites, the infection levels and number of witches' brooms were determined every two months. The increase in number of witches' brooms per bush ranged between 0 and 282 within one year, with an average increase per bush of 28 (SE  $\pm$  4.8) and 39 (SE  $\pm$  9.2) during two years. The average simple interest rate ( $r_s$ ) increase of infection levels for all bushes was 0.015 month<sup>-1</sup> (s.e.  $\pm$  0.0041, n = 72) and 0.0098 month<sup>-1</sup> (s.e.  $\pm$  0.0073, n = 43) during two years.

Aecidioid teliospores germinated between 10 and 20°C, with 15°C as optimum. Light, and particularly near-uv light, stimulated germination. A period of 6 to 8 hours of light was needed to obtain optimum germination levels. The temperature requirements for basidiospore development differed from that of aecidioid teliospore germination. Optimum was at 15°C, but a rapid decrease in basidiospore production occurred at higher temperatures, few developed at 19°C. Two nuclear divisions occurred within 12 hours of germination to produce a metabasidium with three or four nuclei. A third nuclear division occurred in the basidiospores between 24 and 48 hours. Plants inoculated under controlled conditions took 5 to 24 months before witches' brooms began to develop.

A Geographic Information System (GIS) approach was used to model the potential distribution of *E. osteospermi* in South Africa, based on monthly average climate surfaces with parameters derived from the above experiments. The same model was applied to Australia to suggest a potential distribution of the rust if released in

Australia. This potential distribution was similar to one generated using the climate matching computer programme CLIMEX<sup>®</sup>, but gave greater spatial accuracy. Both approaches indicate that *E. osteospermi* should establish in temperate Australia.

*Chrysanthemoides* species, as well as other South African asteraceous plants, were monitored for *E. osteospermi* between 1992 and 2003. *Endophyllum osteospermi* was recorded on *C. monilifera* ssp. *monilifera*, *C. monilifera* ssp. *pisifera*, *C. monilifera* ssp. *rotundata*, *C. monilifera* ssp. *canescens*, *C. monilifera* ssp. *subcanescens*, *C. incana*, an undescribed taxon of *Chrysanthemoides*, *Osteospermum ciliatum*, *O. polygaloides* and *O. potbergense*. *Endophyllum dimorphothecae* sp. nov. is described on *Dimorphotheca cuneata*. *Aecidium elytropappi*, which was recorded on *Elytropappus rhinocerotis* and *Stoebe plumose*, is transferred to *Endophyllum* as *E. elytropappi* comb. nov.

Germination of aecidioid teliospores and penetration by basidiospores were observed on the surface of excised leaves of 32 plant species at 4 days after inoculation. Germinating aecidioid teliospores aborted on 14 plant species, whilst no penetration was attempted on a further 12. Penetration only occurred on 9. Therefore only these 9 plant species need to undergo traditional host specificity testing. Pending these results, *E. osteospermi* could be safely released in Australia for the biological control of *C. monilifera* ssp. *monilifera*.

## OPSOMMING

*Chrysanthemoides monilifera* ssp. *monilifera* 'n meerjarige wat inheems in Suid Afrika is, het 'n belangrike onkruid in Australië geword. *Endophyllum osteospermi* 'n mikrosikliese, autoecious roesswam, induseer heksebesems op *C. monilifera* ssp. *monilifera*. Hierdie roesswam word as 'n potensiële biologiese beheeragent teen *C. monilifera* ssp. *monilifera* in Australië beskou.

Die vegetatiewe groei en voortplanting van gesonde takke op struik met verskillende vlakke van *E. osteospermi* infeksies is by drie lokaliteite gemeet. Groei van gesonde takke op geïnfekteerde bosse was 26–81% minder as die van gesonde takke op ongeïnfekteerde bosse. Die aantal okselknoppe, blomende capitulum, vrugdraende capitulum en pitvrugte op individuele gesonde takke van geïnfekteerde bosse was onderskeidelik 35–75%, 45–90%, 15–99%, en 15–90% minder, as die op ongeïnfekteerde bosse.

By vyf lokaliteite is die infeksievlakke en die aantal heksebesems elke twee maande vasgestel. Die toename in heksebesems van elke plant was tussen 0 en 282 binne een jaar, met 'n gemiddel van 28 (SE ± 4.8) en 39 (SE ± 9.2) gedurende twee jaare. Die gemiddelde eenvoudige rentekoers ( $r_s$ ) toename in infeksievlakke van al die struik was 0.015 maand<sup>-1</sup> (s.e. ± 0.0041, n = 72) en 0.0098 maand<sup>-1</sup> (s.e. ± 0.0073, n = 43) gedurende twee jaare.

Ontkieming van aecidioidteliospore het tussen 10°C en 20°C met 15°C as die optimum. Lig en veral naby-uv lig het ontkieming gestimuleer, terwyl ontkieming relatief swak was onder donker toestande. 'n Periode van 6 tot 8 uur lig was nodig vir optimale ontkiemingsvlakke. Die temperatuurvereistes vir basidiospore ontwikkeling het verskil van die van aecidioid teliospore ontkieming. Optimale was by 15°C, maar 'n vinnige afname in basidiosporeproduksie het by hoër temperature voorgekom, min het by 19°C voorgekom. Twee kernverdelings het binne 12 ure van die begin van ontkieming voorgekom om 'n metabasidium te produseer met drie of vier kerne. 'n Derde kernverdeling het in die basidiospore tussen 24 en 48 uur voorgekom. Plante wat onder beheerde toestande geïnokuleer is het heksebesems 5 tot 24 maande na inokulasie ontwikkel.

‘n Geografiese Inligtings Sisteem (GIS) benadering is gebruik om ‘n model vir die potensiële verspreiding van *E. osteospermi* in Suid Afrika te ontwikkel, gebaseer op die maandelikse gemiddelde klimaatoppervlaktes met parameters wat vanaf bogenoemde eksperimente verkry is. Dieselfde model is in Australië toegepas om ‘n potensiële verspreiding van die roesswam voor te stel. Hierdie potensiële verspreiding was soortgelyk aan ‘n program wat met die klimaats vergelykende rekenaarsprogram CLIMEX<sup>®</sup> ontwikkel is, maar dit het groter ruimtelike akkuraatheid gemaak. Beide benaderings het aangedui dat *E. osteospermi* in Australië behoort te vestig.

*Chrysanthemoides* spesies, asook ander inheemse plante van die Asteraceae, is tussen 1992 en 2003 vir die voorkoms van *E. osteospermi* in Suid Afrika waargeneem. *Endophyllum osteospermi* is op *C. monilifera* ssp. *monilifera*, *C. monilifera* ssp. *pisifera*, *C. monilifera* ssp. *rotundata*, *C. monilifera* ssp. *canescens*, *C. monilifera* ssp. *subcanescens*, *C. incana*, ‘n onbeskryfde taxon van *Chrysanthemoides*, *Osteospermum ciliatum*, *O. polygaloides* en *O. potbergense* waargeneem. *Endophyllum dimorphothecae* sp. nov. is beskryf op *Dimorphotheca cuneata*. *Aecidium elytropappi*, wat op *Elytropappus rhinocerotis* en *Stoebe plumosa* voorkom, is by *Endophyllum* ingesluit as *E. elytropappi* comb. nov.

Ontkieming van aecidioidteliospore en penetrasie deur basidiospore op die oppervlak van verwyderde blare van 32 plant spesies 4 dae na inokulasie is waargeneem. Ontkiemende aecidioidteliospore het op 14 toets plant spesies ge-aborteer, terwyl geen penetrasie op ‘n verdere 12 gepoog is nie. Penetrasie het slegs op 9 voorgekom. Derhalwe hoef slegs die 9 plant spesies tradisionele gasheer spesifisiteitstoetse te ondergaan. Afhange van die resultate kan *E. osteospermi* dus met veiligheid in Australië vir die biologiese beheer van *C. monilifera* ssp. *monilifera* vrygelaat word.

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# 1. The biology of *Endophyllum osteospermi*, and its use for the biological control of *Chrysanthemoides monilifera* ssp. *monilifera*

## Biological control of weeds using rust fungi.

Biological control of weeds involves using organisms to control or reduce the population of weed species (Watson, 1991). The introduction of a host-specific organism from one geographic area where that organism occurs naturally, to another geographic area where its host plant has naturalised and become an invasive weed, is known as the “classical strategy” of weed biological control (Hasan & Ayers, 1990). The first pathogen deliberately used for the classical biological control of a weed was *Puccinia chondrillina* Bubák, used against *Chondrilla juncea* L. in Australia (Hasan, 1974). Field observations that *P. chondrillina* was damaging to its host in its native range (Hasan & Wapshere, 1973), led to it being chosen for use as a biological control agent. Following extensive host specificity testing (Hasan 1974), the rust was released in Australia (Cullen *et al.*, 1973). This rust fungus rapidly brought the dominant narrow-leaved biotype of *C. juncea* under control, but did not infect two other biotypes of the plant in Australia. Unfortunately the intermediate-leaved biotype of the weed then increased in abundance (Burdon *et al.*, 1981; 1984). Additional strains of *P. chondrillina* were subsequently released against the intermediate-leaved biotype of the weed in Australia (Hasan, 1981; 1984), and a further strain has been identified for release against this biotype (Hanley & Groves, 2002). Following the Australian experience, attempts to introduce the strain of *P. chondrillina* used in Australia into the USA failed, but two different strains from Italy were introduced (Lee, 1986) and both strains have had a significant impact on the two most common biotypes of *C. juncea* in the USA (Emge *et al.*, 1981; Supkoff *et al.*, 1988).

Early reviews on the use of pathogens in classical biological control of weeds include Templeton (1982), Adams (1988), Cullen and Hasan (1988), Hasan and Ayers (1990), Bruckart and Hasan (1991), Watson (1991) and Te Beest *et al.* (1992). Much of the early work on weed biological control was done on perennial weeds of natural rangelands or pastures. Presently there is an increasing awareness of the potential

usefulness of biological control of weeds in natural ecosystems (e.g. Mack *et al.*, 2000; Headrick & Goeden, 2001). Rust fungi have been the most extensively used group of fungi employed for classical biological control of weeds. The reasons for this are that: 1) they are obligatorily biotrophic and host specific; 2) they can be highly damaging to their hosts; 3) they are wind-dispersed and capable of rapid dispersal; and 4) they are relatively well documented (Watson, 1991). All rust fungi used for classical weed biocontrol up to the present are autoecious, requiring only one host for the completion of their life cycle (Charudattan & Dinooor, 2000).

The *P. chondrillina* project has been the model on which subsequent classical weed biocontrol programmes using fungi have been based. All have used the same protocol. Watson (1991) listed five species of rust fungi that had been released for the biological control of weeds (*P. chondrillina*, *P. carduorum* Jacky, *Phragmidium violaceum* (Schultz) G. Winter, *Uromyces galegae* (Opiz) Sacc., and *Uromycladium tepperianum* (Sacc.) McAlpine), and another 14 were under consideration for release. Apart from *U. galegae*, these early biological control programs have been highly successful (Charudattan and Dinooor 2000). Since then a further 10 rust fungus species have been released (Table 1), another two are expected to be released shortly (Table 2), whilst three have been rejected for use as their host ranges have included crop or indigenous plants closely related to the target weed (Table 3). An additional three rust fungus species have colonised countries where they were undergoing host specificity testing prior to release (Table 4). A number of rust fungi have followed their host plants as they have been distributed around the world, and exert at least some control (Table 5). Finally a few rust fungi have been considered as biocontrol agents of weeds in their native range (Table 6). These last lists (Tables 4–6) could be greatly expanded, but reference has been restricted to publications where rust fungi have been specifically mentioned as biological control agents.

### **Biocontrol of *Chrysanthemoides monilifera* in Australia.**

The perennial shrub *Chrysanthemoides monilifera* (L.) Norl. (Asteraceae: Calendulae), is native to southern Africa, with one subspecies extending to Tanzania. *Chrysanthemoides* is distinguished from other genera of the Calendulae by the possession

of a fleshy cypsela (often referred to as a drupe) as opposed to a dry cypsela (Norlindh, 1943). Currently there are two recognised species, *C. monilifera* and *C. incana* (Burm. f.) Norl., of which the former has five subspecies, *C. monilifera* ssp. *monilifera*, *C. monilifera* ssp. *pisifera* (L.) Norl., *C. monilifera* ssp. *rotundata* (DC.) Norl., *C. monilifera* ssp. *canescens* (DC.) Norl., *C. monilifera* ssp. *subcanescens* (DC.) Norl., and *C. monilifera* ssp. *septrionalis* Norl. The taxonomy of these two species is however in need of revision, in particular *C. monilifera* ssp. *pisifera* and *C. incana*, both of which as currently circumscribed consist of several distinct taxa (Griffieon, 1995). In addition, the separation of *Chrysanthemoides* from several species of *Osteospermum*, in particular *O. junceum* P.J. Bergius and some members of *Osteospermum* sect. *Homocarpa* and sect. *Polygalina*, is unresolved. This is due to the fact that these latter species have a small amount of fleshy tissue surrounding their cypselas, an adaptation to ant dispersal (Bond & Slingsby, 1983), which is contrary to the single character used to separate these two genera (Wood & Nordenstam, 2003). Other species of *Osteospermum* are wind dispersed and have no fleshy tissue around their cypselas (Bond & Slingsby, 1983). The generic status of *Chrysanthemoides* as currently circumscribed will probably be amended in the future.

*Chrysanthemoides monilifera* is naturalised in south-eastern Australia, where two of the described subspecies occupy distinct climatic zones. *Chrysanthemoides monilifera* ssp. *monilifera* occurs in cool-temperate winter rainfall areas along the coast and inland in Victoria, South Australia, Tasmania and New South Wales. In contrast *C. monilifera* ssp. *rotundata* occurs in warm-temperate to subtropical summer rainfall areas along the coast of New South Wales and southern Queensland (Weiss, 1986; Parsons & Cuthbertson, 1992; Stahle, 1997). *Chrysanthemoides monilifera* ssp. *monilifera* has invaded 780 000 ha in varying densities, and *C. monilifera* ssp. *rotundata* infects over 660 km of coastline in Australia (Stahle, 1997), making them serious invaders of natural vegetation.

Following surveys for natural enemies of *C. monilifera* in South Africa, several insects have been released or are currently under investigation for the biological control of these weeds in Australia (Scott & Adair, 1995; Adair & Edwards, 1996). Insects released include two moths (*Comostolopsis germana* Prout, Geometridae, and ‘*Tortrix*’

sp., Tortricidae) (Adair & Scott, 1989; Scott & Brown, 1992; Anon., 2003), two fruit flies (*Mesoclanis magnipalpis* Bezzi and *M. polana* Munro, Tephritidae) (Edwards & Brown, 1997; Edwards *et al.*, 1999), and four beetles (*Cassida* sp., *Chrysolina* spp. and *C. picturata* (Clark), Chrysomelidae) (Adair & Scott, 1991; Kleinjan & Scott, 1996; Adair & Scott, 1997). Unfortunately, none of the beetles have established successfully (Adair & Edwards, 1996). The moths are herbivores feeding on branch growth tips, and the flies feed on capitulae or developing cypselas. Of the four established insects, only *C. germana* has been in Australia long enough for its impact to be evaluated. It has caused a decrease in flowering and seed production in populations of *C. monilifera* ssp. *rotundata* by up to 50 and 70% respectively, however, it has failed to establish on *C. monilifera* ssp. *monilifera* (Stahle, 1997). Of the four insects that have established, only *M. magnipalpis* was specifically introduced for the control of *C. monilifera* ssp. *monilifera*. Initial surveys of pathogens occurring naturally in Australia have also been done with a view to developing a bioherbicide using *Sclerotinia sclerotiorum* (Lib.) de Bary against *C. monilifera* ssp. *rotundata* (Cothier *et al.*, 1996; Cothier, 2000). Therefore, a destructive agent that specifically targets *C. monilifera* ssp. *monilifera* is required for the biological control of this weed in Australia.

Differences in the population biology of *C. monilifera* ssp. *monilifera* between Australia and South Africa may explain the plant's weediness in Australia. Viable seed densities and seedling recruitment into existing populations in Australia are high (Weiss, 1986), whereas in South Africa they are low (Scott, 1996). The low number of viable seed in South Africa is despite a high production of seed, as the majority of seed produced is non-viable (Weiss & Milton, 1984; Scott, 1996). The high viable seed densities and seedling recruitment in Australia result in continuously expanding populations of the plant, in terms of both distribution and density. This results in the invasion of natural areas, replacement of indigenous vegetation and a disruption of natural ecosystem functioning (Stahle, 1997).

A rust fungus, formerly known as *Aecidium osteospermi* Doidge, occurs commonly on *C. monilifera* ssp. *monilifera* in South Africa, at times becoming locally abundant (Morris 1982b). It is a microcyclic, autoecious rust fungus, inducing galls on stems from which witches' brooms grow. The witches' brooms are perennial. The rust

fungus systemically invades new growth of the witches' brooms. No mycelium occurs in branches and leaves that are not part of the galls or witches' brooms (Morris 1982b). Pycnia and aecidioid teliospores are produced on the leaves and stems of the witches' brooms, the latter in abundance. On germination the aecidioid teliospores, though morphologically identical to typical aeciospores, produce a metabasidium with basidiospores, and therefore it was suggested that this rust fungus be transferred to *Endophyllum* (Morris, 1982b). The taxonomic position of this species was re-assessed, and has subsequently been transferred to *Endophyllum* as *E. osteospermi* (Doidge) A.R. Wood (Wood, 1998).

Branches of the host plant bearing witches' brooms often die back within a few years of infection (Morris, 1982b). Abnormal capitulae may occasionally be produced on witches' brooms, but no viable seeds were produced from these capitulae (Morris, 1982b). Bushes with high levels of witches' brooms have noticeably fewer capitulae than those without or with low levels of witches' brooms, and appear to die more readily (Neser & Morris, 1984). *Endophyllum osteospermi* is believed to have potential as a biological control agent for *C. monilifera* ssp. *monilifera* in Australia (Scott & Adair, 1995; Adair & Edwards, 1996).

### **The biology of *Endophyllum* species.**

The genus *Endophyllum*. (Uredinales, Pucciniaceae), with approximately 30 species (Buriticá, 1999; Kirk *et al.*, 2001), represents a polyphyletic group of taxa derived by a reduction in life cycle from macrocyclic rust fungi with *Aecidium*-like aecia (Cummins & Hiratsuka, 2003). The genus is defined as having aecidioid telia and aecidioid teliospores that are morphologically identical to typical *Aecidium* aecia (cupulate with a distinct peridium) and aeciospores (catenulate, verrucose). However, on germination, the "aeciospores" behave as teliospores in that the germ tube is a metabasidium from which basidiospores are produced. In addition they have type-4 pycnia, which are defined as having determinate growth, a well developed bounding structure, and a strongly convex hymenium (Hiratsuka & Cummins, 1963). This type of life cycle, where spores that are morphologically aeciospores but that germinate as teliospores, is termed endocyclic. Other endocyclic genera of rust fungi include

*Endocronartium*, *Gymnoconia*, and *Monosporidium*. Endocyclic reduction in life cycles has possibly led to the origin of genera of rust fungi with catenulate teliospores (Buriticá & Hennen, 1980; Hiratsuka, 1988).

In certain cases, the rust fungus species from which *Endophyllum* species were derived by life cycle reduction can be surmised, as morphologically similar macrocyclic species still occur on the same species. Examples of this are *E. tuberculatum* (Ellis & Kellerm.) Arthur & Fromme derived from *Puccinia graminella* (Speg.) Dietel & Holw. (Cummins & Hiratsuka, 1983) and *E. alaskanum* Savile derived from *P. veratri* Duby (Savile, 1962). An interesting example of the process of speciation is shown by the biteliomorphic rust fungus on *Capsicum* spp. in South America. Initially aecidioid telia that germinate in the typical endocyclic manner to produce basidiospores are produced (life cycle stage known as *E. pampeanum* (Speg.) J.C. Linq.), yet typical teliospores are produced later on the same witches' brooms (life cycle stage known as *P. pampeana* Speg.) (Hennen *et al.*, 1984).

The unusual life cycle of *Endophyllum* intrigued early uredinologists, and was well studied. This early work was summarised by Ashworth (1935). The life cycle of *E. sempervivi* (Alb. & Schwein) de Bary, the type species of *Endophyllum*, is as follows: 1) basidiospores, uninucleate when first produced, becoming binucleate whilst still attached to the sterigma; 2) the basidiospores are released, germinate and directly penetrate the host epidermal cells; 3) the binucleate infection hyphae develops into a perennial systemic uninucleate inter- and intracellular mycelium; 4) substomatal pycnia develop and following cross fertilisation the uninucleate aecidioid telium primordia become binucleate by nuclear migration; 5) binucleate aecidioid teliospores are produced, but become uninucleate by fusion of the nuclei as they mature; 6) upon germination of the aecidioid teliospores four nuclei are produced (either before or during germination) each of which is separated from one another by septa in the metabasidium; and 7) each nucleus migrates through a sterigmatum into a developing basidiospore (Ashworth, 1935).

The above nuclear cycle is the same as in the majority of macrocyclic rust fungi (Stevens, 1974). Variations on this cycle in *Endophyllum* species have been described, in which the two nuclei of the aecidioid teliospores do not fuse but rather undergo a single division (Thirumalachar & Govindu, 1954; Stevens, 1974), or do not divide at all

(Thirumalachar & Narasimhan, 1950). Pycnia have been reported as either not forming (Arthur & Fromme, 1915; Olive & Whetzel, 1917), or not maturing (Stevens & Mendiola, 1931; Thirumalachar & Narasimhan, 1950; Thirumalachar & Govindu, 1954). These variations indicate that homothallicism may be common in *Endophyllum*, in contrast to the proven or assumed heterothallicism of macrocyclic rust fungi. With regards to this, it is interesting that Ashworth (1935) obtained healthy aecidioid telia whose spores germinated as above even when the pycnia had been destroyed prior to cross-fertilisation. In general, most microcyclic (including endocyclic) rust fungi have become either homothallic if retaining a sexual process or if having lost a sexual process then they are apomitic or asexual (Ono, 2002).

Little is known of the effect of *Endophyllum* species on their host plants. The temperate northern hemisphere species (*E. euphorbiae-sylvaticae* (DC.) G. Winter, *E. lacus-regis* Savile & Parmelee, *E. sempervivi*, and *E. tuberculatum*) are systemic, producing etiolated shoots and reduce or eliminate flowering (Arthur & Fromme, 1915; Savile & Parmelee, 1956; Wilson & Henderson, 1966). Other microcyclic systemic rust fungi also eliminate flowering, such as *Gymnoconia nitens* (Schwein.) F. Kern & Thurst. (Kleiner & Travis, 1991), *Melampsorella cerastii* (Pers.) J. Schröt. (Pady, 1946), *Puccinia monoica* Arthur (Roy, 1993), *P. punctiformis* (F. Strauss) Röhl. (Thomas *et al.*, 1994), and *Puccinia* sp. nr. *consimilis* (Flint & Thomson, 2000).

Other rust fungi causing systemically infected witches' brooms on their hosts include *Aecidium asclepiadinum* Speg. (Charudatan *et al.*, 1978b), *Chrysomyxa arctostaphyli* Dietel (Hansen, 1997), *Endoraecium acaciae* Hodges & D.E. Gardner (Hodges & Gardner, 1984), *Gymnosporangium nidus-avis* Thaxt. (Parmelee, 1971), *Melampsorella caryophyllacearum* (DC.) J. Schröt. (Pady, 1942), *M. cerastii* (Pady, 1942; 1946), *Puccinia vitata* J.F. Hennen & Hodges (Hennen & Hodges, 1981), *Racospermyces angustiphylloides* (D.E. Gardner) J. Walker (Gardner, 1991), and several species of *Ravenelia* (Hernández & Hennen, 2002). Several of these have been recorded as causing reduced growth, death of infected branches and increased mortality of their hosts, including *C. arctostaphyli* (Hansen, 1997), *M. caryophyllacearum* and *M. cerastii* (Pady, 1942). Rust fungi that produce perennial systemically infected galls or cankers, and which have been recorded as causing damage to their hosts, include *Cronartium* spp.

(Devine & Clutter, 1985; Giels & Jacobi, 1993; Peterson & Jewell, 1968), *Melampsora pinitorqua* Rostr. (Martinsson, 1985), and *Uromycladium tepperianum* (Morris, 1997).

These observations suggest that microcyclic (including endocyclic) rust fungi producing galls or witches' brooms have a high potential as biological control agents, as the impact of single infections are greater than that of species producing localised, small lesions. They support the field observations made by several workers (see above) that *E. osteospermi* is damaging to its host plant and therefore has potential as a candidate biocontrol agent. The success of *U. tepperianum* as a biocontrol agent against its host plant *Acacia saligna* (Labill.) H.L. Wendl. in South Africa (Morris, 1997) is testament to this general observation.

### **Molecular techniques in biological control of weeds.**

Despite the widespread use of molecular techniques by mycologists and plant pathologists, weed biological control practitioners have made little use of them. Where these techniques have been applied in weed pathology, they have been predominantly applied to fungi used as bioherbicides. Molecular taxonomic techniques have been most commonly used, for several purposes, namely 1) initial identification to fungal species and differentiation from other isolates of the same species; 2) ensuring that the purity of the specific isolate is maintained during mass production (quality control); 3) for risk assessment of the pathogen once released; 4) to be able to identify the specific isolate after release especially if it is genetically modified; and 5) to identify pathogens that could be vectored by insect biocontrol agents (e.g. Avis *et al.*, 2001; Hintz *et al.*, 2001; Schaad *et al.*, 2001; Davis *et al.*, 2002). Other molecular techniques can be used to investigate host-pathogen interactions to optimise the efficacy of bioherbicide fungi (e.g. Goodwin, 2001). Of these listed purposes, identification and differentiation of isolates is the most relevant to classical biocontrol against weeds. A number of techniques have been used for this purpose, but this largely reflects the development of molecular techniques with time.

As part of a classical biological control programme against a weed using a rust fungus, isozymes were used to distinguish between strains of the candidate agent *P. carduorum*. This data supported morphological and host range studies demonstrating that

the rust fungus strain on the target host plant *Carduus thoermeri* Weinm. differed from other strains of that rust species (Bruckart & Peterson, 1991). Later amplification fragment length polymorphism (AFLP) (of the internal transcribed spacer (ITS) region) was used to distinguish *P. carduorum* from closely related rust fungus species, and also between the rust fungus strain used and another strain on other *Carduus* species (Berthier *et al.*, 1996). This work was necessary to demonstrate that the rust fungus strain introduced into the USA from Europe was different to strains of *P. carduorum* already present in the USA, but on different host plant species. Subsequently, by sequencing the ITS region, it has been demonstrated that the released strain has spread throughout the USA. Sequencing allowed discrimination of the released strain from the strain indigenous to the USA (Luster *et al.*, 2000). Sequencing of the ITS region has also been used to determine the relationship of a *Puccinia* species attacking *Isatis tinctoria* L. in the USA. No rust fungus is known to attack this plant in its native range in Europe. The rust fungus was originally identified as *P. thlaspeos* C. Schub., but later was shown to be closely related to *P. consimilis* Ellis & Everh. The identity of this rust species remains unresolved, but it has been assumed that it is a new association by a rust species native to the USA (Kropp *et al.*, 1997). It has been demonstrated that there are a number of cryptic rust fungus species amongst the *P. monoica* species complex, which includes these two *Puccinia* species, attacking the northern hemisphere crucifers (Roy *et al.*, 1998; Roy, 2001). Other species of rust fungi have been differentiated by the use of specific internal transcriber spacer (ITS) region primers, including *Cronartium* and *Peridermium* species (Vogler & Bruns, 1998), the *Uromyces pisi* (Pers.) de Bary species complex (Pfundner *et al.*, 2001), and grass rust fungi (Zambino & Szabo, 1993; Virtudazo *et al.*, 2001).

In addition to AFLP, restriction fragment length polymorphism (RFLP) of microsatellite DNA can be used to distinguish between isolates within a rust fungus species. This technique was used to demonstrate that the strain of *Phragmidium violaceum* officially released in Australia had not established well in the field but that the illegally imported strain dominated (Evans *et al.*, 2000). Other techniques used to differentiate between isolates within rust fungus species include selectively amplified microsatellites (SAM) and sequence-specific amplification polymorphism (S-SAP) (Keiper *et al.*, 2003).

An interesting adaptation of a molecular technique normally used for taxonomic purposes was that of amplification by PCR of part of the rDNA large sub-unit (LSU) region so that the presence of a *Puccinia* species attacking *I. tinctoria* was readily detected in asymptomatic plants in the USA (Kropp *et al.*, 1995). This technique was used to study the infection process and development of the systemic infection of plants. The host is biennial, infection occurred in the first year but the plants remained asymptomatic for 3 to 9 months. Infection occurred in the leaves of the host plant, and the rust fungus eventually systemically infected the whole plant, growing at a rate of 0.25 cm per week. After overwintering in the persistent roots, the fungus grew with the new growth flush of the host plant and sporulated profusely (Kropp *et al.*, 1996; 1999).

### **Aims of the project.**

Two aspects are of importance in any biological control programme, namely that there is a good potential for the candidate agent to reduce the target weed population, and that there is a low risk of cross-infection to either crop plants or plants indigenous to the area invaded by the weed. Field studies are preferable to glasshouse studies to determine the potential impact of the selected agent before introduction onto a new continent, as studies under artificial conditions may give an overestimate (Watson, 1991). For rust fungi, the complete life cycle needs to be known as well as its host range in its natural distribution range, and the risk of infection to plants not previously exposed (both crop and indigenous plants) by means of host specificity testing, is necessary before the candidate biocontrol species can be released.

The specific aims of the present study were to:

- 1) Quantify the impact of *E. osteospermi* on its host under natural conditions in South Africa;
- 2) Demonstrate that populations of *E. osteospermi* undergo epidemic increases under natural conditions in South Africa;
- 3) Quantify the effect of environmental conditions (temperature and light) on aecidoid teliospore germination and development of basidiospores;

- 4) Re-examine the nuclear cycle and so confirm the complete life cycle of *E. osteospermi*;
- 5) Determine the natural host range of the rust fungus as occurring in South Africa, and determine relationships between any related rust fungi found on plants other than *Chrysanthemoides* with morphological and molecular techniques;
- 6) Determine the infection process of *E. osteospermi* on its host plant, and complete preliminary host specificity testing.

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**Table 1. Rust fungi that have been released for the biological control of weeds.**

Rust species	Target weed species	Country	References
<i>Diabole cubensis</i>	<i>Mimisa pigra</i> var. <i>pigra</i> (giant sensitive plant, Fabaceae)	Australia	Seier and Evans 1996
† <i>Maravalia cryptostegiae</i>	<i>Cryptostegia grandiflora</i> (rubber vine, Periplocaceae)	Australia	Evans 2000 Tomley and Hardwick 1996 Vogler and Lindsay 2002
† <i>Phragmidium violaceum</i>	<i>Rubus constrictus</i> <i>Rubus ulmifolius</i> (Zarzamora, Rosaceae)	Chile	Oehrens and Gonzalez 1974 Oehrens and Gonzalez 1977
	<i>Rubus fruticosus</i> (aggregate sp.) (European blackberry, Rosaceae)	Australia	Mahr and Bruzzese 1998
<i>Prosopidium tuberculatum</i>	<i>Lantana camara</i> (Lantana, Verbenaceae)	Australia	Thomas and Ellison 2000 Tomley and Riding 2002
† <i>Puccinia abrupta</i> var. <i>partheniicola</i>	<i>Parthenium hysterophorus</i> (Parthenium weed, Asteraceae)	Australia	Parker <i>et al.</i> 1994 Tomley and Evans 1995
<i>Puccinia cardui-pycnocephali</i>	<i>Carduus pycnocephali</i> <i>Carduus tenuiflorus</i> (Slender thistles, Asteraceae)	Australia	Burdon <i>et al.</i> 2000
† <i>Puccinia carduorum</i>	<i>Carduus thoermeri</i> (Musk thistle, Asteraceae)	USA	Baudoin <i>et al.</i> 1993 Baudoin and Bruckart 1996 Woods <i>et al.</i> 2002
† <i>Puccinia chondrillina</i>	<i>Chondrilla juncea</i> (Skeleton weed, Asteraceae)	Australia	Cullen <i>et al.</i> 1973 Burdon <i>et al.</i> 1981 Hasan 1981
		USA	Emge <i>et al.</i> 1981 Supkoff <i>et al.</i> 1988
<i>Puccinia evadens</i>	<i>Baccharis halimifolia</i> (Groundsel, Asteraceae)	Australia	Tomley and Willsher 2002
<i>Puccinia melampodii</i>	<i>Parthenium hysterophorus</i> (Parthenium weed, Asteraceae)	Australia	Evans 2000
† <i>Puccinia myrsiphylli</i>	<i>Asparagus asparagoides</i> (Bridal creeper, Asparagaceae)	Australia	Morin <i>et al.</i> 2002
<i>Uromyces galegae</i>	<i>Galega officianalis</i> (Goats rue, Fabaceae)	Chile	Oehrens and Gonzalez 1975
<i>Uromyces heliotropii</i>	<i>Heliotropium europaeum</i> (Common heliotrope, Boraginaceae)	Australia	Delfosse <i>et al.</i> 1995
† <i>Uromycladium tepperianum</i>	<i>Acacia saligna</i> (Port jackson willow, Fabaceae)	South Africa	Morris 1997

† recorded as being successful biological control agents.

**Table 2. Rust fungi under consideration, or that have been considered, as biological control agents against weeds.**

Rust species	Target weed species	Country	References
<i>Cionothrix praelonga</i>	<i>Chromolaena odorata</i> (Chromolaena, Asteraceae)	pantropical	Elango <i>et al.</i> 1993
<i>Melampsora euphorbiae</i>	<i>Euphorbia</i> spp. (Leafy spurge, Euphorbiaceae)	USA	Holden and Mahlberg 1995 Bruckart <i>et al.</i> 1986
<i>Prosopidium tumefasciens</i>	<i>Aloysia gratissima</i> (Whitebush, Verbenaceae)	USA	Cordo and Deloach 1995
<i>Puccinia barbeyi</i>	<i>Asphodelus fistulosus</i> (Onion weed, Asphodelaceae)	Australia	Hasan 1991
<i>Puccinia centaureae</i>	<i>Centaurea maculosa</i> (Spotted knapweed, Asteraceae)	Canada	Clement and Watson 1985
<i>Puccinia hieracii</i>	<i>Hieracium</i> spp. (Hawkweeds, Asteraceae)	USA	Hasan and Fornasari 1996
* <i>Puccinia jaceae</i>	<i>Centaurea solstitialis</i> (Yellow starthistle, Asteraceae)	USA	Bruckart 1989 Shishkoff and Bruckart 1993 Bruckart and Eskadari 2001
<i>Puccinia lantanae</i>	<i>Lantana camara</i> (Lantana, Verbenaceae)	Australia	Thomas and Ellison 2000
<i>Puccinia nassellae</i>	<i>Nasella trichotoma</i> <i>Nasella neesiana</i> (Serrated tussock grass, Chilean needle grass, Poaceae)	Australia	Anderson <i>et al.</i> 2002
* <i>Puccinia spegazzini</i>	<i>Mikania micrantha</i> (Mile-a-minute weed, Asteraceae)	India	Cock <i>et al.</i> 2000
<i>Puccinia xanthii</i>	<i>Ambrosia artemisiifolia</i> <i>Ambrosia trifida</i> (Annual ragweed, Giant ragweed, Asteraceae)	Australia	Batra 1981 Tomley and Evans 1995
<i>Uredo eichhorniae</i>	<i>Eichhornia crassipes</i> (Water hyacinth, Pontederiaceae)	USA	Charudattan <i>et al.</i> 1978a Charudattan <i>et al.</i> 1981
<i>Uromyces rumicis</i>	<i>Rumex crispus</i> (Curly dock, Polygonaceae)	USA	Inman 1971 Schubinger <i>et al.</i> 1985
<i>Uromyces salsolae</i>	<i>Salsola kahli</i> (Russian thistle, Chenopodiaceae)	USA	Hasan <i>et al.</i> 2001
<i>Uromyces scutellatus</i>	<i>Euphorbia</i> spp. (Leafy spurge, Euphorbiaceae)	USA	Defago <i>et al.</i> 1985 Holden and Mahlberg 1995

\*Expected to be released in the near future.

**Table 3. Rust fungi rejected for use as biological control agents against weeds.**

Rust species	Target weed species	Country	Reason for rejection	References
<i>Gymnoconia nitens</i>	<i>Rubus argutus</i> (Florida blackberry, Rosaceae)	Hawaii	Indigenous <i>Rubus</i> susceptible	Gardner <i>et al.</i> 1997
	<i>Rubus cuneifolius</i> (American bramble, Rosaceae)	South Africa	Indigenous and cultivated <i>Rubus</i> susceptible	Morris <i>et al.</i> 1999
<i>Aecidium asclepiadinum</i> and <i>Puccinia araujae</i>	<i>Morrenia odorata</i> (Milkweed vine, Asclepiadaceae)	USA	The bioherbicide DeVine ( <i>Phytophthora palmivora</i> ) has proved so successful that no additional agents are required	Charudattan <i>et al.</i> 1978b
<i>Puccinia canaliculata</i>	<i>Cyperus esculentus</i>	Netherlands	not sufficiently host specific	Scheepens and Hoogerbrugge 1991
<i>Uromyces</i> sp.	<i>Cytisus scoparius</i> (Scotchbroom, Fabaceae)	Australia	not sufficiently host specific	Morin <i>et al.</i> 2000
<i>Uromyces rumicis</i>	<i>Emex australis</i> (Doublegee, Polygonaceae)	Australia	found to occur in Australia, it is having no impact on weed whereas <i>Phomopsis emicis</i> together with insects are bringing weed under control	Morris 1982a Shivas 1987 Scott and Shivas 1998

**Table 4. Rust fungi which dispersed naturally in countries where they had been proposed for use as biological control agents.**

Rust species	Weed species	Country	References
<i>Puccinia hieracii</i> var. <i>piloselloidarum</i>	<i>Hieracium pilosella</i> (Mouse-ear hawkweed, Asteraceae)	New Zealand	Morin and Syrett 1996
<i>Puccinia jaceae</i> var. <i>diffusa</i>	<i>Centaurea diffusa</i> (Diffuse knapweed, Asteraceae)	Canada	Mortensen <i>et al.</i> 1989 Mortensen <i>et al.</i> 1991
† <i>Puccinia xanthii</i>	<i>Xanthium</i> spp. (Noogoora burr, Asteraceae)	Australia	Julien <i>et al.</i> 1979 Morin <i>et al.</i> 1996

† recorded as being a successful biological control agent.

**Table 5. Rust fungi that adventitiously arrived in countries and may have potential as biological control agents.**

Rust species	Target weed species	Country	References
<i>Melampsora hypericorum</i>	<i>Hypericum androsaemum</i> (Tustan, Clusiaceae)	Australia	McLaren <i>et al.</i> 1997
<i>Phragmidium violaceum</i>	<i>Rubus fruticosus</i> (aggregate sp.) (European blackberry, Rosaceae)	New Zealand	Pennycook 1998
<i>Puccinia abrupta</i> var. <i>partheniicola</i>	<i>Parthenium hysterophorus</i> (Parthenium weed, Asteraceae)	South Africa	Wood and Scholler 2002
<i>Puccinia acroptili</i>	<i>Acroptilon repens</i> (Russian knapweed, Asteraceae)	Canada	Mortensen <i>et al.</i> 1991 Palm and Vesper 1991 Dugan and Carris 1992
<i>Puccinia carduorum</i>	<i>Carduus tenuiflorus</i> (Slenderflower thistle, Asteraceae)	USA	Watson and Brunetti 1984
<i>Puccinia coronata</i> f. <i>sp. avenae</i>	<i>Avena barbata</i> <i>Avena fatua</i> (Wild oats, Poaceae)	USA	Carsten <i>et al.</i> 2000 Carsten <i>et al.</i> 2001
<i>Puccinia cnici</i>	<i>Cirsium vulgare</i> (Spear thistle, Asteraceae)	Australia	Bruzzese <i>et al.</i> 1989
<i>Puccinia jaceae</i> var. <i>diffusa</i>	<i>Centaurea diffusa</i> (Diffuse knapweed, Asteraceae)	USA	Palm and Richard 1992 Dugan and Carris 1992
<i>Puccinia lagenophorae</i>	<i>Senecio vulgaris</i> (Groundsel, Asteraceae)	Europe	Frantzen and Hatcher 1997 Paul <i>et al.</i> 1993
<i>Puccinia lygodii</i>	<i>Lygodium japonicum</i> (Climbing fern, Schizaeaceae)	USA	Rayachhetry <i>et al.</i> 2001a
<i>Puccinia psidii</i>	<i>Melaleuca quinquenervia</i> (Melaleuca, Myrtaceae)	USA	Rayachhetry <i>et al.</i> 2001b
<i>Puccinia punctiformis</i>	<i>Cirsium arvense</i> (Canada thistle, Asteraceae)	Canada	Thomas <i>et al.</i> 1994
<i>Uromyces euphorbiae</i>	<i>Euphorbia heterophylla</i> (Milk weed, Euphorbiaceae)	Brasil	Yorinori 1984 Barreto and Evans 1998

**Table 6. Rust fungi indigenous in countries which may be of use as biological control agents against introduced weeds.**

Rust species	Target weed species	Country	References
<i>Puccinia canaliculata</i>	<i>Cyperus esculentus</i> (Yellow nutsedge, Cyperaceae)	USA	Phatak <i>et al.</i> 1983 Beste <i>et al.</i> 1992
	<i>Cyperus rotundus</i> (Purple nutsedge, Cyperaceae)	India	Gupta <i>et al.</i> 2002
<i>Puccinia</i> sp. nr. <i>consimilis</i>	<i>Isatis tinctoria</i> (Dyer's woad, Brassicaceae)	USA	Flint and Thomson 2000 Kropp <i>et al.</i> 2002
<i>Puccinia expansa</i>	<i>Senecio alpinus</i> <i>Senecio jacobea</i> (Ragwort, Asteraceae)	Switzerland	Alber <i>et al.</i> 1985 Alber <i>et al.</i> 1986
<i>Puccinia obliqua</i>	<i>Calotropis procera</i> (Rubber bush, Asclepiadaceae)	Brazil	Barreto <i>et al.</i> 1999

## **2. Infection of *Chrysanthemoides monilifera* ssp. *monilifera* by the rust fungus *Endophyllum osteospermi* is associated with a reduction in vegetative growth and reproduction**

### **ABSTRACT**

The perennial shrub *Chrysanthemoides monilifera* ssp. *monilifera* (Asteraceae: Calendulae), indigenous to South Africa, has become a serious weed of native vegetation in cool-temperate winter rainfall areas in south-eastern Australia. The microcyclic, autoecious, rust fungus *Endophyllum osteospermi* induces the production of witches' brooms on *C. monilifera* ssp. *monilifera*. Field observations indicate that the presence of witches' brooms may reduce the growth and reproduction of the remaining uninfected parts of the host bushes, and therefore this rust fungus is currently being considered as a biocontrol agent for use against *C. monilifera* ssp. *monilifera* in Australia. The vegetative growth and reproductive output of healthy branches (without witches' brooms) on bushes with different levels of *E. osteospermi* infections were measured at three South African sites with naturally infected populations of *C. monilifera* ssp. *monilifera*. The growth of healthy branches on infected bushes was 26–81% less than that of healthy branches on uninfected bushes. The number of buds, flowering capitulae, fruiting capitulae, and drupes on individual healthy branches of infected bushes was 35–75%, 45–90%, 15–99%, and 15–90% less, respectively, than those on uninfected bushes. As these measurements were made under natural conditions, other factors (not measured) may have influenced the results, however the presence of witches' brooms was consistently associated with reduced growth and reproductive output and is therefore presumed to be an important factor. Very little flowering and drupe production occurred on witches' brooms themselves.

### **INTRODUCTION**

The shrub *Chrysanthemoides monilifera* (L.) Norl. (Asteraceae: Calendulae), which is native to southern Africa (with one subspecies extending to Tanzania), is naturalised in south-eastern Australia, where two of the described subspecies (Norlindh 1943) occupy distinct climatic zones. *C. monilifera* ssp. *monilifera*, occurs on the coast and inland in Victoria, South

Australia, Tasmania and New South Wales in cool-temperate winter rainfall areas, whereas *C. monilifera* ssp. *rotundata* (DC.) Norl. occurs along the coast of New South Wales and southern Queensland in warm-temperate to subtropical summer rainfall areas (Stahle 1997; Weiss 1986). It is estimated that 780 000 ha in Victoria are affected by *C. monilifera* ssp. *monilifera* (Stahle 1997). Several insects have been released or are currently under investigation for the biological control of these weeds in Australia (Adair and Edwards 1996; Edwards *et al.* 1999). Initial surveys of pathogens occurring naturally in Australia have also been done with a view to developing a bioherbicide (Cother *et al.* 1996; Cother 2000).

The rust fungus *Endophyllum osteospermi* (Doidge) A.R. Wood (syn. = *Aecidium osteospermi* Doidge) occurs commonly on *C. monilifera* ssp. *monilifera* in South Africa, at times becoming locally abundant (personal observations). It is a microcyclic, autoecious rust fungus, that induces galls on stems from which witches' brooms grow. The witches' brooms are perennial. The rust fungus systemically infects the galls and witches' brooms, including new growth of the witches' brooms. Pycnia and aecidioid telia are produced on the leaves and stems of the witches' brooms, the latter in abundance (Morris 1982). Several other rust fungi causing similar systemically infected witches' brooms on their hosts have been recorded as causing reduced growth, death of infected branches and increased mortality of their hosts, including *Chrysomyxa arctostaphyli* Dietel (Hansen 1997), *Melampsorella caryophyllacearum* (DC.) J. Schröt. (Pady 1942), and *M. cerastii* (Pers.) J. Schröt. (Pady 1942). The gall and witches' broom inducing rust fungus *Uromycladium tepperianum* (Sacc.) McAlpine, deliberately introduced into South Africa as a biocontrol agent, caused at least an 80% decrease in tree density of *Acacia saligna* (Labill.) H.L. Wendl. at eight sites in South Africa within seven years of release, by increasing mortality of large plants, and decreasing recruitment of seedlings (Morris 1997).

*Endophyllum osteospermi* is believed to have potential as a biological control agent for use against *C. monilifera* ssp. *monilifera* in Australia (Adair and Edwards 1996, Scott and Adair 1995). Branches bearing witches' brooms often die back within a few years of infection, and severely infected plants usually die within several years of becoming severely infected (Morris 1982). Abnormal capitulae may occasionally be produced on witches' brooms, but no viable seeds were observed on these capitulae (Morris 1982). Bushes with high levels of infection have noticeably fewer capitulae than those without or low levels of infection

(personal observations). Observations by the author indicate that witches' brooms have a deleterious effect on the remaining uninfected parts of host plants. Field studies were undertaken on *E. osteospermi* and its host under natural conditions in South Africa, to determine whether differences in growth and reproductive output of healthy branches (those without witches' brooms) occurred between bushes with different levels of infection. These data would help to assess the potential of *E. osteospermi* as a biological control agent.

## METHODS

**Effect of infection on host growth.** During the growing seasons (Mar - Nov) of 1994 and 1995, the growth of naturally infected bushes of *C. monilifera* ssp. *monilifera* was measured at monthly intervals at three sites in the Western Cape Province, South Africa. The sites were (i) 4 km north west of Scarborough, Cape Peninsula (34°12'S 18°23'E), (ii) Heuningberg Nature Reserve, Bredasdorp (34°32'S 20°02'E), and (iii) Potberg Education Centre, De Hoop Nature Reserve, east of Bredasdorp (34°23'S 20°32'E) during 1994. In 1995 the Scarborough site was replaced with a site 2 km south of Simon's Town, Cape Peninsula (34°13'S 18°28'E), as most of the plants at the Scarborough site had died.

The Potberg site used in this study had a density of 0.52 plants/m<sup>2</sup> in a total area of 500 m<sup>2</sup> (in 1995), and the Scarborough site had a density of 0.035 plants/m<sup>2</sup> in a total area of 6900 m<sup>2</sup> (in 1992). The plant density of the Heuningberg and Simon's Town sites were not measured, but had densities intermediate between that of the above two sites.

In February (Scarborough) or March (Heuningberg, Potberg) of 1994, 15 bushes at each site were chosen and their infection level determined by visually estimating the volume of the bush occupied by witches' brooms, (classes of 0, 10, 25, 50, 75, or 90% were utilised). There were 5 bushes from each of three infection levels (no infection, medium infection, and heavy infection) at each site. The infection levels used varied between sites depending on availability. For example at Scarborough in 1994 the medium infection level bushes were 10% infected, whereas the heavy infection level bushes were 25% infected; at Heuningberg the medium infection level bushes were 25% infected, whereas the heavy infection level bushes were 75% infected. Ten healthy branches without witches' brooms per bush were arbitrarily

chosen and marked. The length of each marked branch, from its base on the branch from which it grew to the tip of its longest leaf, was measured at monthly intervals. Any marked branches that died were replaced. In 1995 the same procedure was used.

The means and SE for each month's accumulated growth for each infection level at each site were calculated. The averages of the total growth for each bush (accumulated growth of the 10 branches per bush), of those which survived throughout the year, were analysed with two-way ANOVAs to determine if differences occurred between the sites in each year, and between the years for each site. Linear functions were fitted to the average growth per infection level (average of 5 bushes) for the combined site and year data by means of the computer program Table Curve 2D. The Scarborough 1994 data and the Potberg 1994 - 90% infection level point which were excluded as outliers. This was done as an initial exploration to determine which function would be most suitable to use for linear regression. Linear regressions with the polynomial and exponential functions using  $x^{0.5}$  and  $x$  (where  $x$  = infection level) were then fitted for (i) the average growth per infection level (average of 5 bushes) for the combined site and year data (with same exclusions as above), (ii) the average growth per bush for the combined site and year data (with same exclusions as above), and (iii) for the average growth per bush for each individual site and year.

Furthermore, 20 uninfected branches from each bush used for the growth measurements at the Heuningberg and Potberg sites were arbitrarily chosen and destructively sampled at the beginning of the 1995 season. The number of leaves, the length of the branch, and the dry weights of the leaves and branches were recorded. Also the length and dry weight of the largest leaf present on each branch was recorded. Any buds, flowering capitulae, fruiting capitulae and drupes occurring on these branches were also recorded. From the Simon's Town site, 20 arbitrarily chosen branches from each bush used for the growth measurements were destructively sampled near the end of the 1995 season. The number of leaves and branch length were recorded. No buds or capitulae were present on these branches.

**Effect of infection on host flowering.** At the Potberg site, and another site in the De Hoop Nature Reserve (34°27'S 20°26'E) in 1994, many of the bushes flowered almost synchronously. This opportunity was therefore used to determine whether infection was associated with reduced flowering.

The number of buds, flowering capitulae, fruiting capitulae, drupes and branch length

was recorded for 20 arbitrarily chosen branches without witches' brooms of five bushes per infection level (not necessarily the same bushes used in the growth study). The infection levels used were 0, 25, 50 and 75%.

The sum of the average number of buds, flowering and fruiting capitulae per bush for each infection level were analysed with two-way ANOVAs to compare between Potberg 1994 and 1995, and Potberg 1994 and De Hoop 1994. The polynomial and exponential functions using  $x^{0.5}$  and  $x$  (where  $x$  = infection level) were fitted for each site and year.

In addition, the numbers of buds, flowering capitulae, fruiting capitulae and drupes were recorded for 20 branches of witches' brooms on each of 20 infected bushes (i.e. 400 branches in total) at Potberg in 1995.

## RESULTS

**Effect of infection on host growth.** In all but one case (Potberg 1994), the growth of the healthy branches of bushes with witches' brooms was 26 - 81% less compared with growth of uninfected bushes, depending on infection level, site and year (Figure 1). The higher the infection level the greater was this reduction.

A good fit was obtained for the polynomial function ( $R^2 = 0.894$ ), and a slightly better fit was obtained for the exponential function ( $R^2 = 0.911$ ), using  $x^{0.5}$ , for the average growth per infection level for the combined site and year data (using the average for all bushes which gave few data points), however no good fits were obtained for the average growth per bush for each infection level for the combined site and year data (using all data points) ( $R^2 \leq 0.425$ ). Likewise regression lines fitted to each individual site for the average growth per bush for each year were poorly fitted ( $R^2 = 0.137$  to  $0.7$ ). Because of the poor fits to regression lines, analysis of variance was used to test the significance of effects of disease severity on growth parameters.

In the two-way ANOVAs, a significant difference in growth between sites occurred in 1994 ( $P = 0.014$ ) but not in 1995 ( $P = 0.479$ ). There were, however, highly significant differences between the infection levels for each year ( $P < 0.0001$  for 1994 and 1995). There were no significant differences between years for each site, although there were significant ( $P = 0.02$  at Scarborough) or highly significant ( $P < 0.01$  at Heuningberg and Potberg) differences between the infection levels for each year. In none of these analyses was the

interaction significant.

In the destructively sampled branches collected in 1995 from Heuningberg and Potberg (Tables 1 and 2), plants with witches' brooms had reduced branch length and dry weight, reduced branch dry weight/cm, and lower numbers and dry weight of leaves, in comparison with uninfected bushes. The higher the level of infection the greater was the reduction. Average dry weight per leaf, and length and dry weight of the largest leaf were not greatly reduced. Branch length and number of leaves per branch were also reduced on infected bushes at Simon's Town compared with uninfected bushes (Table 3).

**Effect of infection on host flowering.** The average number of buds (35 - 75%), flowering capitulae (45 - 90%), fruiting capitulae (15 - 99%), and drupes (15 - 90%) per branch on bushes with witches' brooms was less than that of uninfected bushes (Figure 2). The overall tendency was for a greater reduction with increasing infection levels. No significant differences occurred in the comparison between Potberg 1994 and 1995 using a two-way ANOVA, for either between sites or between infection levels. Highly significant differences occurred in the comparison between Potberg 1994 and De Hoop 1994 data for both between sites and between infection levels ( $P < 0.01$  and  $0.0001$ , respectively).

The average numbers of buds, flowering and fruiting capitulae, and drupes were lower on infected bushes in comparison with that of uninfected bushes, in the destructively sampled branches collected in 1995 (Tables 1 and 2), except in the case of the 25% infection level at Potberg.

Of the 20 bushes on which flowering on witches' brooms was recorded, there was a low production of buds [mean =  $0.176$  (s.e.  $\pm 0.03$ ) buds per branch] and flowering capitulae [mean =  $0.208$  (s.e.  $\pm 0.03$ ) flowers per branch] on 19 of these bushes. This is considerably less than that which occurred on uninfected branches, even at the highest levels of infection. Only one bush had a higher production than the others. It averaged  $2.55$  (s.e.  $\pm 1.02$ ) buds per branch and  $0.65$  (s.e.  $\pm 0.23$ ) flowering capitulae per branch. There were only 9 fruiting capitulae recorded on the 400 branches observed, with a total of 15 drupes. The viability of these drupes was not determined.

## DISCUSSION

The presence of witches' brooms caused by *E. osteospermi* was associated with lower vegetative growth of the remaining uninfected branches of its host *C. monilifera* ssp. *monilifera*, and in general the higher the infection level the greater this reduction. The reduced growth may have been due to one of two possible mechanisms. Either, some unknown factor that reduces the growth of the bushes predisposes them to high levels of infection, or infection leads to a reduction in growth. Of these two possibilities, the latter is the more likely.

The populations of *C. monilifera* ssp. *monilifera* used in this study were well established, consisting mainly of mature bushes in areas that had not been burnt for approximately six to ten years, and are examples of dense host populations occurring naturally in South Africa. Populations of the host plant are even-aged in South Africa, as they establish following fire (or other major disturbance) with little subsequent recruitment (Scott 1996). Due to the maturity of the host plant stands the populations of *E. osteospermi* studied had had an opportunity to become established. The rainfall occurring at these sites in the two previous and during the study years was favourable for successful infection, allowing an increase in the pathogen populations during the study years (data not presented). Most witches' brooms developed from infections during these years, with only very heavily infected bushes having been infected during earlier years (data not presented). These sites therefore represent the natural impact of *E. osteospermi* on its host under ideal conditions in its native range. All sites were in diverse mixed stands of natural scrub vegetation.

Infection levels on different bushes were very variable. This may be partly due to variation in susceptibility between bushes, variation in host susceptibility to rust fungi is known in several natural pathosystems (Kojwang 1994; Lawrence and Burdon 1989). The variation may also be partly due to unequal distribution of the primary inoculum infecting plants after establishment of each population (Jacobi *et al.* 1993), and to unequal subsequent spread of the pathogen within each host plant population because of variation in environmental factors (Pady 1942; van der Kamp 1988) and position of host plants relative to infected hosts and intervening non-host plants (Dinoor and Eshed 1984).

The anomalous data of Potberg 1994 can be explained by the effect of the very heavily infected bushes undergoing rapid growth of their few uninfected branches in an attempt to compensate for the high levels of die-back that occurred. Of the five bushes used, two died

during the course of the study and the other three died later that same summer. The die-back was probably caused by secondary pathogens (Cother *et al.* 1996) or the combination of infection and drought stress (Neser and Morris 1984).

Infection by *E. osteospermi* was also associated with lower reproductive output (buds, flowering capitulae, fruiting capitulae, and drupes) of uninfected branches of its host *C. monilifera* ssp. *monilifera* compared with that of uninfected bushes. The reduction in flowering and fruiting of individual branches shown here, however, does not adequately describe the full effect of infection on a whole bush's reproductive output. There are three complementary effects of infection: (i) the volume of the bush occupied by witches' brooms is almost capitulum free, (ii) individual uninfected branches produce fewer capitulae, and (iii) a decrease in vegetative growth translates in following years to a smaller plant reproducing less. Infection can thus have a large impact on potential total seed production over the lifespan of individual host bushes. Witches' brooms or whole host plants systemically infected by rust fungi often do not produce flowers and seed, such as with those caused by *M. cerastii* (Pady 1946) and *Puccinia punctiformis* (F. Strauss) Röhl. (Thomas *et al.* 1994).

This study gives data on the potential impact of a candidate biocontrol agent on its host in its native land. The author is aware of only one other example where the impact of a pathogen on its host under natural conditions in its native land was assessed before its use as a classical biocontrol agent. This was *Puccinia chondrillina* Bubák used against *Chondrilla juncea* L. In its native range in Europe, the rust fungus *P. chondrillina* substantially increased *C. juncea* mortality compared with uninfected plants (Hasan and Wapshere 1973). Such studies would be useful in determining the differences between natural and biocontrol pathosystems, and thereby possibly increase the accuracy of predicting whether an agent would be successful as a biocontrol agent or not. Field studies are also preferable to glasshouse studies to determine the potential impact of the selected agent before introduction onto a new continent, as studies under artificial conditions may give an overestimate (Watson 1991).

The results indicate that *E. osteospermi* is potentially a good biocontrol agent for use against *C. monilifera* ssp. *monilifera*, reducing the growth and reproduction of its host. To maximise its effect, *E. osteospermi* should, however, be used in conjunction with other agents such as the recently released seed fly *Mesoclanis magnipalpis* Bezzi (Edwards *et al.* 1999)

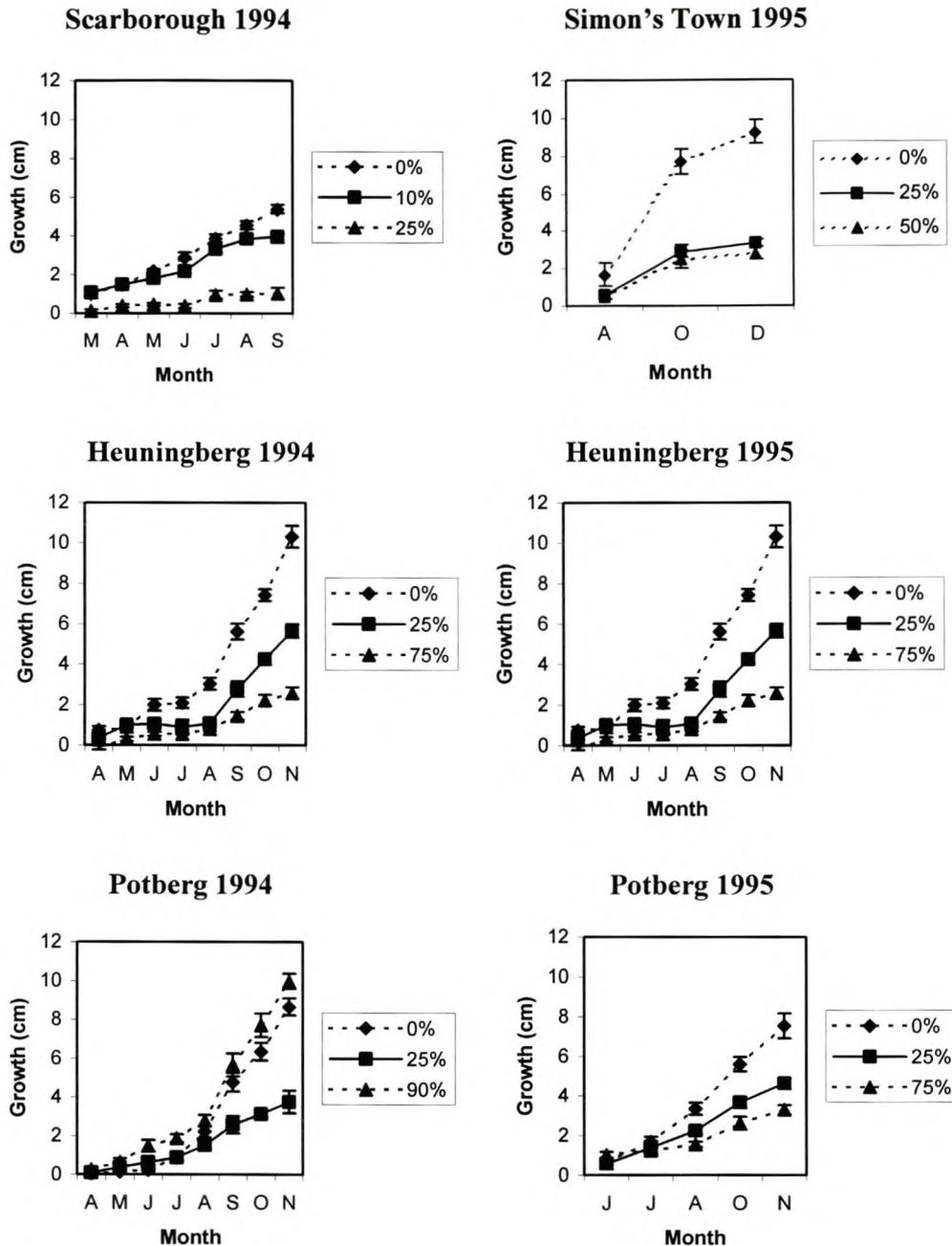
where the reduction in seed production caused by the combined action of both agents should have a significant impact on dispersal of the weed into new areas and recruitment into present stands.

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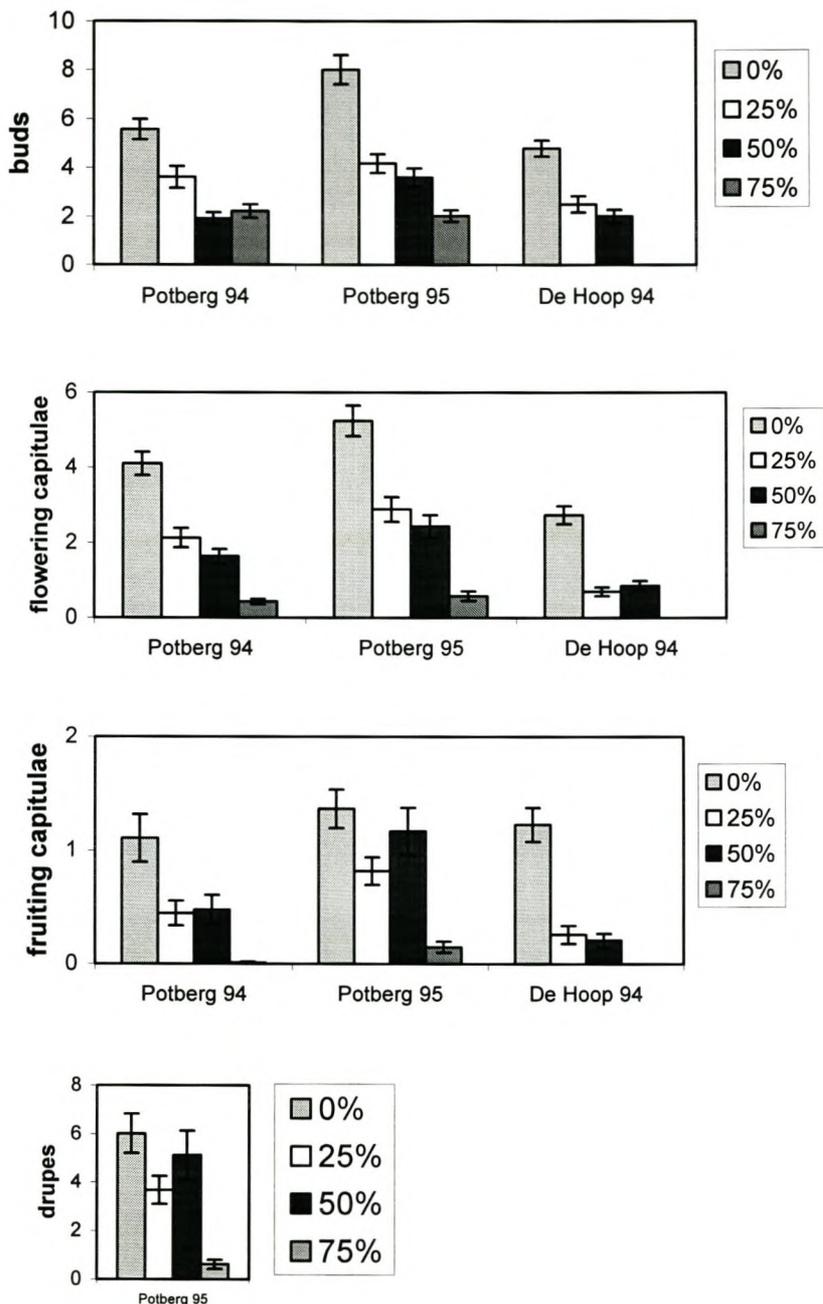
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**Figure 1.** Average accumulated growth in length (with standard errors) of branches of *Chrysanthemoides monilifera* ssp. *monilifera* bushes with different levels of infection by *Endophyllum osteospermi*, at three sites in the Western Cape Province during 1994 and 1995. (The Simon's Town site was substituted for the Scarborough site in 1995, as most plants at the latter site had died by 1995).



**Figure 2.** Average ( $\pm$ SE) numbers of buds, flowering capitulae, fruiting capitulae and drupes per branch of *Chrysanthemoides monilifera* ssp. *monilifera* with different levels of infection by *Endophyllum osteospermi*, at two sites in the Western Cape Province during 1994 and 1995.

**Table 1. Average<sup>A</sup> measurements and dry weights ( $\pm$  standard error) of symptomless branches on bushes of *Chrysanthemoides monilifera* at Potberg<sup>B</sup> with witches' brooms caused by *Endophyllum osteospermi*.**

	0 % infection <sup>C</sup>	25 % infection	75 % infection
branch length (cm)	13.17 ( $\pm$ 0.47)	11.24 ( $\pm$ 0.55)	9.53 ( $\pm$ 0.42)
branch dry weight (g)	0.315 ( $\pm$ 0.0248)	0.229 ( $\pm$ 0.0239)	0.155 ( $\pm$ 0.0194)
dry weight/ cm branch length (g/cm)	0.021 ( $\pm$ 0.0011)	0.017 ( $\pm$ 0.0009)	0.014 ( $\pm$ 0.001)
Total number of leaves / branch	24.42 ( $\pm$ 1.15)	18.39 ( $\pm$ 1.11)	14.65 ( $\pm$ 1.08)
Total dry weight of leaves / branch (g)	0.943 ( $\pm$ 0.0592)	0.602 ( $\pm$ 0.0494)	0.521 ( $\pm$ 0.0426)
Dry weight / leaf (g)	0.04 ( $\pm$ 0.0017)	0.032 ( $\pm$ 0.0017)	0.035 ( $\pm$ 0.0012)
Length of largest leaf (mm)	40.42 ( $\pm$ 0.75)	37.23 ( $\pm$ 0.744)	37.51 ( $\pm$ 0.585)
Dry weight of largest leaf (g)	0.087 ( $\pm$ 0.0035)	0.062 ( $\pm$ 0.0026)	0.063 ( $\pm$ 0.0022)
Number flower buds / branch	8.84 ( $\pm$ 0.81)	7.44 ( $\pm$ 0.65)	2.5 ( $\pm$ 0.3)
Number flowering capitulae / branch	0.91 ( $\pm$ 0.16)	1.34 ( $\pm$ 0.17)	0.13 ( $\pm$ 0.04)
Number of fruiting capitulae / branch	0.04 ( $\pm$ 0.02)	0.24 ( $\pm$ 0.07)	0.01 ( $\pm$ 0.01)
Total number of drupes produced on 100 branches	39	63	4
Total number of fruiting capitulae	7	13	1
Average number of drupes / fruiting capitulum	5.57	4.85	4

<sup>A</sup>Averages are of 20 branches per bush, and 5 bushes per infection level.

<sup>B</sup>Branches were collected 28/6/1995, and consisted of a single branch from the second growth flush of the 1994 growth season, together with any growth that had occurred during 1995.

<sup>C</sup>Level of infection, calculated as the volume of each bush consisting of witches' brooms.

**Table 2. Average<sup>A</sup> measurements and dry weights ( $\pm$  standard error) of symptomless branches on bushes of *Chrysanthemoides monilifera* at Heuningberg<sup>B</sup> with witches' brooms caused by *Endophyllum osteospermi*.**

	0 % infection <sup>C</sup>	25 % infection	50 % infection
branch length (cm)	16.5 ( $\pm$ 0.69)	11.7 ( $\pm$ 0.49)	11.4 ( $\pm$ 0.96)
branch dry weight (g)	0.53 ( $\pm$ 0.04)	0.25 ( $\pm$ 0.02)	0.2 ( $\pm$ 0.02)
dry weight/ cm branch length (g/cm)	0.028 ( $\pm$ 0.0013)	0.022 ( $\pm$ 0.0028)	0.018 ( $\pm$ 0.0024)
Total number of leaves / branch	27.15 ( $\pm$ 1.57)	20.95 ( $\pm$ 1.17)	16.38 ( $\pm$ 1.01)
Total dry weight of leaves / branch (g)	1.043 ( $\pm$ 0.0711)	0.662 ( $\pm$ 0.049)	0.65 ( $\pm$ 0.0653)
Dry weight / leaf (g)	0.038 ( $\pm$ 0.0014)	0.033 ( $\pm$ 0.0026)	0.041 ( $\pm$ 0.0044)
Length of largest leaf (mm)	44.46 ( $\pm$ 0.898)	37.78 ( $\pm$ 0.666)	37.03 ( $\pm$ 0.879)
Dry weight of largest leaf (g)	0.078 ( $\pm$ 0.0039)	0.062 ( $\pm$ 0.0032)	0.064 ( $\pm$ 0.0042)
Number flower buds / branch	9.19 ( $\pm$ 0.74)	7.9 ( $\pm$ 0.66)	4.34 ( $\pm$ 0.34)
Number flowering capitulae / branch	2.38 ( $\pm$ 0.25)	0.56 ( $\pm$ 0.1)	0.27 ( $\pm$ 0.08)
Number of fruiting capitulae / branch	0.68 ( $\pm$ 0.12)	0.18 ( $\pm$ 0.05)	0.15 ( $\pm$ 0.05)
Total number of drupes produced on 100 branches	192	46	51
Total number of fruiting capitulae	32	14	11
Average number of drupes / fruiting capitulum	6	3.3	4.6

<sup>A</sup>Averages are of 20 branches per bush, and 5 bushes per infection level.

<sup>B</sup>Branches were collected 31/7/1995, and consisted of a single branch from the second growth flush of the 1994 growth season, together with any growth that had occurred during 1995.

<sup>C</sup>Level of infection, calculated as the volume of each bush consisting of witches' brooms.

**Table 3. Average<sup>A</sup> branch length and number of leaves ( $\pm$  standard error) of symptomless branches on bushes of *Chrysanthemoides monilifera* at Simon's Town<sup>B</sup> with witches' brooms caused by *Endophyllum osteospermi*.**

	0 % infection <sup>C</sup>	25 % infection	50 % infection
Branch length (cm)	25.03 ( $\pm$ 0.804)	18.16 ( $\pm$ 0.729)	13.54 ( $\pm$ 0.518)
Number of leaves	21.02 ( $\pm$ 0.697)	17.39 ( $\pm$ 0.639)	15.6 ( $\pm$ 0.56)

<sup>A</sup>Averages are of 20 branches per bush, and 5 bushes per infection level.

<sup>B</sup>Branches were collected 7/11/1995, and consisted of a single branch from the first growth flush of the 1995 growth season.

<sup>C</sup>Level of infection, calculated as the volume of each bush consisting of witches' brooms.

### 3. Epidemic increase of *Endophyllum osteospermi* (Uredinales, Pucciniaceae) on *Chrysanthemoides monilifera*

#### ABSTRACT

The epidemiology of the rust fungus *Endophyllum osteospermi* was investigated. This rust fungus is considered to be a candidate biological control agent for *C. monilifera* ssp. *monilifera*, which is an invasive alien weed of native vegetation in south-eastern Australia. Between 10 and 20 plants of *C. monilifera* were marked at each of five sites in the Western Cape Province of South Africa, where both organisms are native. The infection levels and number of witches' brooms were determined every two months over a two year period. Additionally, at three of these sites, the infection levels and number of witches' brooms of all bushes in the host population was determined annually over four years. The increase in number of witches' brooms per bush ranged between 0 and 282 within one year, with an average increase per bush of 28 (SE  $\pm$  4.8) in 1993, and 39 (SE  $\pm$  9.2) in 1994. The average  $r_s$  for all bushes during 1993 was 0.015 month<sup>-1</sup> (SE  $\pm$  0.0041, n = 72) and 0.0098 month<sup>-1</sup> (SE  $\pm$  0.0073, n = 43) during 1994. When host bushes that either died back or died during the course of each year were excluded, then the average  $r_s$  during 1993 was 0.023 month<sup>-1</sup> (SE  $\pm$  0.0048, n = 45) and 0.0348 month<sup>-1</sup> (SE  $\pm$  0.0106, n = 20) during 1994. Under suitable conditions in South Africa, *E. osteospermi* undergoes epidemic increase within its host plant's populations. This rust fungus should therefore be considered as a suitable candidate biological control agent for use in Australia against *C. monilifera* ssp. *monilifera*.

#### INTRODUCTION

Selection of successful candidate classical biological control agents in their native range is fraught with difficulties. Candidate agents should be damaging under natural conditions, in addition to being host specific (Watson, 1991). Candidate agents in their native range should also preferably have a high incidence and be generally widespread on their host plants (Evans, 2000). In the case of several highly successful classical biological control programmes using rust fungi, selection of the agents were based primarily on field observations that revealed the pathogens to be widespread

and occurring at high incidences in their natural range. Subsequent to release, they rapidly increased and reduced their weed host's density. Examples include *Maravalia cryptostegiae* (Cummins) Y. Ono. released in Australia (Evans, 1993, 2000; Vogler *et al.*, 2002), *Puccinia chondrillina* Bubák (Hasan and Wapshere, 1973) employed in Australia (Burdon *et al.*, 1981) and the U.S.A. (Supkoff *et al.*, 1988), and *Uromycladium tepperianum* (Sacc.) McAlpine used in South Africa (Morris, 1991, 1997).

Unfortunately, anticipated success has not always been achieved. The perennial systemic rust fungus *Puccinia punctiformis* (F. Strauss) Röhl. can cause reduced growth and reproduction (Thomas *et al.*, 1994) and high mortality of infected *Cirsium arvense* (L.) Scop. plants (Watson and Keogh, 1981), and has long been considered a potential biocontrol agent (Frantzen, 1994). An investigation into its incidence in its native range demonstrated that this rust fungus generally occurred at low incidences (1–14% in four naturally infected populations over 2 years), though it was recorded having localised foci of infection with high incidence (Frantzen, 1994). It was therefore concluded that this rust fungus species would have to be used in an augmentative programme for it to be a successful biocontrol agent (Frantzen, 1994).

The autoecious, microcyclic rust fungus *Endophyllum osteospermi* (Doidge) A.R. Wood causes systemic infections, resulting in witches' brooms on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera* (Asteraceae, Calenduleae) (Morris, 1982). This host plant is a woody, perennial bush that is common in the Western Cape Province of South Africa (Norlindh, 1943). Abundant aecidioid telia are produced on the witches' brooms (Morris, 1982). The aecidioid teliospores germinate to produce vesicle-like modified basidiospores, which germinate to directly penetrate epidermal cells of immature host leaves. Penetration takes up to 72 hours after germination is initiated (Morris, 1982). Initial development of the witches' brooms is between 5 and 24 months after penetration, but they can persist for several years (Chapter 4). This rust fungus may therefore be considered as being monocyclic within years. The rust fungus survives through unfavourable climatic conditions in the perennial witches' brooms. Infection by this rust fungus is associated with a reduction in growth and reproduction of host plants under field conditions, especially when a large proportion of the host bush consists of witches' brooms. The growth of healthy branches (without witches' brooms) on infected bushes was 26 - 81% less than that of healthy branches on uninfected bushes. The number of buds, flowering capitulae,

fruiting capitulae, and drupes on individual healthy branches of infected bushes was 35 - 75%, 45 - 90%, 15 - 99%, and 15 - 90% less, respectively, than those on uninfected bushes (Chapter 2). Severely infected plants often die (Morris, 1982; Naser and Morris, 1984).

*Chrysanthemoides monilifera* spp. *monilifera* invades coastal and inland districts of Victoria, South Australia, New South Wales and Tasmania in cool-temperate winter-rainfall areas (Stahle, 1997). As the rust is primarily adapted to a temperate climate (Chapter 4), *E. osteospermi* is a candidate agent for the biological control of *C. monilifera* spp. *monilifera* in Australia (Scott and Adair, 1995; Adair and Edwards, 1996).

Field observations throughout the native range of *E. osteospermi* indicated that this rust fungus was either rare or absent in many populations of its host, despite being generally widespread, and having a high incidence and severity in certain host plant populations (Chapter 2). Thus although *E. osteospermi* has been shown to be potentially damaging to its host plant (Morris, 1982; Chapter 2), its variable incidence in its native range is of some concern. The present study was therefore aimed at determining if *E. osteospermi* increased its incidence and severity each growth season (i.e. epidemic increase), or remained at constant levels. The incidence and severity of *E. osteospermi* in selected, wild populations of *C. monilifera*, was determined during 1992 to 1995. The study was conducted in the Fynbos Biome in the winter rainfall region of the Western Cape Province of South Africa. This study provides a basis for comparison between a biocontrol agent in its natural and introduced range, if it is released in Australia in future.

## MATERIALS AND METHODS

**Increase of witches' brooms on individual host plants.** Five populations of *E. osteospermi* naturally infecting *C. monilifera* were studied, namely: 1) Heuningberg Nature Reserve, Bredasdorp 34°32'S 20°02'E (*C. monilifera* ssp. *monilifera*); 2) De Hoop Nature Reserve, E of Bredasdorp 34°32'S 20°02'E (*C. monilifera* ssp. *monilifera*); 3) Potberg Education Centre, De Hoop Nature Reserve, E of Bredasdorp 34°23'S 20°32'E (*C. monilifera* ssp. *monilifera*); 4) near Mossel Bay 34°10'S 22°05'E (1993 only) (*C. monilifera* ssp. *pisifera* (L.) Norl.); and 5) between

Bellvidere and Brenton-on-Sea, Knysna 34°05'S 23°00'E (*C. monilifera* ssp. *pisifera*).

The number of witches' brooms per plant on between ten and twenty selected host plants were recorded from within each study population, every two months from January 1993 until November 1994. The proportional volume of each bush consisting of witches' brooms (infection level) was also determined on each occasion. Bushes were selected so that there was a range of infection levels from each locality at initiation of the observations.

The simple interest infection rate ( $r_s$ ) was calculated annually for the estimated proportion of witches' brooms per bush (infection level) using the formula:

$$r_s = \frac{1}{t_2 - t_1} \cdot \log_e \frac{1 - x_1}{1 - x_2}$$

where  $t$  = time and  $x$  = infection level, and  $t_1$  and  $t_2$  were January and November respectively, and  $r_s$  was calculated to be expressed as per month (Vanderplank, 1963; Zadoks and Schein, 1979).

**Rust incidence within host populations.** At three sites (De Hoop and Potberg, and another site 4 km NW of Scarborough, Cape Peninsula 34°12'S 18°23'E (*C. monilifera* ssp. *monilifera*)), the area occupied by each plant population was divided into 5 m × 5 m quadrants and the position and relative size of each bush was recorded. The number of witches' brooms and infection level was determined for each bush annually from 1992 until 1995.

## RESULTS

**Increase of witches' brooms on individual host plants.** The larger the host bush the higher the initial number of witches' brooms that were recorded. However, considerable variation was observed in the number of witches' brooms recorded throughout the size (height) range of the shrubs assessed (Figure 1). The increase in number of witches' brooms for each bush during both years varied greatly between bushes, between sites, and between years. The average increase in witches' brooms per bush was 28 (SE ± 4.8, range of 0 to 282) in 1993, and 39 (SE ± 9.2, range of 1 to 251) in 1994. The maximum number of witches' brooms recorded on any single host

plant was 484 on a bush that initially had 202 witches' brooms, thus increasing by 282. This particular bush subsequently died during 1994. The maximum number of witches' brooms occurring in 1993 and 1994 against the initial number at the beginning of 1993 for each bush is shown in Figure 2. The total number of witches' brooms recorded at each site is given in Table 1.

Some bushes suffered die-back, or died during the period of observation, with a far higher proportion of bushes exhibiting die-back during 1994 than in 1993. Die-back and death was in many cases associated with a high infection level of *E. osteospermi*. Of the 15 bushes at the Bredasdorp, De Hoop, Potberg, and Bellvidere sites that had infection levels of 50% or more in January 1993, three died and seven suffered severe die-back during the course of the two years of monitoring. Of eight bushes at the Mossel Bay and Potberg sites that had infection levels of 50% or more, three died and one suffered severe die-back during 1993.

Specifically at the De Hoop site, bushes began to die of an unknown cause during 1994, and the entire population had died by the end of 1996. This was not associated with high levels of infection by *E. osteospermi*, as was the above die-back and death of bushes. The 1994 data obtained from this site was therefore excluded from analysis.

The number of bushes of *C. monilifera* with an increase, no change, or a decrease in infection level is given in Table 2. Except for the Bellvidere site, there were greater numbers of host bushes that had an increase in the number of witches' brooms rather than no increase or a decrease.

The average  $r_s$  for individual bushes during 1993 was  $0.015 \text{ month}^{-1}$  ( $\text{SE} \pm 0.0041$ ,  $n = 72$ , range  $-0.0693$  to  $0.1281 \text{ month}^{-1}$ ) and  $0.0098 \text{ month}^{-1}$  ( $\text{SE} \pm 0.0073$ ,  $n = 43$ , range  $-0.1609$  to  $0.1099 \text{ month}^{-1}$ ) during 1994. When host bushes that either died or died-back were excluded, then the average  $r_s$  during 1993 was  $0.023 \text{ month}^{-1}$  ( $\text{SE} \pm 0.0048$ ,  $n = 45$ , range  $0$  to  $0.1281 \text{ month}^{-1}$ ) and  $0.0368 \text{ month}^{-1}$  ( $\text{SE} \pm 0.0099$ ,  $n = 20$ , range  $0$  to  $0.1099 \text{ month}^{-1}$ ) during 1994.

**Rust incidence within host populations.** The incidence of symptomatic bushes for each of the three sites is given in Table 3. Incidence of witches' brooms increased at Scarborough (33 to 76.5%) and Potberg (13 to 48%), but remained approximately constant at the De Hoop site. There was a slight decrease in overall incidence at the Potberg site between 1994 and 1995, as there were approximately 55 newly recruited bushes. However, if the new recruits are excluded, then the incidence

increased to 54%. At the Scarborough site, an outbreak of a tortricid moth (Lepidoptera, Tortricidae) occurred during 1993 and continued into 1994. A high host plant mortality was associated with the severe feeding damage, though the feeding may not be directly responsible for the mortality, as reflected by the decrease in number of host plants present.

## DISCUSSION

A candidate biological control agent should preferably be damaging to its host (Watson, 1991) and be widespread and have a high incidence (Evans, 2000) in its native range, in addition to being host specific. *Endophyllum osteospermi* has been shown to be damaging to its host plant *C. monilifera* ssp. *monilifera* (Chapter 2) and is widespread in its native range. However, it has been observed to occur with a high incidence in only a limited number of host plant populations. Certain biocontrol agents which have limited incidences in their native range have subsequently had little success in controlling their target weed, such as *P. punctiformis* on *C. arvensis* (Frantzen, 1994). This raised a concern as to the potential of *E. osteospermi* as a biocontrol agent in Australia.

The increase in number of witches' brooms on individual host plants, the positive simple infection rates, and the within host plant population increase in both incidence and severity at all sites indicated that *E. osteospermi* undergoes epidemic increase under favourable environmental conditions. The generally low incidences of *E. osteospermi* under natural conditions in South Africa may therefore be due to various historical and climatic factors. The relatively frequent fires (usually at 5–15 yr intervals) that sweep through the areas where *C. monilifera* occurs (van Wilgen *et al.*, 1992) may be particularly important as they would cause local extinction of the rust after each fire. Populations of *C. monilifera* ssp. *monilifera* and ssp. *pisifera* in the Western Cape Province seldom exceed 15 years in age (Scott, 1996). The Scarborough site had been burnt approximately five years before this study was initiated. Despite the rapid increase in incidence of witches' brooms at the Scarborough site between 1992 and 1993, the severity remained fairly low in terms of both number of witches' brooms and infection level. This suggests that the rust had just begun to establish at this site. This population was subsequently decimated by an outbreak of a tortricid moth, preventing follow-up assessments of the development of

this epidemic. It does, however, indicate the speed with which this rust fungus can become established within populations of the host plant during periods of favourable environmental conditions. Rainfall over the known distribution of *E. osteospermi* is variable, both in terms of total precipitation and variation between years (Schulze, 1997). Localities where a high incidence of *E. osteospermi* have been observed have a minimum of 40 mm average rainfall per month in at least one month of the year, though most had more than 80 mm. However much of the area within the distribution of *E. osteospermi* has less than 40 mm average rainfall per month over the year (Chapter 4). In these low rainfall areas the rust generally occurred at low incidence or was absent. These two factors may well be the most important factors contributing to the observed limited number of host plant populations with a high incidence of *E. osteospermi* in South Africa.

Morris (1982) observed that host bushes with heavy infection levels of *E. osteospermi* frequently died. A number of heavily infected bushes died during the course of this study. These observations strongly suggest that high disease incidence is associated with a greater host mortality. The actual cause of death of the plant may be a greater susceptibility to drought (Neser and Morris, 1984), or invasion by secondary pathogens (Cother *et al.*, 1996; Cother, 2002).

*Endophyllum osteospermi* is capable of epidemic increase under suitable climatic conditions. It also reduces the growth and reproductive output of, and may lead to increased mortality rates, of its host plant. It therefore has potential as a biological control agent against *C. monilifera* ssp. *monilifera* in Australia. Data presented here provides a baseline for a comparison with the performance of *E. osteospermi* if it is released as a biocontrol agent. Such a comparison between incidence of an agent in its native and introduced range may assist in selection of other candidate agents that are ultimately successful.

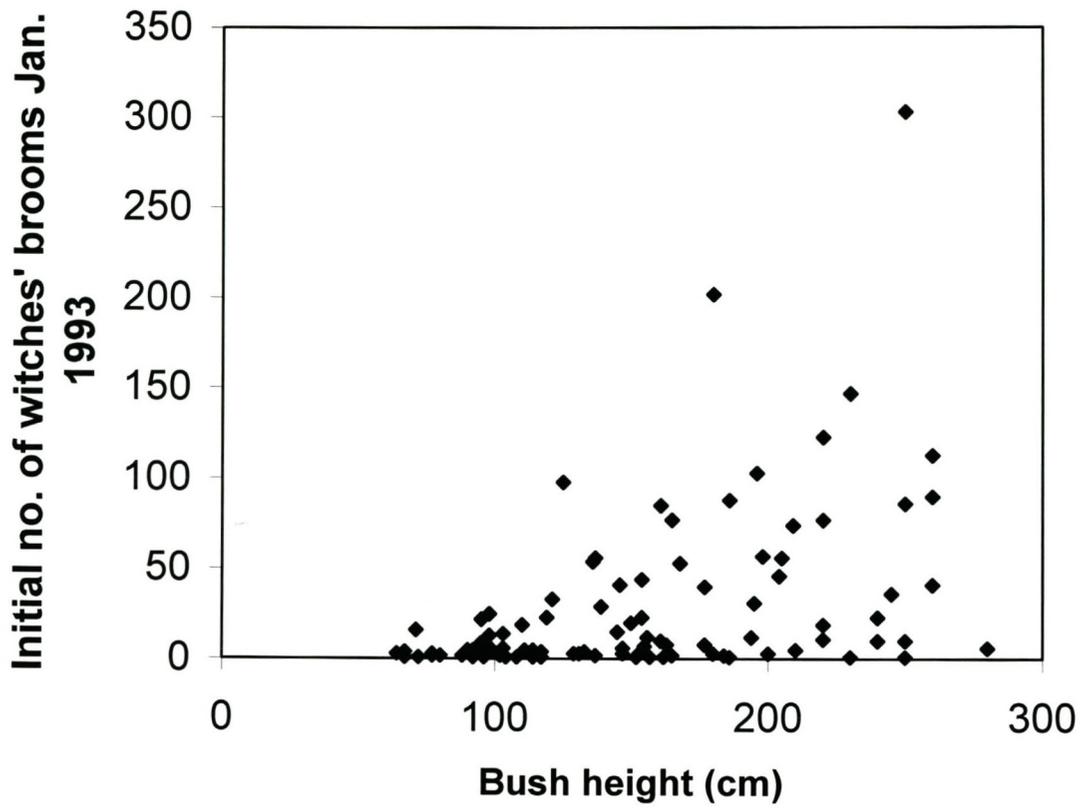
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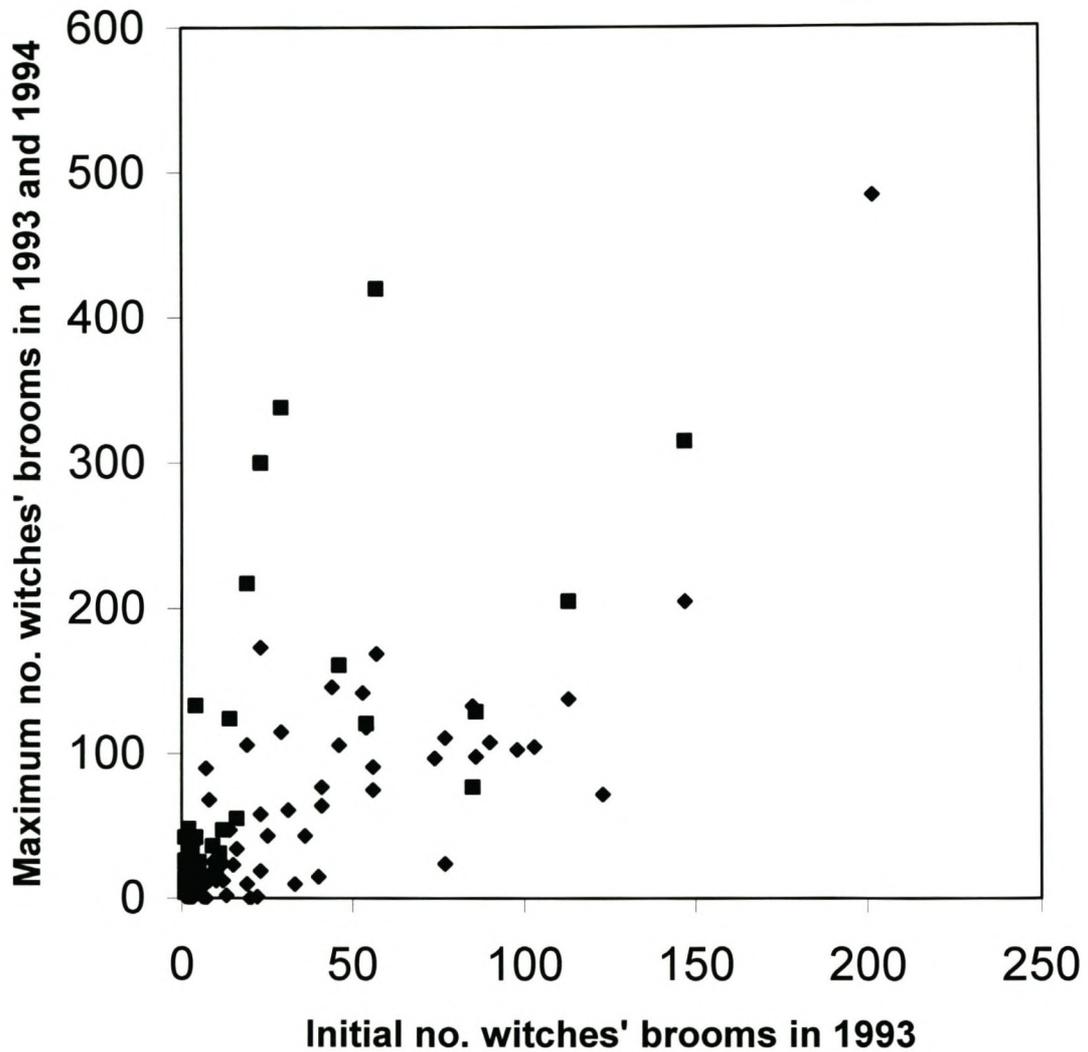
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**Figure 1.** The number of witches' brooms caused by *Endophyllum osteospermi* on individual bushes of *Chrysanthemoides monilifera* at five sites in the Western Cape, South Africa, during January 1993.



**Figure 2.** The maximum number of witches' brooms caused by *Endophyllum osteospermi* on marked bushes of *Chrysanthemoides monilifera* recorded during 1993 (diamonds) and 1994 (squares), against the initial number in January 1993, at five sites in the Western Cape, South Africa. Bushes that died or suffered die-back are excluded.

**Table 1. Total number of witches' brooms caused by *Endophyllum osteospermi* occurring on selected bushes in naturally infected populations of *Chrysanthemoides monilifera*.**

Year	Site	No. of bushes <sup>a</sup>	No. of witches' brooms					
			Jan.	Mar.	May	Jul.	Sep.	Nov.
<b>1993</b>	Bredasdorp	17	n.d. <sup>b</sup>	n.d.	165	295	429	542
	De Hoop	12	248	251	289	433	462	477
	Potberg	13	562	581	585	758	1276	1354
	Mossel Bay	8	182	195	335	351	388	449
	Bellvidere	13	356	400	348	446	497	558
<b>1994</b>	Bredasdorp	12	429	535	649	737	n.d.	822
	Potberg	5	421	525	594	733	1004	857
	Bellvidere	10	382	337	381	464	634	597

<sup>a</sup>Bushes that died or had die-back were excluded, total number of bushes monitored are indicated in Table 2.

<sup>b</sup>n.d. = not determined

**Table 2. Average simple interest rate of increase (month<sup>-1</sup>) of *Endophyllum osteospermi* on selected bushes of *Chrysanthemoides monilifera* at five sites in the Western Cape Province, South Africa, and the number of bushes with a positive, no, or negative change in severity of infection.**

Site	Year	Height of host bushes (cm) <sup>a</sup>	Total no. bushes	Change in infection level. <sup>b</sup>			No. bushes died	Average $r_s$ ( $\pm$ SE) month <sup>-1</sup>
				+	0	-		
Bredasdorp	1993	(64-) 104 (-220)	20	15	3	1	1	0.0127 ( $\pm$ 0.0053)
	1994		20	7	7	2	4	0.0137 ( $\pm$ 0.0101)
De Hoop	1993	(92-) 165 (-260)	15	8	3	1	3	0.0146 ( $\pm$ 0.0102)
	1994		15	2	2	1	10	n.d. <sup>c</sup>
Potberg	1993	(114-) 191 (-250)	10	7	3	0		0.0337 ( $\pm$ 0.0142)
	1994		10	6	2	2		0.037 ( $\pm$ 0.0165)
Mossel Bay	1993	(77-) 150 (-240)	10	5	4	1		0.0254 ( $\pm$ 0.0152)
Bellvidere	1993	(71-) 198 (-260)	17	5	9	3		-0.0007 ( $\pm$ 0.0067)
	1994		17	3	8	6		-0.0099 ( $\pm$ 0.0109)

<sup>a</sup>The (minimum-) average (-maximum) height of marked bushes.

<sup>b</sup>Number of plants with a change in infection levels, where the infection level is the proportion of each bush consisting of witches' brooms caused by *E. osteospermi*, in classes of 5, 10, 25, 50, 75 and 100% infected; + = increase, 0 = no change, - = decrease.

<sup>c</sup>n.d. = not determined

**Table 3. Percentage of *Chrysanthemoides monilifera* ssp. *monilifera* bushes in three populations in the Western Cape Province, South Africa, naturally infected with *Endophyllum osteospermi* at various infection levels.**

Site	Year	Total no. bushes	No. infected bushes	No. witches' brooms <sup>a</sup>	Infection level classes <sup>b</sup>						
					0%	5%	10%	25%	50%	75%	100%
Scarborough	1992	244	79	303	67 <sup>c</sup>	25	5	2	1	0	0
	1993	200	152	999	23.5	40	25	10	1	0.5	0
	1994	36	25	234	31	17	31	19	3	0	0
De Hoop	1992	128	65	—	49	35	9	3	1.5	2 <sup>d</sup>	0
	1993	119	71	872	40	29	19	10	1	1 <sup>d</sup>	0
	1994	116	64	873	45	30	10	11	3	0	0
Potberg	1992	154	20	168	87	11	1	1	1	0	0
	1993	164	54	1463	67	23	7	5	2	0	0.5
	1994	169	81	2478	52	31	8	5	2	1	2
	1995	216	92	2276	57.5	26	6	7	3	0.5	2 <sup>e</sup>

<sup>a</sup>Total number of witches' brooms on all infected bushes.

<sup>b</sup>Infection level classes determined as volume of host bush occupied by witches' brooms.

<sup>c</sup>Expressed as percentage of total number of bushes per infection level.

<sup>d</sup>Bushes died before the following year's monitoring.

<sup>e</sup>Excludes five additional bushes in this class that died during the year before the mapping was carried out.

#### **4. Predicting the distribution of *Endophyllum osteospermi* (Uredinales, Pucciniaceae) in Australia based on its climatic requirements and distribution in South Africa**

##### **ABSTRACT**

The perennial bush *Chrysanthemoides monilifera* ssp. *monilifera* (Asteraceae) is infected by the autoecious, microcyclic rust fungus *Endophyllum osteospermi*. Both organisms are native to South Africa, whilst the plant has also become naturalised in Australia where it is the target of a biological control programme. *Endophyllum osteospermi* is under consideration as a biocontrol agent for this programme. Temperature and light requirements for aecidioid teliospore germination and basidiospore development were studied, as was the nuclear cycle during germination. Aecidioid teliospores germinated between 10 and 20°C, with 15°C as optimum temperature. Light, and particularly near-uv light, stimulated germination whereas germination was poor under dark conditions. A period of 6 to 8 hours of light was the minimum needed to obtain germination levels equivalent to continuous light. The temperature requirements for basidiospore development differed from that for aecidioid teliospore germination. Optimal basidiospore production was at 15°C, but a rapid decrease occurred at higher temperatures, with few developing at 19°C, despite a high germination rate at this temperature. Two nuclear divisions occurred within 12 hours of germination initiation to produce a metabasidium with three or four nuclei. A third nuclear division occurred in the basidiospores that then germinated between 24 and 48 hours. Plants inoculated under controlled conditions took 5 to 24 months after inoculation for witches' brooms to begin to develop. The detailed life cycle of *E. osteospermi* is presented.

A Geographic Information System (GIS) approach was used to develop a model of the potential distribution of *E. osteospermi* in South Africa. This was based on monthly average climate surfaces with parameters derived from the above experiments. The parameters were modified so that the majority of all recorded localities of *E. osteospermi* in South Africa were included, whilst at the same time including only the minimum geographic area. The same model was applied to

Australia to suggest a potential distribution of the rust fungus if released in Australia for the biological control of *C. monilifera* ssp. *monilifera*. This potential distribution was similar to one generated using the climate matching computer programme CLIMEX<sup>®</sup>, but gave greater spatial accuracy, at least in South Africa. Both approaches indicate that *E. osteospermi* should establish in temperate south-eastern Australia, where *C. monilifera* ssp. *monilifera* is an invasive weed.

## INTRODUCTION

The perennial bush *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera* (Asteraceae: Calendulae), is native to the winter rainfall region of the Western Cape Province of South Africa. This plant is susceptible to an autoecious, microcyclic (endocyclic) rust fungus native to South Africa, which produces localised systemic infections causing witches' brooms (Morris, 1982). A reduction in growth and seed production of naturally infected host bushes is associated with the presence of witches' brooms (Chapter 2), and mortality of host bushes is associated with high levels of infection (Neser and Morris, 1984; Chapter 3). Pycnia and aecidioid telia develop predominantly on abaxial leaf surfaces and stems, and less commonly on adaxial leaf surfaces (Morris, 1982). Following germination of aecidioid teliospores, septa develop to produce 3–4 celled metabasidia (promycelia). Subsequently 2–3, and only rarely 4, vesicle-like structures develop from these cells, which are separated by septa from the metabasidium (Morris, 1982; Wood, 1998). These structures have been postulated as representing modified basidiospores (Gardner, 1988; Chen *et. al.*, 1996). The modified basidiospores are not dispersive structures, and do not separate from the promycelia (Morris, 1982; Wood, 1998). The production of basidiospores by what are morphologically aeciospores is typical of an endocyclic rust fungus. Originally this fungus was described as *Aecidium osteospermi* Doidge (Doidge, 1927), but because of its endocyclic nature it was transferred to *Endophyllum* as *E. osteospermi* (Doidge) A.R. Wood (Wood, 1998).

*Chrysanthemoides monilifera* ssp. *monilifera* is naturalised in south-eastern Australia. It is the target of a biological control programme, for which *E. osteospermi* is considered to be a suitable candidate organism (Scott and Adair, 1995; Adair and Edwards, 1996). Morris (1982) determined the effect of temperature on aecidioid teliospore germination, and also the nuclear cycle during germination. The work

reported on here was undertaken to better elucidate the effect of environmental conditions on spore germination and the development of basidiospores, as well as to verify the nuclear cycle of *E. osteospermi*.

The climate matching computer programme CLIMEX<sup>®</sup> (CSIRO, Australia) (Sutherst *et al.*, 1999) has been used to predict the potential distribution of both weeds (e.g. McFadyen and Skarratt, 1996; Holt and Boose, 2000) and biological control agents (e.g. Scott, 1992). A model was developed using CLIMEX, based on the epidemiological parameters determined in this study, that corresponded as closely as possible with the actual distribution of *E. osteospermi* in South Africa. This same model was then used to determine the potential distribution of *E. osteospermi* in Australia.

There are rapid changes in climatic variables over short geographical distances in South Africa within the distribution range of *E. osteospermi*, due to rapid altitude changes over the Cape Fold Mountains and the Drakensberg. There was a limited correspondence between the most accurate prediction of the CLIMEX model and the actual distribution of *E. osteospermi* in South Africa, because of these rapid changes in climate variables. Therefore a Geographic Information System (GIS) approach, using the computer programme ArcView<sup>®</sup> (Environmental Systems Research Institute, USA) was used to develop a model giving a more spatially explicit prediction of the potential distribution in South Africa, and this model was again applied to determine the potential distribution of *E. osteospermi* in Australia.

## METHODS

**Collection of inoculum.** Naturally infected plant material was collected from a site on the Cape Peninsula (2 km south of Simon's Town, 34°13'S 18°28'E), and placed in plastic bags. This material was stored at 5°C for 4–7 days after collection. Dry aecidioid teliospores were obtained from this material by means of a homemade collection devise. This devise was made by placing a small piece of filter paper over the end of a halved 2 ml plastic syringe, and placing this in one end of a 10 mm diam plastic pipe attached to an air pump. The spores were stored in plastic screw-top vials at 5°C until used.

**Effect of temperature and light on acidoid teliospore germination and basidiospore development.** Dry acidoid teliospores were sprinkled over the surface of water agar (1.5% agar, Biolab, Midrand, South Africa) in 6 cm diam Petri dishes, and incubated at 15°C for 24 h in an incubator with fluorescent white and near-uv light ( $13.63 \mu \text{ mol s}^{-1} \text{ m}^{-2}$ ), except where otherwise indicated. Three Petri dishes were used per treatment. The following experiments were done: 1) germination at 10, 15, 19, and 25°C under continuous light conditions; 2) germination at 15°C under continuous light or dark conditions; 3) germination at 15°C under 0 h light/24 h dark, 1 h light /23 h dark, 2 h light/22 h dark, 3 h light/21 h dark, 4 h light/20 h dark, 6 h light/18 h dark, 8 h light/16 h dark, and 24 h light/0 h dark conditions; and 4) germination at 15°C under continuous white or near-uv light only. One hundred randomly chosen spores were observed per Petri dish and the average percentage germination determined for each treatment in each experiment. Each of these 4 experiments was repeated three times.

In all the above experiments (except 4) both white and near-uv fluorescent lights were used in the incubators. For germination in the dark, the Petri dishes were placed in a cardboard box wrapped in a black plastic bag in the same incubator used for germination in the light.

Dry acidoid teliospores were sprinkled over the surface of water agar in 6 cm diam Petri dishes, and incubated as above, at 15, 17, and 19°C. After 48 h, one hundred randomly chosen germinated spores per Petri dish were observed and the average percentage with basidiospores determined for each temperature. This experiment was repeated three times.

**Longevity of acidoid teliospores, and their viability when stored at different temperatures.** A sample of acidoid teliospores was stored at 5°C for a two-month period. At weekly intervals dry acidoid teliospores were dusted over the surface of water agar in three 6 cm diam Petri dishes, and incubated as above. The average percentage germination for each week was determined after observing 100 randomly chosen spores per Petri dish. This experiment was repeated once.

A sample of acidoid teliospores was divided into four subsamples that were stored at 5, 15, 20 and 25°C. At weekly intervals (for 1 month), spores from each temperature were plated on water agar in three 6 cm diam Petri dishes and incubated as above. One hundred spores from each Petri dish were observed and the average

percent germination was calculated for each subsample stored at the respective temperatures. This experiment was repeated once.

**Nuclear staining of germinating aecidioid teliospores.** Dry aecidioid teliospores were sprinkled on water drops on glass microscope slides, placed on moist pieces of tissue paper in 9 cm diam Petri dishes, after which the Petri dishes were sealed and incubated at 15°C with fluorescent white and near-uv light as above. They were incubated for 2, 3, 4, 6, 9, 12, 24 and 48 h. The germinated spores were heat-fixed onto the slides after their respective incubation time, and then immersed in Carnoy's Solution overnight (25% acetic acid, 75% ethanol). The germinated spores were then hydrated and acidified (10 min immersion sequentially in each of 96% ethanol, 70% ethanol, distilled water, cold 1M HCl, hot (60°C) 1M HCl, and then rinsed several times in distilled water). They were then immersed for 10 min in freshly prepared phosphate buffer solution (55 parts of a stock aqueous solution of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (11.87 g  $\text{l}^{-1}$ ) mixed with 45 parts of a stock aqueous solution of  $\text{KH}_2\text{PO}_4$  (9.07 g  $\text{l}^{-1}$ )), following which they were stained until the nuclei were well differentiated by adding 2 drops of Gurr's improved R66 Giemsa's Stain (Gurr®, BDH Chemicals Ltd., Poole, England) per ml of phosphate buffer solution. The spores were then rinsed with buffer solution and examined with a Zeiss Axioskop (Germany) light microscope. Cross sections of mature aecidioid telia made by hand were also Giemsa-stained using the above method.

**Infection of whole plants.** Seedlings (5–10 cm high) of *C. monilifera* ssp. *monilifera* were collected from the field, potted in 20 cm diam plastic pots with a mixture of 1:1:1 of top soil, river sand and compost, and kept in a shade house. They were watered every second day and fed every 2 weeks with 5 g Chemicult® hydroponic nutrient solution (Chemicult Products, Camps Bay, South Africa). When they were approximately 30 to 50 cm in height they were inoculated with an aqueous aecidioid teliospore suspension by means of an airbrush, and incubated for 3 days in a dew chamber in a growth room at 10–16°C. The spore suspension was applied to all leaf and stem surfaces with particular attention paid to the immature leaves at the growing tip of all branches. A total of 12 plants were inoculated in 1995, and a further 22 were inoculated in 1996. The plants were regularly observed for the development of witches' brooms for up to two years after inoculation. New shoots that appeared to be witches' brooms, but which had not developed pycnia or aecidioid telia, were

confirmed as infected by microscopically examining hand-made cross sections of leaves. The sections were cleared for 30 min in Carnoy's Solution and stained (2 min in 0.05% aniline blue in lactophenol), and then observed with a Zeiss Axioskop light microscope for the presence of hyphae and coiled haustoria (Morris 1982).

**The possible distribution of *E. osteospermi* in Australia.** The distribution of *E. osteospermi* in the Western Cape, Eastern Cape and KwaZulu Natal Provinces of South Africa was determined on an *ad hoc* basis during the period 1992 to 2003, and representative herbarium specimens were deposited in the South African National Collection of Fungi, ARC-PPRI, Pretoria (PREM). A total of 93 localities were recorded.

CLIMEX<sup>®</sup> (CSIRO, Australia) is a dynamic simulation model enabling the prediction of an organism's geographical range using climatic parameters (Sutherst *et al.* 1999). CLIMEX was used to create a model that predicted the distribution of *E. osteospermi* in South Africa, using environmental parameters as determined by the above experiments. The model was then modified until it best approximated the known distribution of *E. osteospermi* in South Africa, and then run to generate a potential distribution for Australia. The method used was described in detail by Scott (1992) and McFadyen and Skarratt (1996).

GIS allows spatially explicit presentation of data. Monthly and annual climate data are available on a 1.6×1.6 km grid scale for South Africa (Schulze, 1997), and on a 2.5×2.5 km or a 25×25 km grid scale for Australia (Bureau of Meteorology, Melbourne, Australia). Various data sets can be combined in any combination. A theoretical GIS approach was used in which the effect of environmental conditions on aecidioid teliospore germination and basidiospore development formed the basis for creating a map, using the computer programme ArcView GIS 3.1<sup>®</sup> (Environmental Systems Research Institute, USA), showing an approximation of the likelihood of infection by *E. osteospermi* occurring during July in South Africa, assuming that both host plant and inoculum are present. It was assumed that the potential geographic distribution of *E. osteospermi* was equivalent to this likelihood of infection. Localities of *E. osteospermi* recorded between the Cape Peninsula and Tsitsikamma were used to verify this map.

The 'average monthly maximum temperature' and 'average monthly rainfall' surfaces for July (Schulze, 1997) were first utilized. This was because the majority of

recorded localities fell within the winter or all-year rainfall regions of South Africa. These surfaces were intuitively reclassified so that the maximum number of localities was incorporated. The starting point for this reclassification was based on the optimum environmental conditions determined above. The generated map was refined using the '80% probability of annual rainfall' surface (Schulze, 1997), which was reclassified so that the predicted distribution incorporated as small a surface area as possible. Classes used were: 1) 'average July maximum temperature'  $\leq 20^{\circ}\text{C}$ ; 2) 'average July rainfall' 21–40 mm, 41–80 mm and  $\geq 81$  mm; 3) '80% probability of annual rainfall'  $\geq 301$  mm. The three average monthly rainfall classes were used to show differing potential for infection, the lower the rainfall the lower the potential and the higher the rainfall the higher the potential. These classes were fitted to where *E. osteospermi* had been observed to be either scarce or abundant for at least several years of observation at certain localities.

Subsequently, using the same classes for the relevant surfaces, maps were generated for the months of September, November and January in South Africa, and for July, September, November and January in Australia. The Australian '10 percentile of annual rainfall' surface was used as the equivalent of the South African '80% probability of annual rainfall' (Bureau of Meteorology, Melbourne). These four months were chosen as representative for the whole year.

## RESULTS

**Field observations on *E. osteospermi*.** Pycnia were produced throughout the year on young growth of witches' brooms in advance of the aecidioid telia on individual leaves, regardless of the age of the witches' broom. Production of aecidioid telia appeared to be dependent on the amount and duration of rainfall in the wet season, as growth of the witches' brooms and production of new aecidioid telia only occurred while the host was actively growing (data not presented). Host plant growth occurs during winter and spring (approximately June to November) in the winter rainfall region of South Africa. It was noted that spores collected during hot summer months, although appearing healthy, often had a low viability. Because of this, aecidioid teliospores were only collected for use during winter and spring. During the rain season aecidioid teliospores were produced in abundance.

**Effect of temperature and light on aecidioid teliospore germination and basidiospore development.** The highest percent germination of aecidioid teliospores occurred at 15°C, with 20°C having the second highest germination (Figure 1). Light, in particular near-uv light, promoted germination (Table 1), and 6–8 h of light was the minimum period needed for optimum germination (Table 2). The optimal temperature for basidiospore production was 15°C. At 17°C basidiospore production was much less than that at 15°C, and very few developed at 19°C (Table 3), even though there was a high percentage of aecidioid teliospores that had germinated at both 17 and 19°C.

**Longevity of aecidioid teliospores, and their viability when stored at different temperatures.** When stored at 5°C the aecidioid teliospores maintained their viability for the eight weeks tested (data not presented). Aecidioid teliospores maintained their viability for two weeks (after collection in the field) regardless of the temperature at which they were stored. After this the viability of spores stored at 20 and 25°C declined rapidly, whereas those stored at 15°C declined less rapidly. Spores stored at 5°C maintained their viability over the four weeks of testing (Figure 2).

**Nuclear staining of germinating aecidioid teliospores.** The spore mother cells within the aecidioid telium were binucleate and divided to produce two binucleate cells. The proximal cell remained binucleate, becoming the intercallary cell. The distal cell expanded and became an aecidioid teliospore, which was binucleate with two small nuclei when immature but became uninucleate with one large nucleus at maturity (Figure 3). All mature spores observed were uninucleate. During spore germination the following sequence of events occurred: 1) after 3 h of incubation the single nucleus had moved out of the spore into the growing metabasidium (Figure 4); 2) after 6 h two nuclei were present in the metabasidium after a first nuclear division (Figure 5); 3) after 9 h a first septum had been produced in the metabasidium and four nuclei were present after a second nuclear division; 4) after 12 h one or two more septa had been produced dividing the metabasidium into three or four cells, and two, three or four vesicle-like modified basidiospores had developed, and single nuclei had migrated into each of the basidiospores (Figure 6); and 5) after 24 h two nuclei were usually present in at least one basidiospore and after 48 h all basidiospores had two nuclei after a third nuclear division (Figure 7).

**Infection of whole plants.** Of the 12 plants inoculated in 1995, four developed witches' brooms during 1996 and a further two developed them during 1997. Of the 22 plants inoculated in 1996, four developed witches' brooms in 1997 and a further 10 developed them during 1998. Of these witches' brooms, the first began to develop on a plant five months after inoculation. The last witches' broom began to develop 24 months after inoculation. Systemic infection of all witches' brooms was confirmed either by the presence of pycnia and aecidioid telia, or by microscopic examination of leaf cross sections to detect the intercellular hyphae and tightly coiled intracellular haustoria produced by this rust fungus.

**The possible distribution of *E. osteospermi* in Australia.** Temperature and rainfall were the most important climate parameters in the model generated using CLIMEX. The distribution predicted in South Africa corresponded to higher rainfall areas of the temperate region (with both winter and all-year rainfall seasons) in South Africa (Figure 8). The predicted Australian distribution corresponded with the temperate region of Australia (Stern *et al.*, 2003) (Figure 9).

The predicted distribution of *E. osteospermi* generated with the GIS approach for the months of July, September, November and January for both South Africa (Figure 10) and Australia (Figure 11) are presented. A total of 75 localities fell within the area of possible distribution for the month of July, and only 18 outside. Most of these latter localities were in the summer rainfall region. The predicted distribution of *E. osteospermi* generated with CLIMEX<sup>®</sup> was similar to the above for Australia, but was more restricted in South Africa.

## DISCUSSION

Morris (1982) provided details on the macroscopic and microscopic symptoms of infection by *E. osteospermi* on its host plant *C. monilifera* ssp. *monilifera*, as well as the effect of temperature on germination of its spores and its nuclear cycle. The work reported on here was carried out to verify and expand on this existing knowledge, as a preliminary step to developing a reliable method of inoculation for host specificity testing of *E. osteospermi*. In a few respects conclusions drawn from the work reported on here differ to those of Morris (1982).

Morris (1982) stated that pycnia production occurred only at the beginning of the growth season, occurring only during March and April, and preceded the

development of any aecidioid telia on whole witches' brooms. This coincides with the onset of the winter rains in the south-western Cape. It was observed, however, that pycnia production occurred throughout the growth season, preceding development of aecidioid telia on each newly produced leaf regardless of when these grew.

Morris (1982) reported 16°C to be the optimal temperature for germination, with good germination occurring at 12 and 20°C as well. This was verified in this study. However, he recorded germination after only 6 h, and therefore did not observe that basidiospore development had a different temperature requirement to aecidioid teliospore germination. Thus despite good germination at 19–20°C, infection is unlikely to occur at these temperatures as few basidiospores develop. Considering the lower levels of germination at 10–12°C, there is a narrow range around 15°C of optimal temperature conditions for infection of the host plant to occur. This is discussed further below.

The nuclear cycle was essentially as reported by Morris (1982), apart from that he stated that typically the modified basidiospores were uninucleate except for the occasional binucleate one due to two nuclei migrating into it. Unfortunately no times of these observations were given. It was found in this study that the basidiospores always became binucleate by means of a nuclear division, this nuclear division taking place between 24 and 48 h. Rust fungi basidiospores are commonly binucleate (Gold and Mendgen, 1991). It was observed that often the nucleus in the proximal cell to the aecidioid teliospore did not undergo a second nucleus division, producing a metabasidium with only three nuclei. In all such cases, only 2 basidiospores were produced.

Field observations indicated that witches' brooms from branches produced during a single year's growth developed only at the end of that growth season, whereas on branches produced during the previous one or two years growth witches' brooms would develop even right at the beginning of the growth season. This indicated that initiation of a witches' broom took from several months to over 2 years from the time at which infection occurred. This was verified by the whole plant inoculations in which witches' brooms took between 5 and 24 months to begin to develop. Field observations indicate that individual witches' brooms survive from several months to a few years once developed. The detailed life cycle of *E. osteospermi* has therefore been elucidated and is shown in Figure 12.

*Endophyllum osteospermi* is well adapted to a temperate climate with seasonal rainfall. The production of localised systemic infections (witches' brooms) on a perennial non-senescent woody host allows for over-summering (during warm to hot dry summers) or over-wintering (during cool dry winters) without the need for producing the typical rust fungus dormant spores (teliospores). This habit was essential in allowing the contraction of the life cycle to an endocyclic one. Microcyclic life cycles commonly occur in rust fungi as an adaptation to harsh environments, such as desert (Anikster and Wahl, 1979), arctic (Savile, 1953) and alpine (Savile, 1964) conditions. These environments all have in common short growth seasons, as does the environment in which *E. osteospermi* and its host plant *C. monilifera* ssp. *monilifera* occur naturally in South Africa. The perennial mycelium also allows infective spores to be produced very rapidly with the onset of suitable weather. Further adaptations to a temperate climate are the light requirements for spore germination, and the low temperature requirement for the production of modified basidiospores. These two adaptations in combination ensure that the spores are more likely to germinate during a rain period rather than in overnight dew. This is particularly important given the length of time (2 to 3 days) necessary for penetration into the host leaf epidermis to occur (Morris, 1982; Wood, 1997).

The theoretical GIS exercise done here to suggest the potential distribution of *E. osteospermi* was possible because of having two data sets. Firstly laboratory studies of the effect of environmental conditions on germination of aecidioid teliospores and production of basidiospores of *E. osteospermi* (and therefore of the climatic conditions necessary for infection), and secondly also details on the distribution of this rust fungus in South Africa. A simple GIS model was created that gave a good approximation to the actual distribution in South Africa. The suggested distribution of this rust fungus is equivalent to the likelihood of infection given that a suitable host plant is present, and the greater the number of months in which a specific locality has a likelihood of infection then the greater the chance of the rust being present and persistent at that locality. Also the higher the rainfall the greater the likelihood of infection occurring. The possible distribution of *E. osteospermi* does not necessarily coincide with the distribution of its host plant in South Africa. The potential distribution includes some areas where the plant is absent, and a large

portion of the distribution of *Chrysanthemoides* is either unsuitable or marginal for *E. osteospermi*.

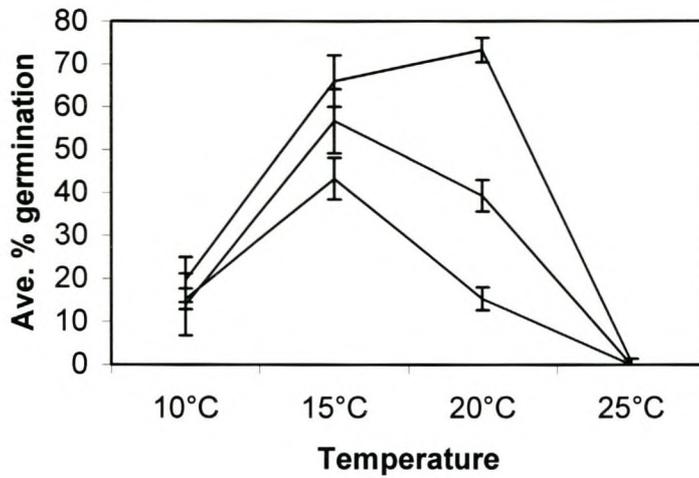
Based on the optimal environmental conditions determined by this study, and verified against the actual distribution of *E. osteospermi* in South Africa, the potential distribution of this rust fungus in Australia has been postulated. This area corresponds to the “No dry season (warm summer)” and “No dry season (mild summer)” subdivisions of the Temperate climatic region of Australia (Stern *et al.*, 2003). The potential distribution (of July only) extended into a limited part of the Tropical climate region of Australia, an unexpected result. This may be due to differences in climate between South Africa and Australia, in particular the high annual rainfall which occurs throughout the year in parts of the Tropical climate region of Australia. No area in South Africa has a comparable climate, and therefore was not a factor in developing the model. It is unlikely that *E. osteospermi* would actually establish in these tropical areas, due to unfavourable temperatures for most of the year. In changing time scales by using average monthly climate data to approximate daily climate data, a degree of error would have been introduced. Interpretation of the results of such an exercise as done above should always be done with caution, but should not detract from its usefulness.

Though largely agreeing with the GIS approach used here, CLIMEX also indicated that the “Distinctly dry (and mild) summer” subdivision of the Temperate climatic region of Australia (Stern *et al.*, 2003) is suitable for infection to occur. This rust fungus, like its host plant *C. monilifera* ssp. *monilifera*, is likely to establish in temperate south-eastern Australia if introduced as a biological control agent. It is interesting to note that apparent ideal environmental conditions for *E. osteospermi* occur over a much larger geographical area in Australia than in South Africa. Another difference is that in Australia the potential for infection shrinks during the year going from winter (July) to summer (January), whereas in South Africa there is a shift eastwards between winter and summer rainfall areas. Although infection will possibly occur in a much wider area, the impact of this rust fungus on the weed would be greatest in Tasmania and southern Victoria, which are apparently climatically suitable for the development of this rust fungus, according to both CLIMEX and the GIS approach used. *Endophyllum osteospermi* may therefore exert considerable control on *C. monilifera* ssp. *monilifera* in these regions.

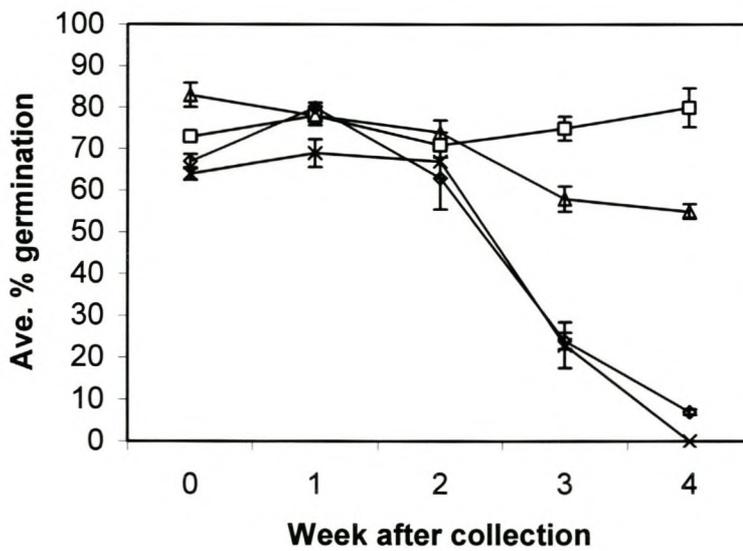
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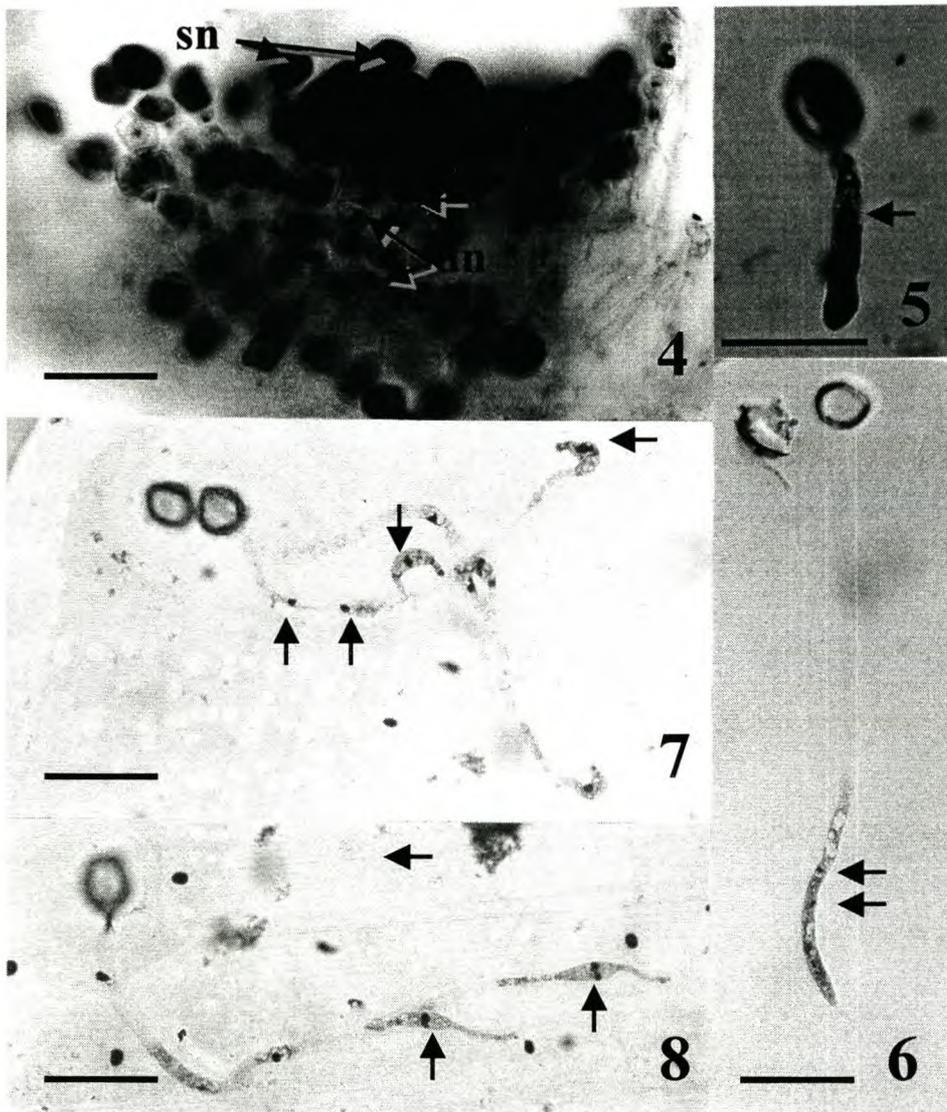
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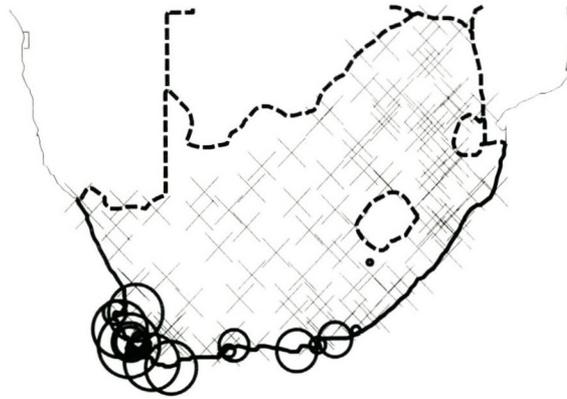
**Figure 1.** Average percent germination ( $\pm$  standard error) of three collections of acidoid teliospores of *Endophyllum osteospermi* at 10, 15, 20 and 25°C after 24 h of incubation under continuous light conditions.



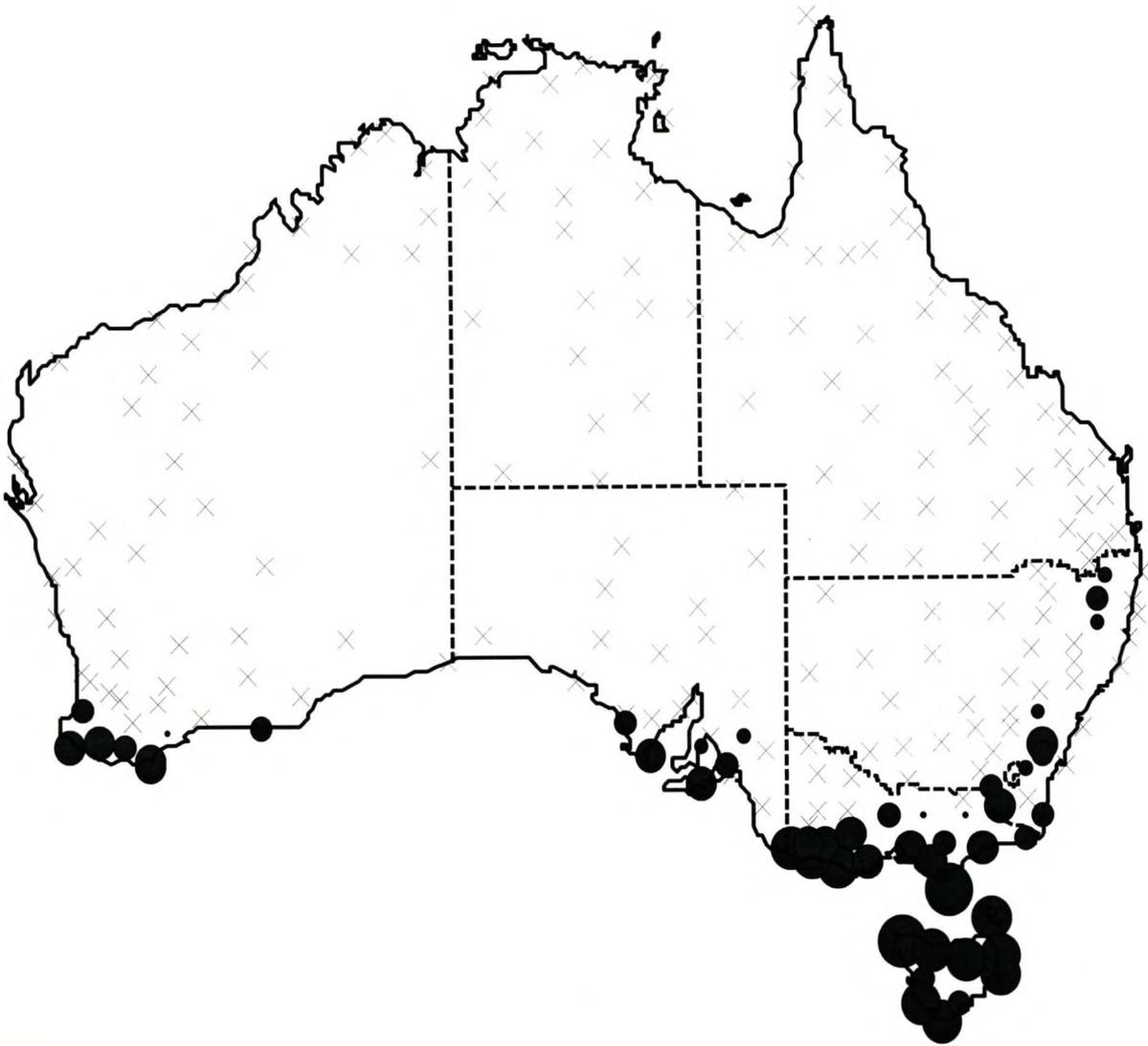
**Figure 2.** Average percent germination ( $\pm$  standard error) of a single collection of acidoid teliospores of *Endophyllum osteospermi* stored at 5 (square), 15 (triangle), 20 (cross) and 25°C (diamond) for 4 weeks after collection. Germination was determined after 24 h incubation at 15°C under continuous light conditions.



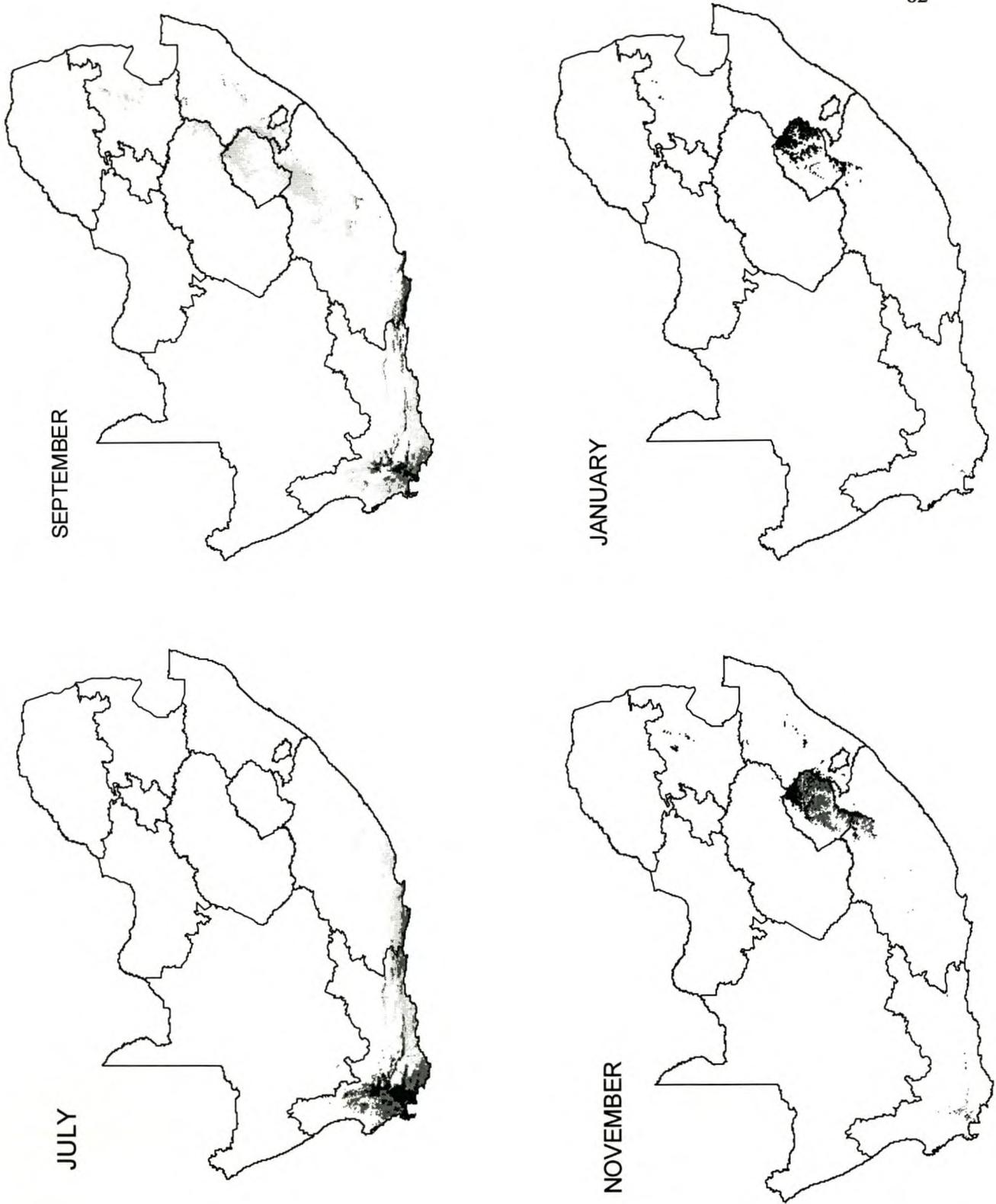
**Figures 3–7.** Aecidioid telia and germinating aecidioid teliospores of *Endophyllum osteospermi*. **Figure 3.** Transverse cross section through an aecidioid telium with stained nuclei, immature spores (dn) and intercallary cells (i) have two nuclei whereas mature spores (sn) have a single nucleus. **Figure 4.** Showing single nucleus (arrow) that has moved from inside the aecidioid teliospore into the germ tube, at 3 h after the onset of germination. **Figure 5.** Showing two nuclei (arrows) in the germ tube, at 6 h after the onset of germination. **Figure 6.** Showing four nuclei (arrows) in the metabasidium of which the distal two have moved into vesicle-like modified basidiospores, at 12 h after the onset of germination. **Figure 7.** Showing two nuclei (arrows) in each of the vesicle-like modified basidiospores which have germinated, at 24–48 h after the onset of germination. Scale bar = 40  $\mu\text{m}$ .



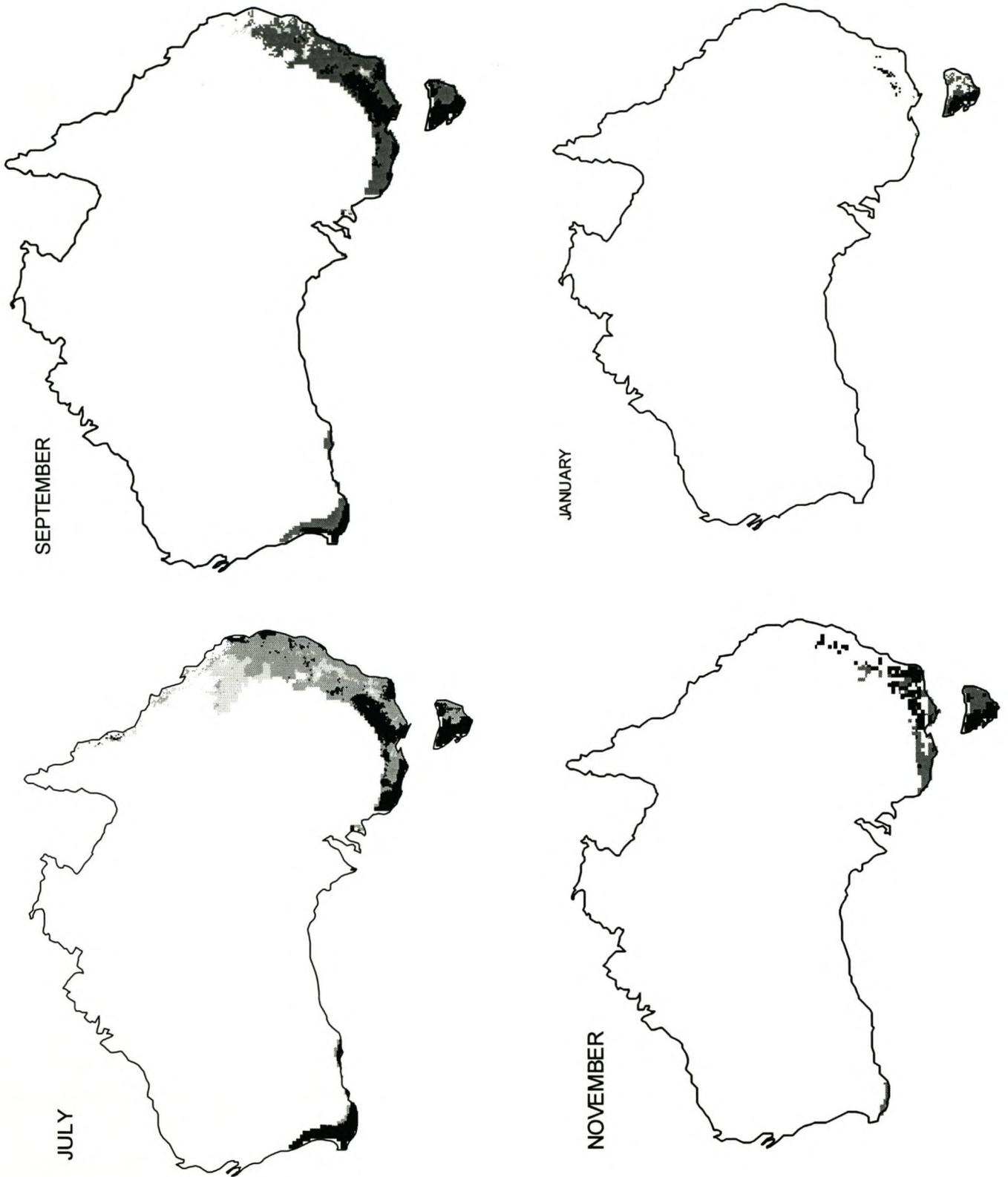
**Figure 8.** Map of South Africa showing locations suitable for infection by *Endophyllum osteospermi* to occur, generated by CLIMEX. Circles indicate a suitable location (the larger the circle the greater the suitability), and crosses indicate unsuitable localities. Each location represents a weather station at the epicenter of the circles or crosses.



**Figure 9.** Map of Australia showing locations suitable for infection by *Endophyllum osteospermi* to occur, generated by CLIMEX. Circles indicate a suitable location (the larger the circle the greater the suitability), and crosses indicate unsuitable localities. Each location represents a weather station at the epicenter of the circles or crosses.

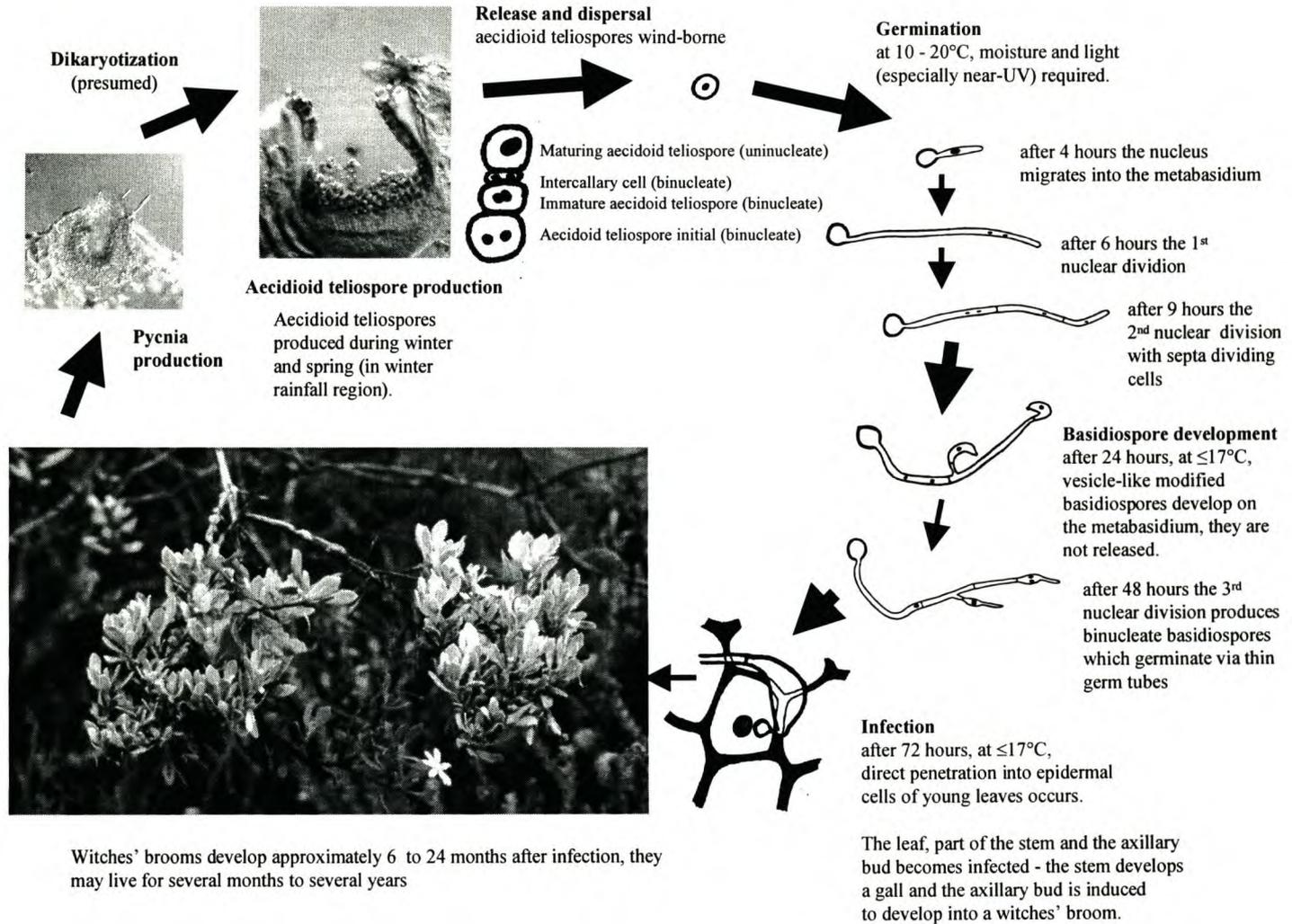


**Figure 10.** Approximation of the potential of *Endophyllum osteospermi* to infect suitable host plants in South Africa for the months of July, September, November and January. Light grey = low potential, medium grey = medium potential, black = high potential, white = unsuitable climate.



**Figure 11.** Approximation of the potential of *Endophyllum osteospermi* to infect suitable host plants in Australia for the months of July, September, November and January. Light grey = low potential, medium grey = medium potential, black = high potential, white = unsuitable climate.

**Figure 12.** Life cycle of *Endophyllum osteospermi* on its host plant *Chrysanthemoides monilifera* ssp. *monilifera*.



**Table 1. Percent germination of *Endophyllum osteospermi* aecidioid teliospores under different light conditions at 15°C.**

Light source	% Spore germination <sup>A</sup>		
	repeat 1	repeat 2	repeat 3
<i>Effect of light or dark conditions</i>			
dark (no light) <sup>B</sup>	40.0** <sup>C</sup>	21.3**	3.3**
continuous white and near-uv light	74.0	83.3	56.7
<i>Effect of light sources</i>			
continuous white light only	49.3*	31.7**	24.7**
continuous near-UV light only	83.7	81.7	58.7

<sup>A</sup>Average of 300 spores per treatment, each experiment was repeated 3 times.

<sup>B</sup>Dark treatments were placed in a cardboard box in a black plastic bag in the lighted incubators to ensure temperature conditions were identical.

<sup>C</sup>Averages within each repeat followed by \* are significantly different ( $P < 0.05$ ), and \*\* are highly significantly different ( $P < 0.01$ ), according to one-way ANOVA. Comparisons are between dark and light conditions, and between the light sources.

**Table 2. Percent germination of *Endophyllum osteospermi* aecidioid teliospores at different durations of light and dark conditions.**

Light source	% Spore germination <sup>A</sup>		
	repeat 1	repeat 2	repeat 3
24 h dark <sup>B</sup>	1.3e <sup>C</sup>	25.3e	32.0e
1 h white and near-uv light/ 23 h dark	10.0d	35.3d	32.0e
2 h white and near-uv light/ 22 h dark	14.7d	41.0c	36.7d
3 h white and near-uv light/ 21 h dark	34.7c	38.0cd	54.0b
4 h white and near-uv light/ 20 h dark	29.0c	54.0b	49.3c
6 h white and near-uv light/ 18 h dark	58.3a	53.7b	56.3b
8 h white and near-uv light/ 16 h dark	43.0b	61.7a	68.7a
24 h white and near-uv light	42.7b	64.3a	69.7a

<sup>A</sup>Average of 300 spores per treatment, each experiment was repeated 3 times.

<sup>B</sup>Dark treatments were placed in a cardboard box in a black plastic bag in the lighted incubators to ensure temperature conditions were identical.

<sup>C</sup>Averages followed by different figures are significantly different according to the LSD calculated for each repeat, repeat 1 LSD = 7.6, repeat 2 LSD = 4.5, repeat 3 LSD = 4.3.

**Table 3. Average percent ( $\pm$  standard error) of germinated *Endophyllum osteospermi* aecidioid teliospores that produced vesicle-like modified basidiospores after 48 hours incubation at different temperatures.**

Temperature (°C)	% Basidiospore development <sup>A</sup>		
	repeat 1	repeat 2	repeat 3
15	80.0 $\pm$ 3.5	84.7 $\pm$ 2.9	73.0 $\pm$ 3.2
17	24.0 $\pm$ 7.2	46.3 $\pm$ 7.3	47.3 $\pm$ 5.7
19	3.7 $\pm$ 0.3	9.3 $\pm$ 1.8	22.0 $\pm$ 3.1

<sup>A</sup>Average of 300 germinated spores per treatment, the trial was repeated 3 times.

## 5. Morphological and molecular characterisation of *Endophyllum* species on perennial asteraceous plants in South Africa

### ABSTRACT

*Endophyllum osteospermi* is an autoecious, endocyclic rust fungus, which had only been recorded from *Chrysanthemoides monilifera* ssp. *monilifera* (Asteraceae, Calendulae), a perennial woody shrub. Both organisms are indigenous to South Africa. Because *E. osteospermi* is being considered for release in Australia as a biological control agent against *C. monilifera* ssp. *monilifera* it was necessary to determine its host range and natural distribution in South Africa. To address this, natural stands of *Chrysanthemoides* species, as well as other South African asteraceous plants, were monitored for *E. osteospermi* between 1992 and 2003. A morphological and molecular comparison of specimens referable to *Endophyllum* was undertaken. Based on these results, *E. osteospermi* was recorded on *C. monilifera* ssp. *monilifera*, *C. monilifera* ssp. *pisifera*, *C. monilifera* ssp. *rotundata*, *C. monilifera* ssp. *canescens*, *C. monilifera* ssp. *subcanescens*, *C. incana* and an undescribed taxon of *Chrysanthemoides*. *Endophyllum osteospermi* was also recorded on *Osteospermum ciliatum*, *O. polygaloides* and *O. potbergense*. Furthermore, a closely related but previously undescribed species, *E. dimorphothecae* sp. nov. is described on *Dimorphotheca cuneata*. *Aecidium elytropappi* is transferred to *Endophyllum* as *E. elytropappi* comb. nov., being recorded on *Elytropappus rhinocerotis* and *Stoebe plumosa*. Results from this study show that in South Africa *E. osteospermi* is restricted to a small group of related plant species in the Calenduleae. This rust species is therefore considered suitable as a candidate agent for the biological control of *C. monilifera* ssp. *monilifera*, and pending the results of host specificity testing, would most likely be safe to introduce into Australia.

## INTRODUCTION

The autoecious, endocyclic rust fungus *Endophyllum osteospermi* (Doidge) A.R. Wood has up to the present been recorded only on the perennial woody shrub *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera* (Asteraceae, Calendulae), both the plant and the fungus species being indigenous to South Africa (Doidge, 1950; Morris, 1982; Crous *et al.*, 2000). Witches' brooms develop on plants that are systemically invaded with mycelium, and from which pycnia and aecidioid telia develop. Upon germination the aecidioid teliospores produce a metabasidium with 2–4 vesicle-like modified basidiospores (Morris, 1982; Wood, 1998). Infection is associated with a reduction in growth and reproduction in host plants growing in the field (Chapter 2), which in severe cases can lead to plant death (Morris, 1982).

*Chrysanthemoides monilifera* ssp. *monilifera* has become naturalised in south-eastern Australia where it is an invasive weed, and is threatening native vegetation and wildlife (Parsons and Cuthbertson, 1992; Stahle, 1997). The invasive success of *C. monilifera* ssp. *monilifera* is due to its capacity for vigorous growth, copious production of seed, and rapid regeneration after fire in the absence of natural enemies (Parsons and Cuthbertson, 1992). A biological control programme targeting this plant has been initiated, as part of which *E. osteospermi* is considered to be a potential biological control agent (Scott and Adair, 1995; Adair and Edwards, 1996).

Records of the distribution of *E. osteospermi* are limited to two specimens lodged at the South African National Collection of Fungi (PREM; ARC-Plant Protection Research Institute, Pretoria) (Doidge, 1950). A better understanding of the natural distribution and host range of *E. osteospermi* in South Africa is therefore necessary to determine the risks posed if this rust fungus is to be introduced into Australia as a biological control agent.

To address this question, natural stands of *Chrysanthemoides* species were observed between 1992 and 2003 in the Western Cape, Eastern Cape, and KwaZulu-Natal Provinces of South Africa. Other indigenous asteraceous plants that occurred within the natural distribution range of *C. monilifera* in these provinces were also observed during the same period.

Currently six subspecies of *Chrysanthemoides monilifera* are recognised (Norlin, 1943), namely *C. monilifera* ssp. *monilifera*, *C. monilifera* ssp. *pisifera* (L.) Norl., *C. monilifera* ssp. *rotundata* (DC.) Norl., *C. monilifera* ssp. *canescens*

(DC.) Norl., *C. monilifera* ssp. *subcanescens* (DC.) Norl., and *C. monilifera* ssp. *septrionalis* Norl.. However, *C. monilifera* ssp. *pisifera* as presently circumscribed encompasses several distinct taxa (Griffioen, 1995), and is in need of revision. Populations of five of the recognised subspecies were observed for the presence of witches' brooms, especially *C. monilifera* ssp. *monilifera* and several forms of *C. monilifera* ssp. *pisifera*. No observations of *C. monilifera* ssp. *septrionalis* were made as this subspecies occurs outside of the area of study. Several forms of *C. incana* (Burm. f.) Norl. were also observed.

Rust fungi causing witches' brooms or galls and with "aeciospores" which upon germination proved to be aecidioid teliospores, and hence were referable to the genus *Endophyllum*, were found on a number of perennial asteraceous plants in addition to *Chrysanthemoides* species. These rust specimens proved to be morphologically similar to *E. osteospermi*. Plant hosts on which they were collected included *Dimorphotheca cuneata* (Thunb.) Less. (Calendulae), *Elytropappus rhinocerotis* (L. f.) Less. (Gnaphalieae), *Osteospermum ciliatum* P.J. Bergius, , *O. polygaloides* L., *O. potbergense* A.R. Wood & B. Nord. (Calendulae), and *Stoebe plumosa* (L.) Thunb. (Gnaphalieae). The rust fungus on *E. rhinocerotis* is currently known as *Aecidium elytropappi* Henn., and in addition to several collections on this host in South Africa (Doidge, 1950), it has also been recorded from *Stoebe kilimandsharica* O. Hoffm. in Tanzania (Henderson, 1972). None of the other plant species listed has previously been recorded as hosts of rust fungi. To resolve the specific identity of the various collections, specimens were compared based on morphological and molecular data. The latter was based on sequences of the internal transcribed spacer (ITS1 – 5.8S – ITS2) region of the ribosomal DNA which was obtained from selected specimens.

## MATERIAL AND METHODS

**Morphology.** Fresh aecidioid teliospores were dusted on water agar (1.5% agar, Biolab, Midrand, South Africa) in 6 cm diam Petri dishes, after which the Petri dishes were sealed and incubated at 15°C for 24 h (Chapter 4). Germinated spores were examined with a Zeiss Axioscop light microscope and photographed using a Zeiss MC63 camera.

Fresh or dried aecidioid telia were mounted in Jung Tissue Freezing Medium™ (Leica Instruments, Germany), and 10 µm thick transverse sections cut with a Leica CM1100 Cryostat microtome. Sections were mounted in 70% lactic acid aqueous solution, and 25 aecidioid teliospores and peridial cells per specimen were measured at 1000× magnification. In addition, 50 aecidioid teliospores from each of selected dried specimens collected throughout the distribution of *E. osteospermi* were also measured. The length to width ratio of each aecidioid teliospore and the 95% confidence limits of the means were calculated. Measurements are stated as the average ± 95% confidence limits with the minimum and maximum given in parentheses. In addition, type specimens of *E. osteospermi* and *A. elytropappi* were also examined. Another species of rust, *A. metalasiae*, is probably closely related (Doidge, 1927). Unfortunately, despite searching for this species it was not collected. The type specimen of *A. metalasiae* was also examined.

Whole and cross sections of aecidioid telia and pycnia were prepared according to the method of Adendorff and Rijkenberg (2000) and observed with a Hitachi S-570 scanning electron microscope (SEM) at 10KV.

**Molecular analysis.** Fresh rust samples were collected in the field, placed in plastic bags and processed in the laboratory within 3 days after collection. Where abundant aecidioid telia were produced on individual large witches' brooms, these were removed from the plant material and used for DNA extraction. Where the above was not possible, parts of developing witches' brooms or galls bearing aecidioid telia were used. The collections used are listed in Table 1, and identified by a † sign in the listings of specimens examined.

Specimens were placed in 1.5 ml Eppendorf tubes in 100 µl extraction buffer, and manually ground using a sterile conical tissue grinder (Bel-Art Products, USA). After grinding, an additional 400 µl extraction buffer was added. The extraction buffer was prepared with equal volumes of 100 mM Tris (pH 9.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), and 2% CTAB (cetyltrimethylammonium bromide). The suspension was passed through three freeze-thaw cycles of 10 min in ice then 3 min in a water bath at 100°C, then incubated in a water bath at 65°C for 60 min. The isolation protocol of Lee and Taylor (1990) was used to extract genomic DNA from these aecidioid telia or infected plant samples. The primer pairs ITS1f and ITS4b (Gardes and Bruns, 1993), and ITS5 (White *et al.*, 1990) and ITS4b were used to amplify part of the nuclear rRNA operon using the PCR conditions recommended by the authors

(White *et al.*, 1990; Gardes and Bruns, 1993). Between two and four PCR products were obtained for each locality, each from individual witches' brooms. The PCR products were separated by electrophoresis at 95 V for 1 h in a 1.5% (w/v) agarose gel in 0.5x TAE running buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualised under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK) following ethidium bromide staining.

The PCR products were purified using a NucleoSpin<sup>®</sup> Extract 2-in-1 kit (Macherey-Nagel, Germany). The purified products were sequenced in both directions using the PCR primers and the cycle sequencing reaction was carried out as recommended by the manufacturer with an ABI PRISM Big Dye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA) containing AmpliTaq DNA Polymerase. The resulting fragments were analysed on an ABI Prism 3100 DNA Sequencer (Perkin-Elmer, Norwalk, CN).

Sequences were assembled and added to sequences obtained from GenBank (Berthier *et al.*, 1996; Weber *et al.*, 2003; L.J. Szabo, unpubl.) using Sequence Alignment Editor v2.0a11 (Rambaut, 2002), and manual adjustments for improvement were made by eye where necessary. Except at one locality (Cape Peninsula), all sequences obtained from each locality were identical to the others from that locality. The phylogenetic analyses of sequence data were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford, 2000). Alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Hillis and Bull, 1993). Other measures including tree length, consistency index, retention index and rescaled consistency index (CI, RI and RC) were also calculated. The resulting trees were printed with TreeView Version 1.6.6 (Page, 1996).

## Taxonomy

*Endophyllum osteospermi* (Doidge) A.R. Wood, *S. Afr. J. Bot.* **64**:146 (1998).  
(Figures 1–7).

Basionym: *Aecidium osteospermi* Doidge, *Bothalia* **2**:171 (1927).

Infections systemic, causing witches' brooms, host stems galled at base of witches' brooms. *Pycnia* amphigenous but mostly hypophyllous, scattered unevenly, not in lesions, preceding the aecidioid telia; 150–210 µm diam; flask-shaped with ostiolar trichomes. *Aecidioid telia* amphigenous and on stems but mostly hypophyllous, scattered unevenly, not in lesions; aecidioid, orange, cylindrical, up to 0.5 mm long, 200–375 µm diam; peridial margin white, reflexed, deeply incised. *Peridial cells* firmly joined together, irregular oblong to sub-rhomboid, (19–)26–27.5(–39) × (12–)19–20(–29) µm; outer wall striate, (7–)10–11(–15) µm thick; inner wall coarsely verrucose, (3–)4(–6) µm thick. *Aecidioid teliospores* irregular angular-globose, ovate to ellipsoid, (18–)21.5–22(–28) × (14–)17–18(–23) µm, length/width ratio 1:1–1.75; spore wall hyaline, evenly verruculose, 1–2(–2.5) µm thick; apex slightly thickened with a distinct germ pore, 2–4 µm thick; upon germination producing 2(–4) vesicle-like modified basidiospores.

*Specimens examined*: **SOUTH AFRICA**. KwaZulu-Natal Province: Monks Cowl Nature Reserve, Cathedral Peak, Drakensberg, 29°44'S 29°12'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *canescens* (DC.) Norl., 26 Jan. 1997, *A.R. Wood* 61 (PREM 56794). – Eastern Cape Province: Grahamstown, 33°18'S 26°31'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 6 Oct. 1998, *A.R. Wood* 82 (PREM 56815); Kabeljou River Nature Reserve, E of Jefferys Bay, 33°59'S 24°56'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 7 Oct. 1998, *A.R. Wood* 83 (PREM 56816); 8 km W of Hankey / Humansdorp intersection on N2, 33°59'S 24°42'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 6 Oct. 1998, *A.R. Wood* 84 (PREM 56817); Zuurberg Pass, N of Addo, 25°35'S 33°19'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 15 Feb. 2000, *A.R. Wood* 92 (PREM 56825); 3 km W of Alexandria along R27,

Eastern Cape, 33°38'S 26°23'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 20 Aug. 2002, *A.R. Wood* 384 (PREM 57889); Kidds Beach, S of East London, 33°09'S 27°41'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *rotundata* (DC.) Norl., 20 Aug. 2002, *A.R. Wood* 385 (PREM 57886); Kaysers Beach, S of East London, 33°12'S 27°36'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *rotundata* (DC.) Norl., 20 Aug. 2002, *A.R. Wood* 386 (PREM 57890). – Western Cape Province: Lower slopes of Lion's Head, Cape Town, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 13 Nov. 1912, *W.J. Saxton* (PREM 6587, TYPE); Mierkraal, Bredasdorp District, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 16 Nov. 1927, *C.A. Smith* (PREM 22839); 2 km S of Simon's Town, 34°13'S 18°23'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 7 Jul. 1997, *A.R. Wood* 65 (PREM 56798); *sin loc.* on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, Oct. 1997, *A.R. Wood* 66 (PREM 56799); Afrikaans Language Monument, Paarl, 33°46'S 18°57'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 27 Jun. 1997, *A.R. Wood* 68 (PREM 56081); near old tunnel along N1, DuToit's Pass, E of Paarl, 33°44'S 19°09'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 18 Jul. 1997, *A.R. Wood* 69 (PREM 56802); Heuningberg Nature Reserve, Bredasdorp, 34°35'S 20°02'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 6 Aug. 1997 *A.R. Wood* 73 (PREM 56806); Caledon Botanical Garden, Caledon, 34°13'S 19°25'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 28 Sep. 1997, *A.R. Wood* 70 (PREM 56803); Potberg Education Centre, De Hoop Nature Reserve, E of Bredasdorp, 34°23'S 30°32'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 29 Sep. 1997, *A.R. Wood* 75 (PREM 56808); Houw Hoek Pass, W of Bot River, 34°13'S 19°11'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 29 Sep. 1997, *A.R. Wood* 76 (PREM 56809); †*sin loc.* on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 5 May 2003, *A.R. Wood* 516 (PREM 57895); 1 km S of homestead, De Hoop Nature Reserve, E of Bredasdorp, 34°28'E 20°24'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 10 Dec. 1997, *A.R. Wood* 74 (PREM 56807); Fernkloof Nature Reserve, Hermanus, 34°23'S 19°15'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 16 Feb. 1998, *A.R. Wood* 71 (PREM 56804); Helshoogte, 1.5 km N of Stellenbosch, 33°55'S 18°54'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 7 Sep. 1998, *A.R. Wood* 72 (PREM 56805); Rhodes Memorial, Cape Town, 33°58'S 18°30'E, 16 on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, Aug. 1999, *A.R. Wood* 67

(PREM 56800); Helderberg Nature Reserve, Somerset West, 34°03'S 18°52'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 10 Dec. 2000, *A.R. Wood* 298 (PREM 57336); *sin loc.* on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 16 Mar. 2003, *A.R. Wood* 509 (PREM 57892); Gydo Pass, N of Ceres, 33°14'S 19°20'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 9 Feb. 2003, *A.R. Wood* 504 (PREM 57891); †Kirstenbosch National Botanic Garden, Cape Town, 34°00'S 18°24'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 2 May 2003, *A.R. Wood* 514 (PREM 57893); Steenbok Park, Bain's Kloof Pass, E of Paarl, 33°32'S 19°08'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, 16 May 2003, *A.R. Wood* 519 (PREM 57896); 12 km from Cango Mountain Resort on road to De Rust, 33°24'S 22°22'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 19 Mar. 1998, *A.R. Wood* 86 (PREM 56819); Swartberg Pass, W of Oudtshoorn, 33°21'S 22°06'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 20 Mar. 1998, *A.R. Wood* 85 (PREM 56818); 2 km E of Gouritz River along N2, E of Albertinia, 34°12'S 21°46'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 5 Oct. 1998, *A.R. Wood* 77 (PREM 56810); 1 km W of Albertinia, 34°12'S 21°34'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 5 Oct. 1998, *A.R. Wood* 78 (PREM 56811); Niekershek / Suurbraak intersection on N1, W of Heidelberg, 34°05'S 20°40'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 5 Oct. 1998, *A.R. Wood* 79 (PREM 56812); road to Karatara, E of Sedgfield, 34°01'S 22°51'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 5 Oct. 1998, *A.R. Wood* 80 (PREM 56813); road to Noetzie, N of Knysna, 34°03'S 23°07'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 5 Oct. 1998, *A.R. Wood* 81 (PREM 56814); Rooiberg Pass, S of Calitzdorp, 33°40'S 21°38'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 3 Feb. 2000, *A.R. Wood* 91 (PREM 56824); 2 km NE of Duiwenhoks River along road to Gysmanshoek Pass, W of Heidelberg, 33°58'S 21°00'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 21 Dec. 2000, *A.R. Wood* 299 (PREM 57335); †between Bellvidere and Brenton-on-Sea, SW of Knysna, 34°05'S 23°00'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 3 Jun. 2002, *A.R. Wood* 376 (PREM 57887); †2 km W of Gouritz R. Bridge along N2, W of Albertinia, 34°12'S 21°44'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 5 Jun. 2002, *A.R. Wood* 380 (PREM 57888); Tsitsikamma National Park, 34°01'S 23°53'E, on

*Chrysanthemoides monilifera* (L.) Norl. ssp. *pisifera* (L.) Norl., 28 May 2003, *A.R. Wood* 524 (PREM 57897); S of Calitzdorp along road to Hot Springs at junction with road to Rooiberg Pass, Little Karoo, 33°38'S 21°42'E, on *Chrysanthemoides monilifera* (L.) Norl. ssp. *subcanescens* (DC.) Norl., 3 Feb. 2000, *A.R. Wood* 90 (PREM 56823); between the ocean and Rietvlei, N of Milnerton, 33°50'S 18°29'E, on *Chrysanthemoides incana* (Burm. f.) Norl., 9 Sep. 1998, *A.R. Wood* 64 (PREM 56797); †*sin loc.* on *Chrysanthemoides incana* (Burm. f.) Norl., 13 Jul. 2003, *A.R. Wood* 525 (PREM 57898); Koeberg Nature Reserve, N of Melkbostrand, 33°40'S 18°26'E, on *Chrysanthemoides incana* (Burm. f.) Norl., 12 Jun. 1997, *A.R. Wood* 62 (PREM 56795); *sin loc.* 9 Sep. 1998, *A.R. Wood* 63 (PREM 56796); Red Hill Pass, Simon's Town, 34°11'S 18°25'E, on *Chrysanthemoides* undescribed taxon, 14 Jul. 1998, *A.R. Wood* 87 (PREM 56820); between Vermaaklikheid and Stillbaai, 34°20'S 21°02'E, on *Chrysanthemoides* undescribed taxon, 9 Oct. 1998, *A.R. Wood* 88 (PREM 56821); Stilbaai, 34°23'E 21°25'E, on *Chrysanthemoides* sp., 9 Oct. 1998, *A.R. Wood* 89 (PREM 56822); †4.7 km NW of Cape Point Nature Reserve, Cape Peninsula, 34°13'S 18°24'E, on *Chrysanthemoides* sp., 2 May 2003, *A.R. Wood* 515 (PREM 57894); 5 km W of Arniston, 34°38'S 20°11'E, on *Chrysanthemoides* sp., 19 Oct. 2003, *A.R. Wood* 530 (PREM 57899); Jonkershoek Nature Reserve, Stellenbosch, 33°59'S 18°59'E, on *Osteospermum ciliatum* Berg., 18 Sep. 1994, *A.R. Wood* 93 (PREM 57903); 2 km S of Simon's Town, 34°13'S 18°28'E, on *Osteospermum ciliatum* Berg., Aug. 1996, *A.R. Wood* 59 (PREM 56856); *sin loc.* on *Osteospermum ciliatum* Berg., 3 Oct. 1996, *A.R. Wood* 56 (PREM 56852); *sin loc.* on *Osteospermum ciliatum* Berg., 7 Jul. 1997, *A.R. Wood* 60 (PREM 56857); near Potberg Education Centre, De Hoop Nature Reserve, E of Bredasdorp, 34°23'S 20°32'E, on *Osteospermum potbergense* A.R. Wood & B. Nord., Jul. 1995, *A.R. Wood* 57 (PREM 56853); *sin loc.* on *Osteospermum potbergense* A.R. Wood & B. Nord., 24 Jun. 1998, *A.R. Wood* 58 (PREM 56855); *sin loc.* on *Osteospermum potbergense* A.R. Wood & B. Nord., Jun. 2002, *A.R. Wood* 374 (PREM 57904); Boesmans Pass, Riviersonderend Mountains, N of McGregor, on *Osteospermum polygaloides* L., 14 Aug. 1976, *L. Hugo* 786 (NBG); Swartberg Pass, S of Prince Albert, 33°19'S 22°02'E, on *Osteospermum polygaloides* L., 21 Mar. 1998, *A.R. Wood* 49 (PREM 57900); †*sin loc.* on *Osteospermum polygaloides* L., 18 Jun. 2002, *A.R. Wood* 383 (PREM 57902); Caledon Wild Flower reserve, Caledon, 34°13'E

19°25'E, on *Osteospermum polygaloides* L., 1 Aug. 1999, A.R. Wood 151 (PREM 57901);

*Notes.* *Chrysanthemoides incana*, *C. monilifera* ssp. *canescens*, *C. monilifera* spp. *pisifera*, *C. monilifera* spp. *rotundata*, *C. monilifera* spp. *subcanescens*, *Osteospermum ciliatum*, *O. polygaloides* and *O. potbergense* are all new host records for this rust fungus.

***Endophyllum dimorphothecae*** A.R. Wood & Crous, **sp. nov.** (Figures 10–17).

Pycnidia amphigena, plerumque hypophylla, dispersa, prius aquam telia aecidioidea apparentia, 135–200 µm diam. Telia aecidioidea amphigena, plerumque hypophylla, dispersa, aurantiaca, cylindrica, ad 0.4 mm alta, 135–200 µm diam. Cellulae peridiales forma variabili, oblongae vel subrhomboideae, (17–)23–26(–35) × (12–)17–19(–23) µm; paries externus striatus, (5–)7–8(–9) µm crassus, paries internus verrucosissimus, (2–)3–4(–5) µm crassus. Teliosporae aecidioideae irregulariter angulares, globosae vel ellipsoideae, (16–)20–21(–28) × (12–)16–17(–22) µm, ratio longitudo/latitudo 1 : 1–1.85; paries hyalinus, verruculosus, 1(–2) µm crassus, apex indistinctus vel modice inspissatus ad 1.5–2 µm; germinantes 2(–4) basidiosporas vesiculiformes proferunt.

Infections systemic, causing witches' brooms. *Pycnia* amphigenous but mostly hypophyllous, scattered unevenly, not in lesions, preceding the aecidioid telia; 135–200 µm diam; flask-shaped with ostiolar trichomes. *Aecidioid telia* amphigenous but mostly hypophyllous, scattered unevenly, not in lesions; aecidioid, orange, cylindrical, up to 0.4 mm high, 135–200 µm diam, peridial margin white, reflexed, incised. *Peridial cells* firmly joined together, irregular oblong to sub-rhomboid, (17–)23–26(–35) × (12–)17–19(–23) µm; outer wall striate, (5–)7–8(–9) µm thick; inner wall coarsely verrucose, (2–)3–4(–5) µm thick. *Aecidioid teliospores* irregular angular-globose to ellipsoid, (16–)20–21(–28) × (12–)16–17(–22) µm, length/width ratio 1:1–1.85; spore wall hyaline, verruculose, 1(–2) µm thick, apex indistinct or apex slightly thickened, 1.5–2 µm thick; upon germination producing 2(–4) vesicle-like modified basidiospores.

**HOLOTYPE.** – **SOUTH AFRICA.** Western Cape Province: Anysberg Nature Reserve, between Laingsburg and Ladismith, Little Karoo, 33°27'S 20°42'E, on *Dimorphotheca cuneata* (Thunb.) Less., 14 Sep. 2003, *A.R. Wood* 527 (PREM 57921) (Isotypes: K (M) 122450, PUR N4781)

*Etymology* – named after the host plant.

*Specimens examined in addition to the type:* **SOUTH AFRICA.** Northern Cape Province: †summit of Verlatekloof Pass, S of Sutherland, 32°31'S 20°38'E, on *Dimorphotheca cuneata* (Thunb.) Less., 16 June 2002, *A.R. Wood* 381 (PREM 57918); Roggeveld escarpment, farm Blesfontein, SW of Sutherland, 32°27'S 20°26'E, on *Dimorphotheca cuneata* (Thunb.) Less., 16 June 2002, *A.R. Wood* 382, (PREM 57920); *sin. loc.* on *Dimorphotheca cuneata* (Thunb.) Less., 23 Feb. 2003, *A.R. Wood* 507 (PREM 57920, PUR N4778); †Roggeveld escarpment, farm Boplaas, SW of Sutherland, 32°31'S 20°24'E, on *Dimorphotheca cuneata* (Thunb.) Less., 23 Feb. 2003, *A.R. Wood* 505 (PREM 57919, K (M) 122451).

***Endophyllum elytropappi*** (Henn.) A.R. Wood & Crous, **comb. nov.** (Figures 18–23)

Basionym: *Aecidium elytropappi* Henn., *Hedwigia* 37: 294 (1898).

Infections systemic, causing fusiform stem galls up to 5 cm long or witches' brooms depending on host. *Pycnia* not seen. *Aecidioid telia* on stems, closely crowded, not in lesions; aecidioid, orange, cylindrical, up to 2 mm long, 225–275 µm diam; peridium extending well beyond epidermis, peridial margin white, not reflexed, not or shallowly incised. *Peridial cells* irregular oblong to sub-rhomboid, (21–)28.5–30(–39) × (15–)21–22(–30) µm; outer wall striate, (10–)13–14(–17) µm thick; inner wall coarsely verrucose, (4–)5(–7) µm thick. *Aecidioid teliospores* irregular angular-globose, ovate to ellipsoid, (21–)25.5–26(–32) × (16–)20.5–21(–25) µm, length/width ratio 1:1–1.81; spore wall hyaline, verruculose, 1.5–2(–3) µm thick; apex slightly

thickened with a distinct germ pore, (2–)3–5  $\mu\text{m}$  thick; upon germination producing (3–)4 vesicle-like modified basidiospores.

*Specimens examined:* **SOUTH AFRICA.** Eastern Cape Province: Bergplaas plateau, Baviaanskloof Wilderness Area, W of Patensie, 33°37'S 24°28'E, on *Elytropappus rhinocerotis* (L. f.) Less., Jan. 2000, *A.R. Wood* 168 (PREM 57913). Northern Cape Province: †farm Blesfontein, SW of Sutherland, 32°27'S 20°26'E, on *Elytropappus rhinocerotis* (L. f.) Less., 23 Feb. 2003, *A.R. Wood* 506 (PREM 57917, K (M) 122448, PUR N4777). Western Cape Province: Bot River, on *Elytropappus rhinocerotis* (L. f.) Less., Nov. 1896, *Schlechter s.n.* (K (M) 110309, TYPE); hills above Darling, 33°22'S 18°22'E, on *Elytropappus rhinocerotis* (L. f.) Less., 1 Jan. 1999, *A.R. Wood* 130 (PREM 57912, K (M) 122447, PUR N4775); Elandsberg Nature Reserve, N of Wellington, 33°27'S 19°04'E, on *Elytropappus rhinocerotis* (L. f.) Less., 2 Feb. 2003, *A.R. Wood* 501 (PREM 57914); †border of farms Kaapzicht and Zewenwacht wine estates, off Botteleray rd., near Kuils River, 33°56'S 18°44'E, on *Elytropappus rhinocerotis* (L. f.) Less., 5 Feb. 2003, *A.R. Wood* 502 (PREM 57915); Anysberg Nature Reserve, between Laingsburg and Ladismith, 33°28'S 20°35'E, on *Elytropappus rhinocerotis* (L. f.) Less., 12 Sep. 2003, *A.R. Wood* 528 (PREM 57916); near old tunnel Du Toit's Pass, NE of Paarl, 33°44'S 19°09'E, on *Stoebe plumosa* (L.) Thunb., 18 Jul. 1997, *A.R. Wood* 371 (PREM 57908); Jan Marais Nature Reserve, Stellenbosch, 33°55'S 18°53'E, on *Stoebe plumosa* (L.) Thunb., 4 Dec. 2000, *A.R. Wood* 257 (PREM 57905); *sin loc.* on *Stoebe plumosa* (L.) Thunb., 23 May 2002, *A.R. Wood* 372 (PREM 57909); near Toll House, Montague Pass, N of George, 33°54'S 22°25'E, on *Stoebe plumosa* (L.) Thunb., 25 Jul. 2001, *A.R. Wood* 336 (PREM 57906); 2 km E of Outeniqua Nature Reserve offices, towards Tierkop, N of George, 33°55'S 22°26'E, on *Stoebe plumosa* (L.) Thunb., 4 Jun. 2002, *A.R. Wood* 377 (PREM 57910); Jonkershoek Plantation, Stellenbosch, 33°58'S 18°56'E, on *Stoebe plumosa* (L.) Thunb., 5 Feb. 2003, *A.R. Wood* 503 (PREM 57911).

*Note.* *Stoebe plumosa* is a new host record for this fungus.

*Aecidium metalasiae* Syd. & P. Syd., *Annal. Mycol.* **10**: 35 (1912). (Figures 8–9).

Infections systemic, causing fusiform galls on host stem, up to 2 cm long. *Pycnia* not seen. *Aecia* closely crowded on galls, deeply immersed; aecidioid, orange, cylindrical, up to 2 mm long, approx. 200 µm diam; peridial margin white, not reflexed, not or shallowly incised. *Peridial cells* firmly joined together, irregular oblong to sub-rhomboid, (23–)25–27(–30) × (13–)18–21(–28) µm; outer wall striate, (5–)6–7(–10) µm thick; inner wall coarsely verrucose, (2–)3–4(–5) µm thick. *Aeciospores* irregular ovate to ellipsoid to angular-globose, orange, (21–)24–26(–32) × (18–)20–22(–24) µm, length/width ratio 1:1.04–1.52; spore wall hyaline, verruculose, (3–)4(–5) µm thick, germination not observed.

*Specimen examined*: **SOUTH AFRICA**. Western Cape Province: Lions Head, Cape Town, on *Metalasia* sp., Dec. 1908, *IB Pole Evans s.n.* (PREM 686, TYPE).

*Notes*. The host was originally identified as *M. muricata* (L.) D. Don. However, since the original report the concept of this plant species has changed, and this epithet is now applied to a plant that is restricted to coastal sands. Furthermore, it does not occur on mountain slopes (Karis, 1989), which is the habitat at the type locality of *A. metalasiae*. It is possible, therefore, that the host is *M. densa* (Lam.) Karis, a common and widespread species that is known from the type locality of *A. metalasiae* (Karis, 1989). However, as the specimen has no flowers its identity remains uncertain.

## RESULTS

**Morphology.** In all specimens observed, fresh aecidioid teliospores germinated to produce metabasidia with two to four vesicle-like modified basidiospores. These basidiospores are not dispersed (Figures 4, 5, 12, 13, 20, 21). *Endophyllum osteospermi* and *E. dimorphothecae* predominantly produced two basidiospores, whereas *E. elytropappi* predominantly produced four basidiospores.

The pycnia of *E. osteospermi* and *E. dimorphothecae* are morphologically typical of type 4 pycnia (Hiratsuka and Cummins, 1963), being defined as subepidermal, with determinate growth and a strongly convex hymenium (Figures 24–26). Pycnia were not observed on *E. elytropappi*, but may well be produced. The

aecidioid telia (Figures 27–28) and aecidioid teliospores (Figure 29) of all four species observed are morphologically typical of the *Aecidium* type of aecia (Sato and Sato, 1985) and aeciospores. The surface ornamentation of the aecidioid teliospores corresponds to the ‘verrucose type’ of Sato and Sato (1982), and type 1 of Savile (1973). This latter type is defined as having uniformly small, or a mixture of small and large warts (verruculae), and with no pore plugs or refractile granules. The verruculae in these four species are all uniformly small (Figure 29). There was some variation in aecidioid teliospore dimensions from different specimens collected throughout the known range of *E. osteospermi* (Table 2). This variation was, however, not associated with geographic location. Aecidioid teliospores from drier localities were slightly smaller than those from wetter localities. These types of pycnia, aecidioid telia and aecidioid teliospore morphologies are typical of the Pucciniaceae (Cummins and Hiratsuka, 2003).

The four species of *Endophyllum* treated here are morphologically very similar, they can be distinguished according to the following key:

**Key to *Endophyllum* spp. with vesicle-like modified basidiospores occurring on taxa in Calendulae, Asteraceae**

- 1 Peridermium extends just beyond host epidermis (usually no more than 0.5 mm, and rarely up to 1 mm), aecidioid teliospore walls 1–2.5  $\mu\text{m}$  thick 2
- 1 Peridermium extends well beyond host epidermis (up to 3 mm), aecidioid teliospore wall 1.5–5  $\mu\text{m}$  thick 3
- 2 (1) Aecidioid teliospore wall (1–)2(–2.5)  $\mu\text{m}$  thick, apex distinct 2–4  $\mu\text{m}$  thick; outer wall of peridial cells 7–15  $\mu\text{m}$  thick **osteospermi**
- 2 (1) Aecidioid teliospore wall 1(–2)  $\mu\text{m}$  thick, apex indistinct or slightly thickened 1.5–2  $\mu\text{m}$  thick; outer wall of peridial cells 5–9  $\mu\text{m}$  thick **dimorphothecae**
- 3 (1) Aecidioid teliospore wall 1.5–2(–3)  $\mu\text{m}$  thick, apex distinct (2–)3–5  $\mu\text{m}$  thick; outer wall of peridial cells 10–17  $\mu\text{m}$  thick **elytropappi**
- 3 (1) Aecidioid teliospore wall uniformly (3–)4(–5)  $\mu\text{m}$  thick, no distinct apex; outer wall of peridial cells 5–10  $\mu\text{m}$  thick **metalasiae**

**Molecular analysis.** Both parsimony and neighbour-joining analysis supported the distinction of three clades, one for each of the species *E. dimorphothecae*, *E. elytropappi* and *E. osteospermum* (Figure 30). The sequence

obtained from the rust fungus on the host plant *Osteospermum polygaloides* fitted within the *E. osteospermi* clade, confirming the morphological analysis. Attempts were made to sequence DNA extractions from herbarium specimens of *Endophyllum* on this and other *Osteospermum* species. Unfortunately, none of these attempts yielded sequences of sufficient quality to include in the analysis.

## DISCUSSION

Prior to this work, published distribution records of *E. osteospermi* consisted only of two specimens on *C. monilifera* ssp. *monilifera* (Doidge, 1950). Morris (1982) recorded populations on *C. monilifera* ssp. *monilifera* from the Cape Peninsula (Constantia), and in the Stellenbosch, Franschhoek and Villiersdorp districts. Unfortunately no voucher specimens from these localities were preserved. Furthermore, these records are all in the winter rainfall areas in the western part of the Western Cape Province, South Africa. The above specimens listed therefore represent a range extension of approximately 1300 km eastwards. They also indicate that *E. osteospermi* is not restricted to a winter rainfall region. All forms of *Chrysanthemoides* are probably susceptible to this rust fungus, but forms that occur in semi-arid regions, or warm humid regions, possibly avoid infection due to a climate unfavourable to infection by *E. osteospermi*.

In addition to *Chrysanthemoides* species, *E. osteospermi* was also found on several species of *Osteospermum*, which are closely related to *Chrysanthemoides*. It appears that *O. potbergense* is not a normal host of *E. osteospermi*, as witches' brooms were only found on plants occurring amongst bushes of *C. monilifera* on which *E. osteospermi* was abundant (Wood and Norsenstam, 2004). The aecidioid telia were not observed to mature and open on *O. potbergense*, neither were witches' brooms found on plants further away from infected *C. monilifera*. A similar situation occurred in *O. ciliatum* at one locality (2 km S of Simon's Town), but at another locality no infected *C. monilifera* plants were found near infected *O. ciliatum* plants (Jonkershoek Nature Reserve). At both these sites the aecidioid telia matured and opened normally. Where infected *O. polygaloides* plants were found, no infected *C. monilifera* plants were found nearby (at least within a 1 km distance). Therefore, *O. ciliatum* and *O. polygaloides* can be considered as true hosts of this rust. Sequence data obtained from *O. polygaloides* confirmed that the species infecting this plant was

indeed *E. osteospermi*. No other *Osteospermum* species were found to be infected with *E. osteospermi*.

Recent field observations on the morphology of cypselas of various species of *Osteospermum* indicate that the single character (fleshy exocarp surrounding the cypselas) used to distinguish *Chrysanthemoides* from *Osteospermum* (Norlindh, 1943) is insufficient to distinguish between these two genera, and that the status of *Chrysanthemoides* must be re-examined (Wood and Nordenstam, 2004). The above-mentioned hosts of *E. osteospermi* may possibly be congeneric, depending on the revised status of *Chrysanthemoides*.

No other host plants of *E. osteospermi* are known, despite many observations made over the years on related plants. The rust fungus on *D. cuneata*, also in the Calendulae, proved to be distinct though closely related, and was therefore described as a new species, *E. dimorphothecae*. This host plant is ecologically distinct from *C. monilifera*, occurring in areas much drier than those where *C. monilifera* grows (Norlindh, 1943). The molecular data supported the distinction between *E. osteospermi* and *E. dimorphothecae*, also suggesting that these two species possibly shared a common ancestor in the recent past. All other asteraceous plants found in South Africa that were hosts of *Endophyllum* species, were host to *E. elytropappi* (*Elytropappus* and *Stoebe* spp.) or *Endophyllum macowanianum* (Thüm.) A.R. Wood (on *Conyza scabrida* DC., Astereae) (Wood, in press). The only other species of rust fungus found in South Africa that caused witches' brooms on asteraceous hosts was an undescribed species of *Uromyces* on a species of *Euryops* (Senecioneae) (A.R. Wood, unpubl. data). Unfortunately, no specimens of *A. metalasiae* were collected, despite a number of surveys made to try and recollect it. The type locality is in the same area (Lion's Head, Cape Town) from which the type of *E. osteospermi* also originated. The latter species, however, was found to still be present in that area. *Aecidium metalasiae* is morphologically very similar to *E. elytropappi*, but is readily distinguished by its thicker, aecidioid teliospore walls, and is therefore maintained as a separate species. However, it may be only an aberrant specimen of *E. elytropappi*, and is possibly best considered as a doubtful species.

A morphological development pattern and nuclear cycle similar to that of the aecidioid teliospores of *E. osteospermi* has been reported for a number of other rust fungi, including *Prospodium transformans* (Shuttleworth, 1953), *Puccinia vitata* (Gardner, 1988), and *Racospermyces koa* (Gardner, 1981; Chen *et al.*, 1996). Vesicle-

like modified basidiospores appear to be rare amongst rust fungi. This, together with the morphology and molecular data presented here indicates that these species form a phylogenetically closely related complex.

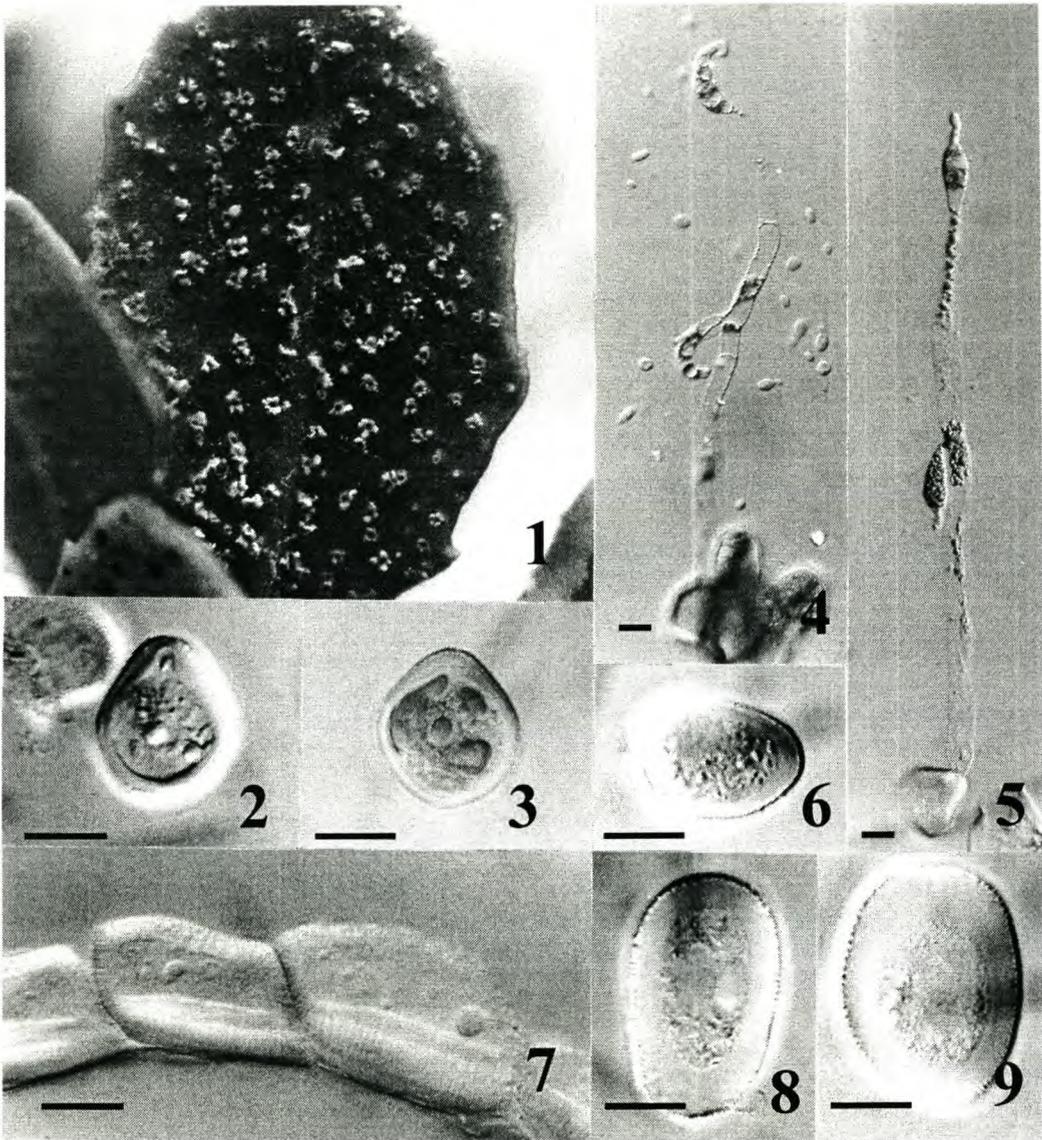
In South Africa *E. osteospermi* is naturally restricted to a small group of closely related plant hosts in the tribe Calenduleae. This rust species is therefore considered to be a candidate agent for the biological control of *C. monilifera* ssp. *monilifera*. Pending host specificity testing, this rust fungus would most likely be safe to introduce into Australia.

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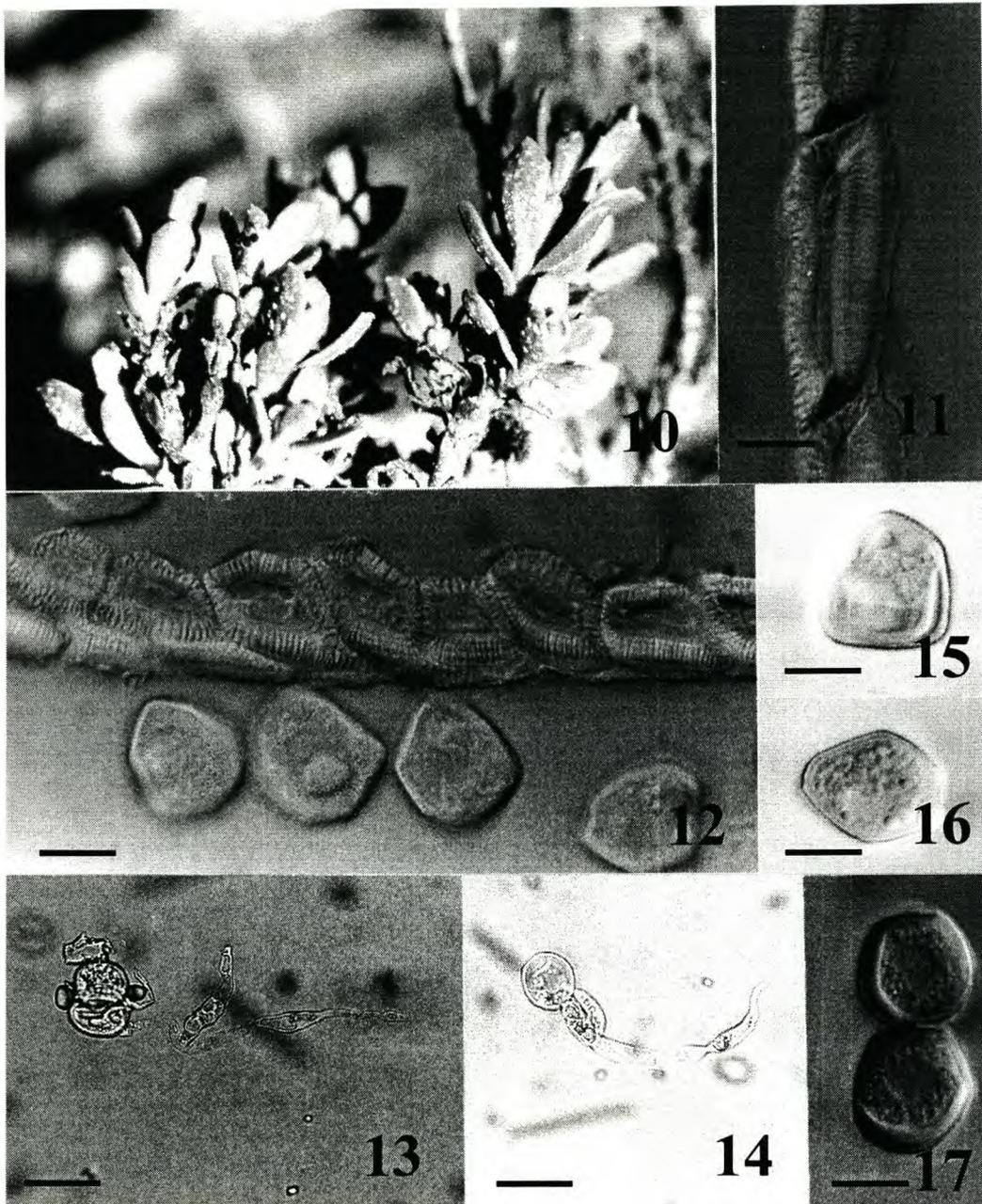
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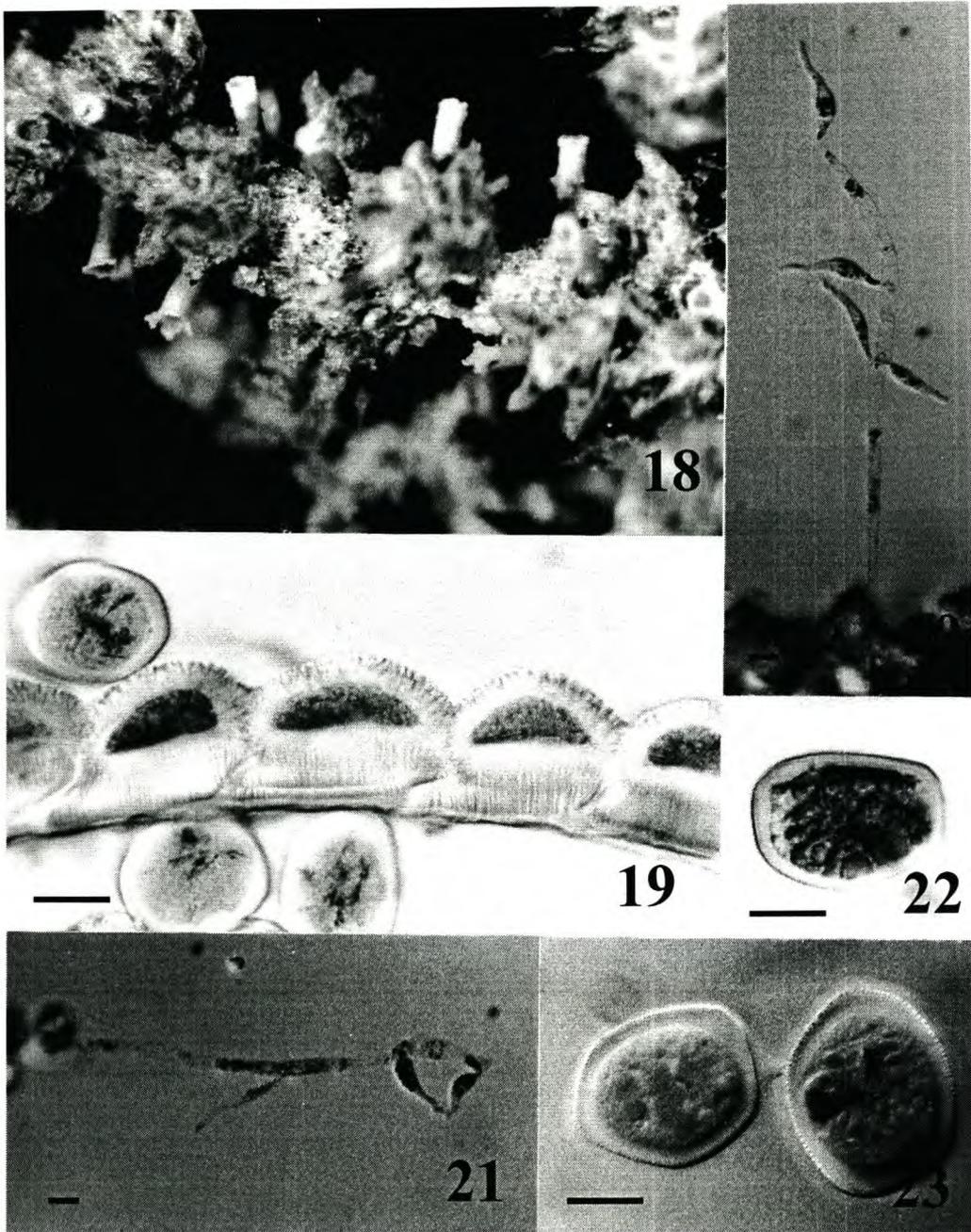
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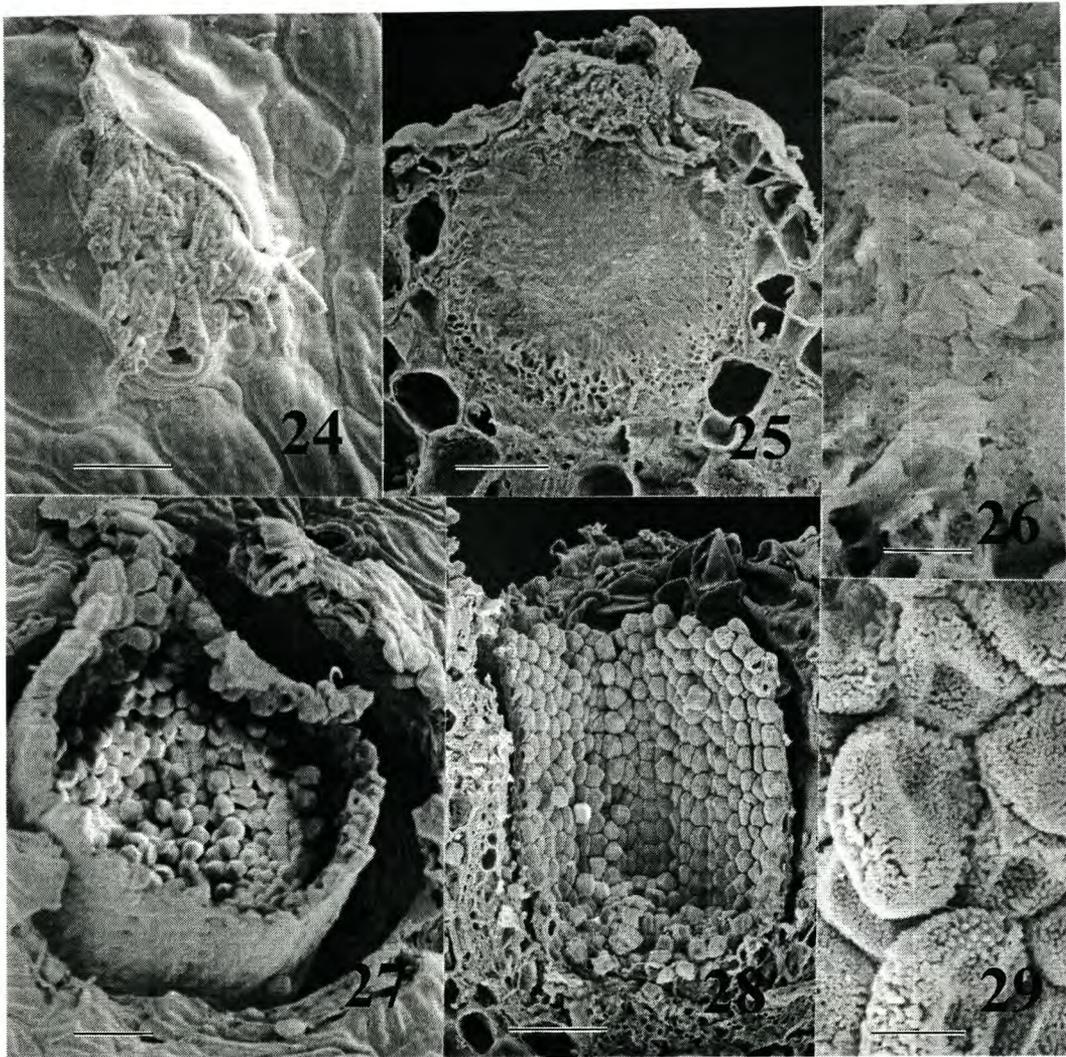
**Figures 1–7.** *Endophyllum osteospermi*. **Figure 1.** Aecidioid telia on the abaxial surface of a leaf from a witches' broom on *Chrysanthemoides monilifera*. **Figures 2–3.** Aecidioid teliospore showing a distinctly thickened apex and germ pore. **Figures 4–5.** Germinated aecidioid teliospore with two vesicle-like modified basidiospores. **Figure 6.** Aecidioid teliospore showing a distinctly thickened apex. **Figure 7.** Peridial cells of an aecidioid telium. **Figures 8–9.** Aecidioid teliospore of *Aecidium metalasiae*. Scale bar = 10  $\mu\text{m}$ .



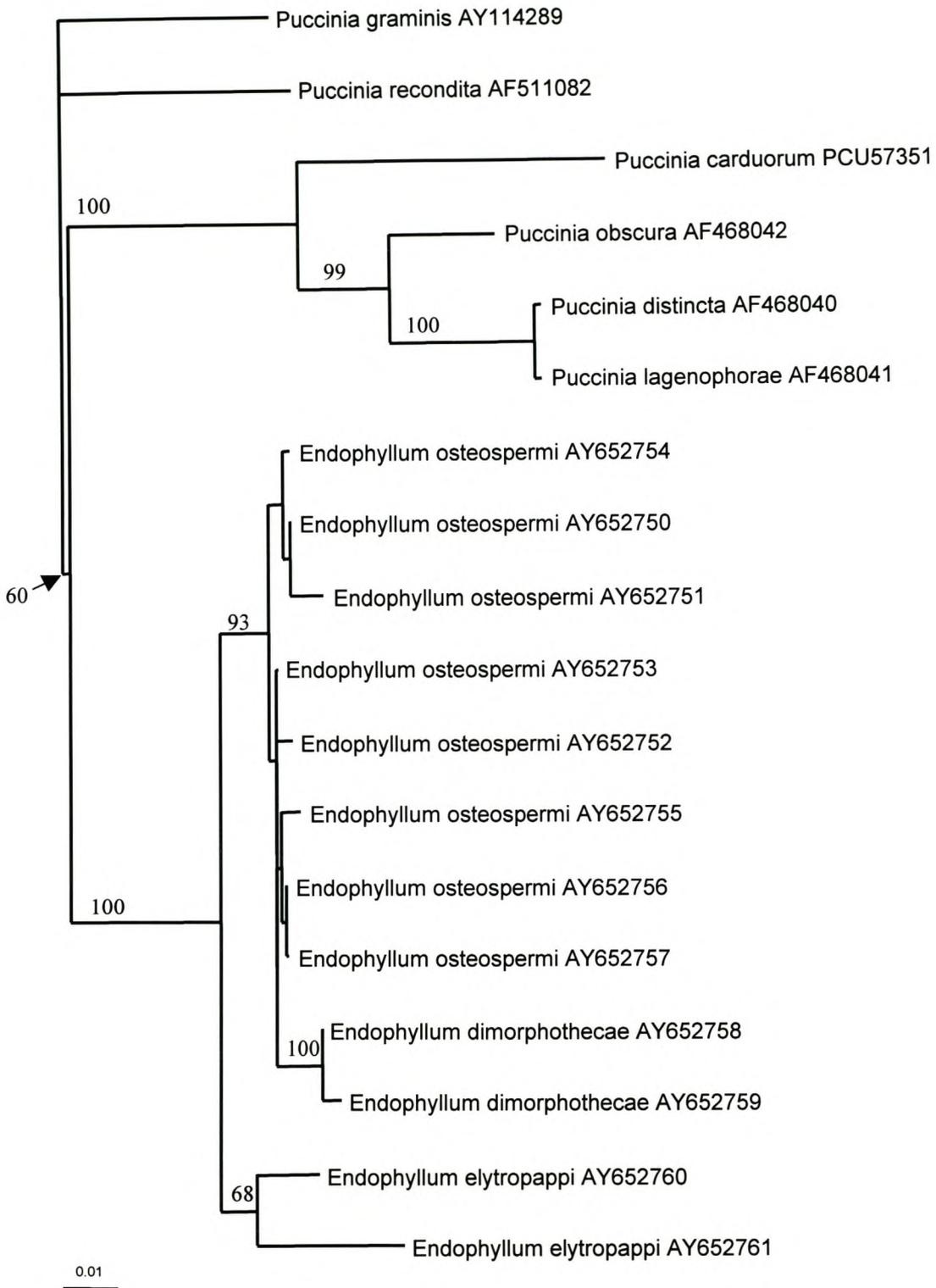
**Figures 10–17.** *Endophyllum dimorphothecae*. **Figure 10.** Aecidioid telia on a witches' broom on *Dimorphotheca cuneata*. **Figures 11–12.** Peridial cells of an aecidioid telium. **Figures 13–14.** Germinated aecidioid teliospore with two vesicle-like modified basidiospores. **Figures 15–17.** Aecidioid teliospore(s). Scale bar: 10–12 = 10  $\mu\text{m}$ , 13–14 = 25  $\mu\text{m}$ , 15–17 = 10  $\mu\text{m}$ .



**Figures 18–23.** *Endophyllum elytropappi*. **Figure 18.** Aecidioid telia on a witches' broom on *Stoebe plumosa*. **Figure 19.** Peridial cells of an aecidioid telium. **Figures 20–21.** Germinated aecidioid teliospore with four vesicle-like modified basidiospores. **Figure 22.** Aecidioid teliospore showing a distinctly thickened apex and germ pore. **Figure 23.** Two aecidioid teliospores. Scale bar = 10  $\mu\text{m}$ .



**Figures 24–29.** *Endophyllum osteospermi* on *Chrysanthemoides monilifera* ssp. *monilifera*. **Figure 24.** Surface view of the epidermis of a leaf showing the emerging flexuous hyphae, pycniospores and honeydew of a pycnium. **Figure 25.** Transverse section through a pycnium. **Figure 26.** Enlarged view of transverse section through a pycnium showing pycniospores and pycniosporophores embedded in a mucous matrix (honeydew). **Figure 27.** Surface view of the epidermis of a leaf showing an acedoid telium. **Figure 28.** Transverse section through an acedoid telium, showing peridium and some acedoid teliospores. **Figure 29.** Acedoid teliospores. Scale bars: 1 = 30  $\mu\text{m}$ , 25 = 40  $\mu\text{m}$ , 26 = 7  $\mu\text{m}$ , 27 = 50  $\mu\text{m}$ , 28 = 68  $\mu\text{m}$ , 29 = 10  $\mu\text{m}$ .



**Figure 30.** Neighbour-joining tree of the ITS1–5.8S–ITS2 rDNA region of *Endophyllum dimorphothecae*, *E. elytropappi*, and *E. oteospermi*. Bootstrap support values (1000 replicates) are shown at the nodes.

**Table 1. Specimens of *Endophyllum* species subjected to DNA analysis.**

Rust species	Host species	Locality	GenBank no.	PREM no.
<i>Endophyllum osteospermi</i>	<i>Chrysanthemoides monilifera</i> ssp. <i>monilifera</i>	Kirstenbosch National Botanic Garden, Cape Town	AY652755	57893
	<i>C. monilifera</i> ssp. <i>monilifera</i>	Houw Hoek Pass, Bot River	AY652752	57895
	<i>C. monilifera</i> ssp. <i>pisifera</i>	Gouritz River, Albertinia	AY652754	57888
	<i>C. monilifera</i> ssp. <i>pisifera</i>	Between Bellvidere and Brenton-on-Sea, Knysna	AY652753	57887
	<i>Chrysanthemoides</i> sp.	Cape Point, Scarborough	AY652750 AY652751	57894
	<i>Chrysanthemoides incana</i>	Rietvlei, Milnerton	AY652756	57898
	<i>Osteospermum polygaloides</i>	Swartberg Pass, Prince Albert	AY652757	57902
<i>Endophyllum dimorphothecae</i>	<i>Dimorphotheca cuneata</i>	Verlatekloof Pass, Sutherland	AY652758	57918
	<i>Dimorphotheca cuneata</i>	Boplaas farm, Sutherland	AY652759	57919
<i>Endophyllum elytropappi</i>	<i>Elytropappus rhinocerotis</i>	Blesfontein farm, Sutherland	AY652761	57917
	<i>Elytropappus rhinocerotis</i>	Border of Kaapzicht and Zewenwacht wine estates, Kuils River	AY652760	57915

**Table 2. Measurements of *Endophyllum osteospermi* aecidioid teliospores from various localities.**

Locality	Host <sup>A</sup>	PREM no. <sup>B</sup>	l x w (min.–max.) <sup>C</sup>	l x w (95% conf. lim.) <sup>D</sup>
Lions Head, Cape Town (TYPE)	<i>C.m.m.</i>	6587	19–28 x 15–21	22–24 x 17–19
Gydo Pass, Ceres	<i>C.m.m.</i>	57891	18–26 x 15–22	22–23 x 18–19
Houw Hoek Pass, Bot River	<i>C.m.m.</i>	57895	19–24 x 16–21	21–22 x 18–19
Kirstenbosch, Cape Town	<i>C.m.m.</i>	57893	18–25 x 16–20	21–22 x 17–18
Miller's Point, Simon's Town	<i>C.m.m.</i>	56799	23–39 x 18–34	30–32 x 25–27
Paarl Rock, Paarl	<i>C.m.m.</i>	56801	20–27 x 15–24	22–23 x 19–20
Potberg, De Hoop Nature Reserve	<i>C.m.m.</i>	56808	22–30 x 18–23	24–25 x 20–21
Grahamstown	<i>C.m.p.</i>	56815	19–29 x 14–20	21–22 x 17–18
Heidelberg	<i>C.m.p.</i>	56812	17–27 x 16–22	21–22 x 18–19
Jeffrey's Bay	<i>C.m.p.</i>	56816	19–27 x 14–22	21–22 x 17–18
Langeberg, Heidelberg	<i>C.m.p.</i>	57333	18–31 x 14–20	21–22 x 17–18
Sedgefield	<i>C.m.p.</i>	56813	18–29 x 16–21	22–23 x 17–18
Cathedral Peak	<i>C.m.c.</i>	56794	22–29 x 16–25	24–25 x 19–20
Calitzdorp	<i>C.m.s.</i>	56823	20–27 x 16–22	23–24 x 19–20
Stilbaai	<i>C.sp.</i>	56822	18–24 x 14–22	21–22 x 18–19
Koeberg Nature Reserve, Melkbosstrand	<i>C.i.</i>	56795	20–28 x 16–24	24–25 x 20–20
Jonkershoek, Stellenbosch	<i>O.c.</i>	57903	19–25 x 14–19	21–23 x 16–18
Swartberg Pass, Prince Albert	<i>O.p.</i>	57900	18–26 x 15–23	20–22 x 17–18

<sup>A</sup>*C.m.m.* = *Chrysanthemoides monilifera* ssp. *monilifera*, *C.m.p.* = ssp. *pisifera*, *C.m.s.* = ssp. *subcanescens*, *C.m.c.* = ssp. *canescens*, *C.sp.* = *Chrysanthemoides* sp., *C.i.* = *Chrysanthemoides incana*, *O.c.* = *Osteospermum ciliatum*, *O.p.* = *Osteospermum polygaloides*.

<sup>B</sup>Accession numbers of specimens deposited at National Collection of Fungi, ARC-Plant Protection Research Institute, Pretoria, South Africa (PREM).

<sup>C</sup>Minimum and maximum range of length and width of 50 spores from each locality.

<sup>D</sup>95% confidence limits of the average length and width of 50 spores from each locality.

## 6. Preliminary host specificity testing of *Endophyllum osteospermi*, a biological control agent against *Chrysanthemoides monilifera* ssp. *monilifera*

### ABSTRACT

*Chrysanthemoides monilifera* ssp. *monilifera*, indigenous to the Western Cape Province of South Africa, is a serious invader of native vegetation in winter-rainfall areas of south-eastern Australia. The rust fungus *Endophyllum osteospermi* causes witches' brooms on *C. monilifera* ssp. *monilifera* and is associated with a reduction in growth and seed production of its host under natural conditions, as well as mortality of severely infected bushes. This rust fungus is considered to be a potential biological control agent for use against *C. monilifera* ssp. *monilifera* in Australia. As *E. osteospermi* has a long latent period, typically between one and two years between infection and the initiation of witches' brooms, the logistics of doing traditional host specificity testing, in which all test plant species are inoculated and observed for symptom development, make such testing unfeasible for this rust fungus. Germination of aecidioid teliospores and penetration by basidiospores were observed on the surface of excised leaves of 32 test plant species at 4 days after inoculation, and compared to that on *C. monilifera* ssp. *monilifera*. Germinating aecidioid teliospores aborted on 14 test plant species, whilst no penetration was attempted on a further 12 test plant species. Penetration only occurred on 9 of the 32 test plant species, in addition to *C. monilifera* ssp. *monilifera*. Inoculation of 9 selected test plant species confirmed the above results. Therefore, only the 9 test plant species in which penetration occurred, or at least was attempted, need to undergo traditional host specificity testing. Pending these results, *E. osteospermi* could be safely released in Australia for the biological control of *C. monilifera* ssp. *monilifera*.

### Introduction

*Chrysanthemoides monilifera* (L.) Norl. ssp. *monilifera*, indigenous to the Western Cape Province of South Africa, is a serious invader of native vegetation in winter-rainfall areas of south-eastern Australia (Parsons and Cuthbertson, 1992). This invasiveness has been attributed to the production of large quantities of viable seed in

Australia, in comparison to that in South Africa (Weiss and Milton, 1984; Scott, 1996), and the greater competitiveness of seedlings compared to native Australian plants (Weiss and Noble, 1984). Mature plants in Australia have a much greater canopy size, and populations have a higher recruitment rate compared to those in South Africa (Scott, 1996). These differences have been attributed to the effects of a number of herbivores and pathogens in South Africa which are absent in Australia. A number of herbivorous insects have been evaluated and released, or are presently being evaluated, for biological control purposes (Scott and Adair, 1995; Adair and Edwards, 1996). At present only two of the seven insect agents released have established and begun to exert control, namely *Comostolopsis germana* Prout (Lepidoptera: Geometridae) (Adair and Edwards, 1996; Stahle, 1997) and *Mesoclanis polana* Munro (Diptera: Tephritidae) (Edwards *et al.*, 1999). Furthermore, a recently released undescribed tortricid moth (Lepidoptera: Tortricidae) is establishing despite current drought conditions in Australia (Anonymous, 2003). These agents are primarily targeted against *C. monilifera* ssp. *rotundata* (DC.) Norl. Four beetles (Coleoptera: Chrysomelidae) have been released but failed to establish for various reasons (Adair and Edwards, 1996). The recently released fruit fly *Mesoclanis magnipalpis* Bezzi is currently the only agent primarily targeted against *C. monilifera* ssp. *monilifera* (Edwards *et al.*, 1999), but it is not yet known whether it has begun exerting control.

The rust fungus *Endophyllum osteospermi* (Doidge) A.R. Wood causes witches' brooms on *C. monilifera* ssp. *monilifera* (Morris, 1982) and is associated with a reduction in growth and seed production of its host under natural conditions (Chapter 2), as well as mortality of severely infected bushes (Neser and Morris, 1984; Chapter 3). During periods of suitable environmental conditions, populations of *E. osteospermi* increase rapidly (Chapter 3). It is microcyclic (endocyclic), autoecious (Morris, 1982; Chapter 4), and previously had not been recorded from any plant other than *C. monilifera* (Doidge, 1950; Crous *et al.*, 2000), though recently it has been found to infect five of the six currently recognised subspecies of *C. monilifera*, and a few closely related species of *Osteospermum* (Chapter 5). This rust fungus is considered to be a potential biological control agent for use against *C. monilifera* ssp. *monilifera* in Australia (Scott and Adair, 1995; Adair and Edwards, 1996).

Field observations and whole-plant inoculations of the host indicated that *E. osteospermi* has a long latent period, typically between one and two years (Chapter 4).

The logistics of doing traditional host specificity testing, in which all test plant species are inoculated and observed for host reaction as presence or absence of symptom development (e.g. Hasan, 1972; Politis *et al.*, 1984; Bruzzese and Hasan, 1986; Bruckart, 1989; Evans and Tomley, 1994; Parker *et al.*, 1994), make such testing logistically unfeasible for this rust fungus, considering its long latent period. Therefore a different approach was proposed to overcome this difficulty. Plants possess many methods of defending themselves against attack by pathogens, including both physical and biochemical methods that can be either present before attack or produced in response to attack (Agrios, 1988). The approach followed in the preliminary host specificity tests reported herein was to do an initial screening of predetermined test plant species to establish whether pre-existing physical or chemical defense mechanisms were present, preventing penetration by the germinating aecidioid teliospores of *E. osteospermi*. If penetration does not occur on a particular plant species, then that plant species is immune to attack by *E. osteospermi* and no further host specificity testing is required for that plant species. In those cases where penetration does occur, this does not mean that the test plant is necessarily a host, but rather that an active rather than a passive defense method may be initiated in response to penetration. These plant species may therefore still prove to be immune non-hosts. Traditional host specificity testing of only those plant species where penetration occurred would still however have to be conducted. Thus the cost of doing traditional host specificity testing is reduced following the initial screening proposed here. The present study reports on the results from preliminary host specificity screening of *E. osteospermi*.

## MATERIALS AND METHODS

**Source of inoculum.** Aecidioid teliospores were obtained from a naturally infected population of *C. monilifera* ssp. *monilifera* occurring approximately 2 km south of Simon's Town on the Cape Peninsula (34°11'S 18°25'E). Plants from the Cape Peninsula appear to be morphologically identical to those found in Australia. Parts of witches' brooms with healthy aecidioid telia were collected and aecidioid teliospores harvested and stored using the same method as described in Chapter 3. The dry aecidioid teliospores were stored in an incubator at 5°C and used within one month of collection. The inoculations were done during the winter to spring growing

season (July to November/January depending on how long the rains continued into spring), while fresh viable aecidioid teliospores were available from the field.

**Penetration of the host plant *C. monilifera* ssp. *monilifera*.** The method of Morris (1982) was followed to inoculate excised leaves of *C. monilifera* ssp. *monilifera* plants grown from seed collected in Australia. Excised soft young leaves (approximately 1–1.5 cm in length; mature leaf length 4–6 cm) were carefully rubbed to remove their covering of hairs, and placed on moist tissue paper in 9 cm diam. Petri dishes. Dry aecidioid teliospores were dusted over the surface of the leaves, and sprayed with water using an air brush until very small droplets were visible to the naked eye. The leaves were incubated in a controlled environment room with a maximum temperature of 16°C for periods of 24, 48, 72 and 98 h after inoculation. After these periods the epidermis was sliced off the leaf using a scalpel, cleared in Carnoy's Solution (25% acetic acid, 75% absolute alcohol) for 15–30 min, stained for 2 min in aniline blue in lactophenol, and mounted in glycerol on glass microscope slides. Inoculated leaves were observed using a Zeiss Axioskop light microscope and microphotographs taken with a Zeiss M63 camera.

**Inoculation of test plants: Detached leaf trials.** Detached immature leaves of the test plants were treated in the same manner as the above method used in the host penetration studies. One leaf from each of five different plants were used per test plant species per inoculation, there were three inoculations for each test plant species. Table 1 lists all test plant species. The leaves were cleared 4 days after inoculation, and stored in Carnoy's Solution until they could be stained and observed.

The only modification to the above method of preparing specimens for microscopic examination was for clearing, which depended on the leaf thickness and hairiness. The epidermis of thicker leaves were sliced off (e.g. *Gazania ringens* (L.) Gaertn. and *Dimorphotheca cuneata* (Thunb.) Less.). For those with a thin leaf blade but thick veins, the veins were sliced off with a scalpel just before clearing (e.g. *Melanthera biflora* (L.) Wild). If possible, all hairs were removed before inoculation. These treatments greatly facilitated microscopic observation.

Controls consisted of inoculated *C. monilifera* ssp. *monilifera* leaves from plants grown from seed collected in Australia. Only if the control leaves were successfully penetrated were the other test plant leaves observed.

The whole of each leaf was observed microscopically (using a 20x stage objective), and all germinated spores found on the leaves were examined (using a 40x

stage objective). It was recorded whether any spores had successfully penetrated into a test plant epidermal cell, or whether the spore germination and development was abnormal compared to that as found on *C. monilifera* ssp. *monilifera*.

Because of the various treatments that had to be done on the leaves to allow microscopic examination, loose spores were lost from the leaves. This was especially true of those plants with smooth leaves (including *C. monilifera* ssp. *monilifera* once the leaf hairs were removed). This prevented quantitative comparisons of germination rates being made on the different test plants.

**Inoculation of test plants: Whole-plant trials.** The following plants were inoculated: *Calendula officinalis* L., *Chrysanthemoides monilifera* ssp. *rotundata*, *Dimorphotheca cuneata*, *D. jucundum* E. Phillips, *Gazania rigens*, *Gerbera jamesonii* Bolus ex Adlam, *Olearia lirata* Hutchinson, *Osteospermum junceum* P.J. Bergius, and *Ozothamnus ferrugineus* (Labill.) Sweet. Test plants were inoculated by dusting dry aecidioid teliospores of *E. osteospermi* onto marked leaves, and then spraying those leaves with water using an air brush. Two leaves on each of five individual plants of each test species were inoculated. The plants were then placed in a dew chamber for 3 days in a controlled environment room with a maximum temperature of 16°C. After 3 days, one leaf from each of the five test plants for each plant species were cleared, stained and observed as above. The inoculations were repeated once.

As controls, plants of *C. monilifera* ssp. *monilifera* were treated in the same way. Only when successful penetration was observed in the control leaves were the leaves of the other plant species observed.

## RESULTS

**Penetration of the host plant *C. monilifera* ssp. *monilifera*.** At 24 h the aecidioid teliospores had germinated and developed to produce 2 (sometimes 3, and only occasionally 4) vesicle-like modified basidiospores (Chapter 4). These structures adhered to the leaf surface. The germ tubes developed in a straight line perpendicular to the germ pore. At 48 h penetration pegs could be observed at the tip of the basidiospores, penetrating directly into a host epidermal cell. At 72 h, a small round vesicle had developed in the host epidermal cell (Figures 1–3), which had expanded by 96 h to be sausage shaped (Figures 4–6). At this time, the basidiospores are empty

of cellular contents. Penetration only occurred on young expanding leaves, no penetration occurred on mature, fully expanded leaves.

**Inoculation of test plant: Detached leaf trials.** Results are summarised in Table 1. The reaction of the germinating aecidioid teliospores on the various test plant species was divided into 4 categories; namely 1) aecidioid teliospores aborted immediately after germination, 2) aecidioid teliospores germinated normally but no sign of attempted penetration observed, 3) penetration is attempted (penetration peg observed) but no vesicle developed within the epidermal cell, and 4) penetration with vesicle observed.

For the purpose of this work, aborted spores were those with germ tube length less than  $\frac{2}{3}$  of that of a normal germ tube, and where there was no sign of basidiospore development. In most cases the germ tubes were very short and thickened. Those test plants that caused aborted spores can be divided into 2 sub-groups; (i) those on which all germinated aecidioid teliospores were aborted, and (ii) those on which more than 35% of germinated spores were aborted with many of the rest having incomplete or misshapen germ tubes. Those in the first sub-group (i) were: *Carthamus tinctorius* L., *Dimorphotheca cuneata* (Figure 7), *Helichrysum petiolare* Hilliard & B.L. Burtt (Figure 8), *Ixodia achillaeoides* R. Br., *Leucophyta brownii* Cass., *Ozothamnus turbinatus* DC., and *O. ferrugineus* (Labill.) Sweet (Figure 9). *Carthamus tinctorius* differed from all the other plants in that the cell contents of the germinated spores had completely collapsed (Figure 10). Those in the second sub-group (ii) included: *Acacia melanoxylon* R. Br., *Brachycome multifida* DC. (Figure 11), *Calendula officinalis*, *Cyanara scolymus* L. (Figure 12), *Helianthus annuus* L., *Sigesbeckia orientalis* L., and *Tanacetum vulgare* L. In all the above plants, glands or glandular trichomes were common or abundant on the leaf surfaces (Table 1). In the second subgroup non-glandular trichomes were also common to abundant, and in general the aborted spores germinated directly on the leaf surface whereas those that developed normally were up amongst the non-glandular trichomes. *Brachycome multifida* and *A. melanoxylon* both showed an age modified response wherein the younger leaves were densely covered with glandular hairs and showed high levels of abortion, whereas as the leaves matured the glandular hairs were less dense and some of the spores developed normally.

Test plants on which aecidioid teliospores germinated normally but did not attempt to penetrate included: *Acacia longifolia* (Andrews) Willd, *Actites*

*megalocarpa* (Hook. f.) N.S. Lander (Figure 13), *Adenstemma lavenia* (L.) Kuntz. (Figure 14), *Banksia integrifolia* Labill. ex Meisn., *Chrysanthemum morifolium* Ramat, *Cotula turbinata* L., *Delairea odorata* Lem., *Lactuca sativa* L. (Figure 15), *Leptospermum laevigatum* (Gaertn.) F. Muell., *Melanthera biflora* (L.) Willd (Fig. 16), *Olearia axillaris* (DC.) Benth., *O. lirata* (Sims) Hutch., *Senecio odoratus* Hornem., and *Vernonia tigna* Klatt. On these leaves the basidiospores showed no sign of having attempted to penetrate the plant epidermis, in that at 96 h they were still full of their cellular contents, there was usually a long thin germ tube emerged from the modified basidiospores, no penetration pegs were observed, and the germ tubes and basidiospores did not adhere to the epidermis. The epidermis of leaves of these plants consisted of small rounded cells in comparison to the large flat cells found in *Chrysanthemoides*.

Penetration pegs were produced, although no further signs of penetration were seen on *Dimorphotheca jucundum* and *Bedfordia arborescens* Hochr. On *B. arborescens*, one vesicle in a leaf epidermal cell was observed but no other penetration events were found notwithstanding that many penetration pegs were produced. Successful penetration, as found in *C. monilifera* ssp. *monilifera*, occurred in *C. monilifera* ssp. *rotundata*, *Eucalyptus cladocalyx* F. Muell., *Gazania rigens*, *Gerbera jamesonii*, *Osteospermum ciliatum* P.J. Bergius, and *O. junceum*.

**Inoculation of test plants: Whole-plant trials.** In all but one case (*D. jucundum*) the observations made on the whole plants were exactly the same as that observed in the detached leaf trials (Table 2). All germinated aecidioid teliospores on *C. officinalis*, *D. cuneata*, *O. lirata*, and *O. ferrugineus* aborted. Successful penetration occurred in *C. monilifera* ssp. *rotundata*, *G. rigens*, *G. jamesonii*, *D. jucundum* and *O. junceum*.

In *D. jucundum* incomplete penetration was observed in the detached leaf trials, although penetration pegs were commonly produced, but successful penetration was observed in the whole plant trials. Slightly younger leaves were used in the whole plant trials than in the detached leaf trials. It was noted that the younger leaves used in the whole plant trials had a more dense covering of glandular hairs than the older leaves used in the detached leaf trials, and a high proportion of germinated spores had aborted on the younger leaves. This reaction was not noted on the older leaves used in the detached leaf trials.

## DISCUSSION

Witches' brooms took between 5 and 24 months to begin to develop after whole plants of *C. monilifera* ssp. *monilifera* were inoculated (Chapter 4). This long latent period makes traditional host specificity testing, in which all test plants are inoculated and observed for symptom development, difficult in practice. The relatively rapid results obtained in the trials reported on in this paper identified those plant species, out of a predetermined list, needing more detailed testing. Of a total of 35 test plant species, only 6 require further testing, namely *B. arborescens*, *D. jucundum*, *E. cladocalyx*, *G. rigens*, *G. jamesonii* and *O. junceum*. These are the only species in which direct penetration into the plant epidermal cells was attempted or was successful. Except for *B. arborescens*, all these species are common indigenous plants or commonly cultivated in the Western and Eastern Cape Provinces, where *E. osteospermi* occurs naturally (Chapter 5), and are therefore exposed to natural infection by *E. osteospermi*. None have been reported as being infected with *E. osteospermi* (Doidge, 1950; Crous *et al.*, 2000). Both *C. monilifera* ssp. *rotundata* and *O. ciliatum* are naturally infected in South Africa (Chapter 5), and therefore further testing of these species is unwarranted.

Of the 9 test plant species in which penetration did occur, or was attempted, all but one belong to the Asteraceae (Calenduleae, Senecioneae, Arctotideae and Mutiseae). The only exception, *E. cladocalyx*, was initially a surprising result. However phylogenetic relatedness itself is not an indicator of whether penetration should occur or not. A lack of correlation between phylogenetic relatedness and susceptibility has been noted in a number of host specificity tests (Wiedemann, 1991). Two of the 5 plant species in the Calenduleae that were tested caused germinating spores to abort. Whether penetration occurred or not depended on the leaf epidermis structure of the plant species. Leaves of all the plants in which penetration occurred, including *E. cladocalyx*, have in common a very smooth epidermal cell layer with large epidermal cells as found in *C. monilifera*. None of the 12 test plant species on which the spores germinated but did not attempt penetration had this leaf morphology. This suggests that for penetration to occur a physically suitable leaf surface on which the modified basidiospores can attach is essential. The necessity for a correct physical surface for rust fungi to penetrate into their hosts is well known (e.g. Heath, 1982).

Germinated aecidioid teliospores aborted on 14 of the test plant species. All these 14 plant species had many to abundant glands or glandular trichomes on the leaf surface. On those that had trichomes in addition to the glands, germinating aecidioid teliospores on the leaf surface aborted whereas those amongst the trichomes germinated normally. It is probable that *Chrysanthemum morifolium* should also be included amongst these species, but due to the dense covering of trichomes on the young leaves of this species not enough observations on the leaf surface were made to be certain. Glands were not observed on *Leucophyta brownii*, due to the dense layer of trichomes, but they probably occurred as all germinating aecidioid teliospores on this species aborted. Such abortion on germination has seldom been reported for a rust fungus, though a number of succulent plant species within the Asclepiadaceae and *Olea paniculata* Roxb. caused plasmolysis either before or during spore germination of *Maravalia cryptostegia* (Cummins) Y. Ono (Evans and Tomley, 1994).

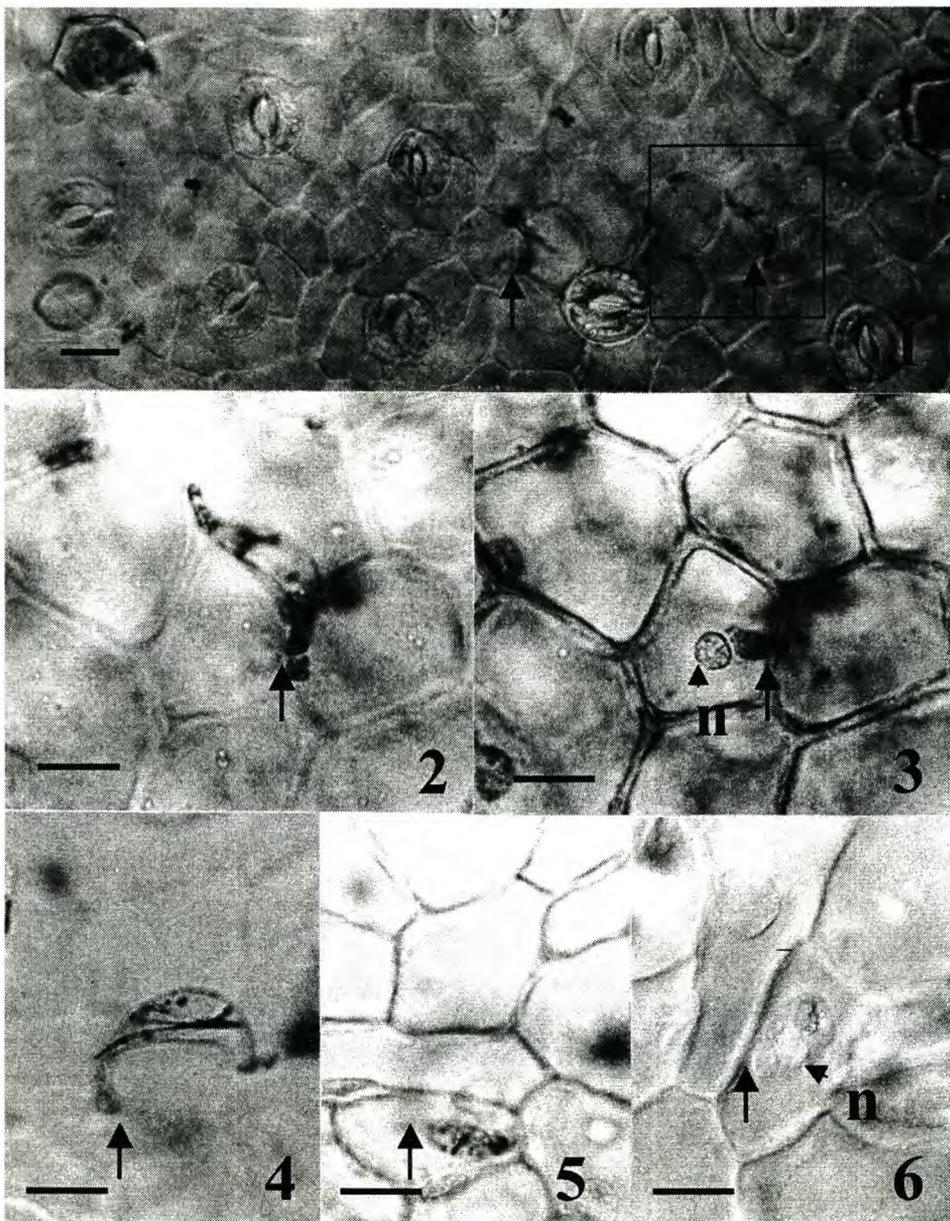
The whole-plant trials confirmed that the results obtained in the detached leaf trials were a true reflection of what happens in whole plants, and not false results because of physiological changes occurring in the leaves after detachment. The results indicate that *E. osteospermi* is likely to prove to be suitably host specific as a candidate biocontrol agent against *C. monilifera* ssp. *monilifera*.

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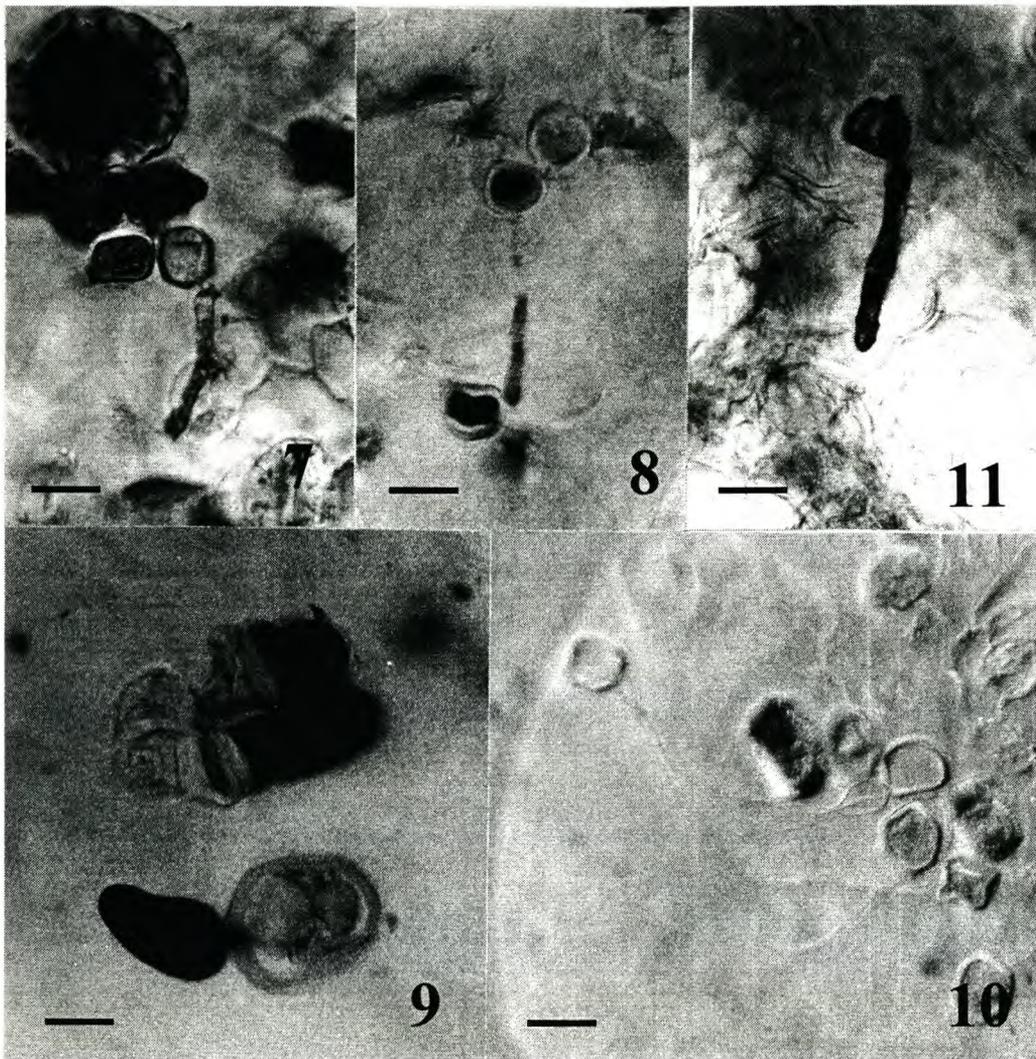
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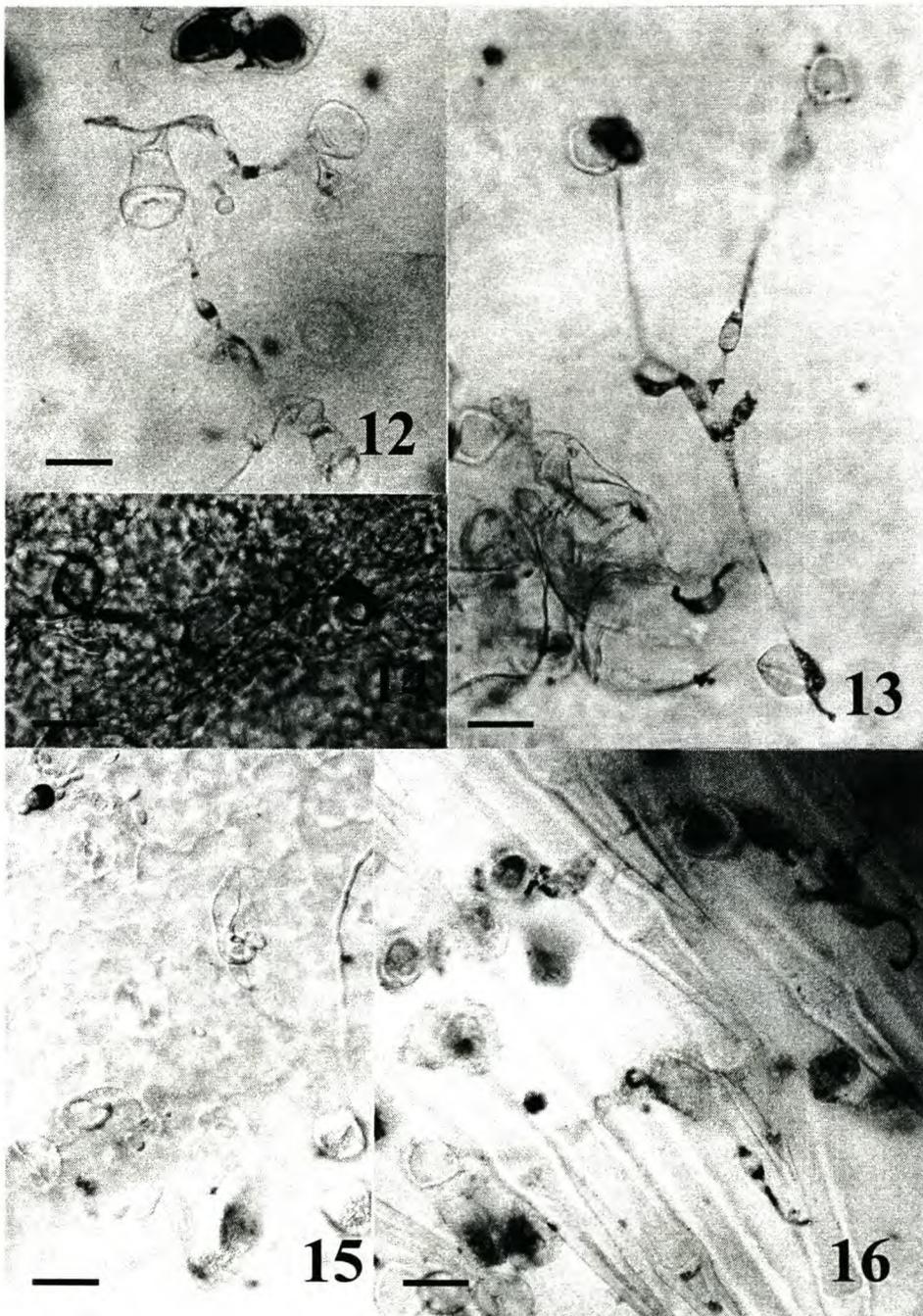
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**Figures 1-6.** Penetration by basidiospores of *Endophyllum osteospermi* into epidermal cells of *Chrysanthemoides monilifera* ssp. *monilifera*. **Figure 1** Germinated aecidioid teliospore with two vesicle-like modified basidiospores, both of which have penetrated directly into epidermal cells of a young leaf. Arrow indicates point of penetration. **Figures 2-3.** Basidiospore and vesicle, indicated by box in Figure 1, which has penetrated into an epidermal cell of a young leaf at 3 days after inoculation. The plane of focus in Figure 2 is above the leaf surface, but is within the epidermal cells in Figure 3. Arrow indicates point of penetration. **Figures 4-5.** Basidiospore and vesicle which has penetrated into an epidermal cell of a young leaf at 4 days after inoculation. The plane of focus in Figure 4 is above the leaf surface, but is within the epidermal cells in Figure 5. Arrow indicates point of penetration. **Figure 6.** Vesicle in an epidermal cell of a young leaf at 4 days after inoculation. Arrow indicates point of penetration. Scale bar: 1 = 25  $\mu\text{m}$ , 2-6 = 15  $\mu\text{m}$ .



**Figures 7–11.** Aborted accidioid teliospores of *Endophyllum osteospermi* on leaf surfaces of various non-host plant species. **Figure 7.** On *Dimorphotheca cuneata*. **Figure 8.** On *Helichrysum petiolare*. **Figure 9.** On *Ozothamnus ferrugineus*. **Figure 10.** On *Carthamnus tinctorius*, cellular contents of spores and metabasidia are completely collapsed. **Figure 11.** On *Brachycome multifida*. Scale bar = 25  $\mu\text{m}$ .



**Figures 12–16.** Germinated aecidioid teliospores of *Endophyllum osteospermi*, on leaf surfaces of various non-host plant species. No sign of attempted penetration was observed on these plant species. **Figure 12.** On *Cyanara scolymus*. **Figure 13.** On *Actites megalocarpa*. **Figure 14.** On *Adenstemma lavenia*. **Figure 15.** On *Lactuca sativa*. **Figure 16.** On *Melanthera biflora*. Scale bar = 25  $\mu\text{m}$ .

**Table 1: Preliminary host specificity testing of *Endophyllum osteospermi*, to determine whether germinating aecidioid teliospores penetrated into the excised leaves of the test plants (++) , attempted penetration (+), did not attempt penetration (N), or aborted (A) on germination<sup>A</sup>, at four days after inoculation.**

Test plant family and tribe	Test plant species	Presence/absence of leaf glands	Inoculation <sup>C</sup>		
			1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>
<i>Asteraceae</i>					
1	<i>Calenduleae</i> <i>Chrysanthemoides monilifera</i> ssp. <i>rotundata</i>	n <sup>B</sup>	++ <sup>A</sup>	++	++
2	<i>Osteospermum ciliatum</i>	n	++	++	++
3	<i>Osteospermum junceum</i>	y, 1	+	++	
4	<i>Dimorphotheca cuneata</i>	y, 2	A	A	A
5	<i>Dimorphotheca jucundum</i>	y, 1	+	+	+
6	<i>Calendula officinalis</i>	y, 3	A	A	A
7	<i>Senecioneae</i> <i>Bedfordia arborescens</i>	n	++	+	+
8	<i>Delairea odorata</i>	n	N	N	N
9	<i>Senecio odoratus</i>	n	N	N	N
10	<i>Arctotideae</i> <i>Gazania rigens</i>	n	++	+	++
15	<i>Anthemideae</i> <i>Chrysanthemum morifolium</i>	y, 3	N	N	N
16	<i>Tanacetum vulgare</i>	y, 2	A	A	A
11	<i>Astereae</i> <i>Brachycome multifida</i>	y, 2	A	A	A
12	<i>Cotula turbinata</i>	n	N	N	N
13	<i>Olearia axillaris</i>	n	N	N	N
14	<i>Olearia lirata</i>	n	N	N	N
17	<i>Cynareae</i> <i>Carthamnus tinctorius</i>	y, 2	A	A	N
18	<i>Cynara scolymus</i>	y, 2	A	A	A
19	<i>Eupatoriae</i> <i>Adenstemma lavenia</i>	n	N	N	N
20	<i>Heliantheae</i> <i>Helianthus annuus</i>	y, 3	A	A	A
21	<i>Melanthera biflora</i>	n	N	N	A
22	<i>Sigesbeckia orientalis</i>	y, 2	A	A	A
23	<i>Inuleae</i> <i>Leucophyte brownsii</i>	n (?)	A	A	A
24	<i>Helichrysum petiolare</i>	y, 3	A	A	A
25	<i>Ixodia achillaeoides</i>	y, 2	A	A	A
26	<i>Ozothamnus turbinatus</i>	y, 2	A	A	A
27	<i>Ozothamnus ferrugineus</i>	y, 3	A	A	A
28	<i>Lactuceae</i> <i>Actites megalocarpa</i>	n	N	N	N
29	<i>Lactuca sativa</i>	y, 1	N	N	N
30	<i>Mutiseae</i> <i>Gerbera jamesonii</i>	n	++	++	++
31	<i>Vernonieae</i> <i>Vernonia tigna</i>	n	N	N	N

**Table 1: continued.**

Test plant family	Test plant species	Presence/ absence of leaf glands	Inoculation		
			1st	2nd	3rd
<i>Leguminosae</i>					
32	<i>Acacia longifolia</i>	y, 1	N	N	N
33	<i>Acacia melanoxylon</i>	y, 3	A	A	A
<i>Myrtaceae</i>					
35	<i>Eucalyptus cladocalyx</i>	n	+	++	++
36	<i>Leptospermum laevigatum</i>	n	N	N	N
<i>Proteaceae</i>					
37	<i>Banksia integrifolia</i>	n	N	N	N

<sup>A</sup>Successful penetration (++) = vesicle observed in leaf epidermal cell, attempted penetration (+) = penetration peg at end of short germ tube of basidiospore observed, unsuccessful penetration (N) = no penetration peg, long germ tube of basidiospore, basidiospore not adhering to epidermis, aborted (A) = short metabasidium, no basidiospore.

<sup>B</sup>n = glands absent, y = glands present, if present then 1 = sparsely, 2 = intermediate and 3 = densely distributed.

<sup>C</sup>Each test plant species was inoculated three times, on each occasion penetration occurred on inoculated control leaves of *C. monilifera* ssp. *monilifera*.

**Table 2: Results of whole plant inoculation trials showing whether germinated aecidioid teliospores of *Endophyllum osteospermi* successfully penetrated (++) epidermal cells of leaves or aborted (A) after germination on various plant species.**

Test plant	1 <sup>st</sup> inoculation	2 <sup>nd</sup> inoculation
<i>Chrysanthemoides monilifera</i> ssp. <i>rotundata</i>	++ <sup>A</sup>	++
<i>Osteospermum junceum</i>	++	++
<i>Calendula officinalis</i>	A	A
<i>Dimorphotheca cuneata</i>	A	A
<i>Dimorphotheca jucundum</i>	++	++
<i>Gazania rigens</i>	++	++
<i>Olearia lirata</i>	A	A
<i>Ozothammus ferrugineus</i>	A	A
<i>Gerbera jamesonii</i>	++	++

<sup>A</sup>See table 1 for legend.