Chapter 9

Investigations into seedling mortality of *Pennisetum clandestinum*

9.1 Introduction

It has been widely reported that one seed of kikuyu grew when first planted at The Royal Botanic Gardens, Sydney. While Eykamp (2003) stated that a total of six seeds entered the country originally, there is no other evidence available to validate this claim. Whatever the original number may have been, it indicated early on that kikuyu grass has a low germination percentage.

There are a number of factors, both externally and internally, even in favourable growing conditions, which can contribute to seedling mortality pre and post germination. Externally, soil fungal damage from *Fusarium, Pythium* and *Rhizoctonia* spp., known as Damping-off, are common problems in young turfgrass seedlings (Beard, 1973; Smiley *et al.,* 2005). Internally, the presence of inhibitors restricting the alpha-amalase enzyme activity can reduce germination. Some forms of turfgrasses which possess seed germination inhibitors have been noted by Beard (1973) as *Digitaria sanguinalis* and some cultivars of *Poa pratensis.*

At an early stage in my work a seedling tray count with cultivar 'Whittet' showed that from a total of 100 seeds sown only 63 germinated. In addition, many seedlings after growing for a short time yellowed and died, further reducing the actual establishment rate. Although this is above the 60% minimum germination set by the National Seed Quality Standards (Zurbo, 2007) it still provides too high a loss of potential new genotypes. This is especially the case for any prospective hybridisation programme on a species capable of producing one major population per year. Early observations on the germination process gave an indication as to why, particularly when carried out on agar slopes for accurate viewing. It was hoped that by viewing the germination process on

agar it would provide an idea as to why in most cases the majority of germinating seeds failed to fully develop into mature plants. These experiments were carried out between March and June, 2006.

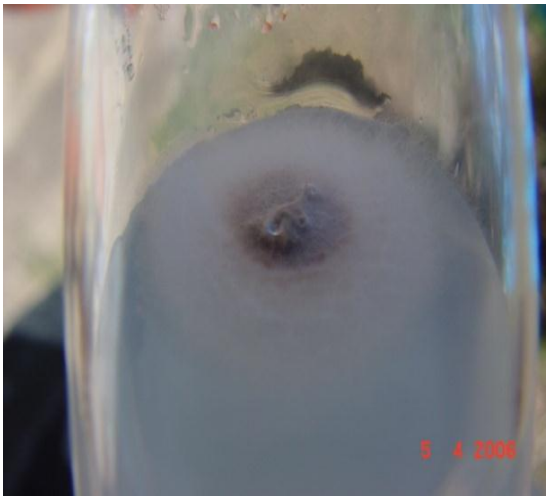
9.2 Seed and Base Agar Mixture

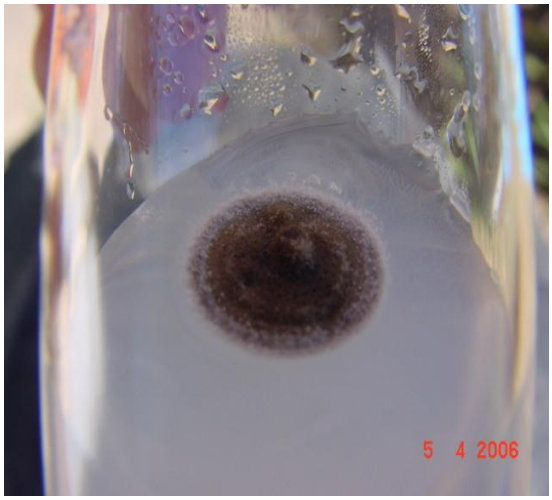
Pennisetum clandestinum c.v. 'Whittet' (Eykamp Kikuyu Co., Quirindi, NSW) was chosen for initial studies into gel establishment as it is the most common form of kikuyu seed commercially available. Working in a laminar flow hood seed was initially surface sterilised starting with immersion in 70% (v/v) ethanol for 2min before being removed and immersed in a 1:1 aqueous dilution of domestic household bleach (Sodium Hypochlorite solution containing 3.5% of active chlorine) and were gently stirred every 10min for a total period of 30min. The seeds were then triple rinsed in autoclaved distilled water and rested in the final rinse water for up to 10min before placing on agar consisting of 10g/L purified agar (Sigma A7921), 20g/L Sucrose (BDH AnalaR® 10274.4B) and 2.4g/L Gamborg's B-5 Basal Medium with Minimal Organics (Sigma G58893). The pH of the medium was adjusted to 5.8 with approximately 300µL 1M NaOH. The prepared medium was then transferred to tubes, 10mL per 30mL tube (Sarstedt Pty. Ltd. 60.9922.212), before autoclaving at 121°C for 15min (Siltex HC2 MK1-94). The tubes were then placed on an angle to cool and create the agar slope. The cultured seeds were then incubated at a temperature range of 16°C (dark)/ 24°C (light) (±1°C) under a 12h photoperiod of white fluorescent light with an intensity of 5µmol m^{-2} s^{-1}.

9.3 Germination

Germination of kikuyu grass, in favourable conditions, occurs rapidly. Imbibition is complete within 24h with subsequent emergence of the radicle at 3d after sowing, providing warm conditions of about 25°C are maintained. However, as soon as the radicle had emerged on the majority of occasions, an unidentified fungus quickly emerged and engulfed the seed. The fungus was not limited to one particular species with several forms being noted and isolated into pure cultures. On most occasions it resulted in rapid seedling mortality, but in others it was slower to emerge, gradually

taking control of the seedling development and resulting in mortality if the seedling was not removed from the medium, Figures 9.1 – 9.4.





Figures 9.1 – 9.2. Examples of fungi emerging from germinating seeds of kikuyu grass.

Figures 9.3 – 9.4. Examples of fungi emerging from germinating seeds of kikuyu grass.

Fearing that an inadequate surface sterilisation technique was responsible for the fungal invasion, seeds of cultivar 'Whittet' were imbibed over 24h in distilled water before surface sterilizing as before, followed by dissection onto fresh agar slopes. Individually, the seed coat, embryo, endosperm, and a portion comprising the embryo/endosperm containing the scutellum were removed, set to grow on agar slopes and observed. Contamination was only observed in the portion containing the scutellum, the timing

indicating that the pathogen becomes active or is ‘released’ at about the same time as the secretion of enzymes during germination, Figure 9.5.

Figure 9.5. Germination of *Pennisetum clandestinum* showing the radical and plumule, with fungi emerging from the scutellum.

9.3.1 Agar and Seed Sterilisation Amendments

To try to reduce the occurrence of the emerging fungi, further seed preparation and agar amendments were made in an attempt to secure a high percentage of clean germination, which included:

9.3.1.1 Hot Water Treatment:

Seeds of *Pennisetum clandestinum* c.v. ‘Whittet’ were placed in a 60°C water bath for 10min before proceeding to surface sterilization, and placement on agar slopes.

9.3.1.2 Mercuric Chloride ($HgCl_2$):

Seeds of *Pennisetum clandestinum* c.v. ‘Whittet’ were immersed in 70% (v/v) ethanol for 2min before immersion in 0.05% and 0.1% $HgCl_2$ for 15min, triple rinsed with autoclaved distilled water and placed on agar slopes.

9.3.1.3 Plant Preservative Mixture (PPM):

Seeds of *Pennisetum clandestinum* c.v. 'Whittet' were surface sterilised and placed on agar slopes amended with 0.05%, 0.1% and 0.2% (v/v) Plant Preservative Mixture (Austratec Pty. Ltd. P 820).

Fungal contamination was still observed during germination in both the hot water and mercuric chloride treatments, so these methods were subsequently dismissed. The biocide PPM provided effective control of the fungal growth at all concentrations, further highlighting that the fungi is of internal origin and not a surface sterilising concern, Figure 9.6.

It was determined that given the control of the unknown fungi by PPM, an addition of 0.1% would be routinely made to the agar mixture in preparation of future sowings of kikuyu seed for experimental purposes.

Figure 9.6. Control of the fungal growth in germinating seeds of *Pennisetum clandestinum* using Plant Preservative Mixture. L-R: Control (x2), PPM 0.05% (x2), PPM 0.1% (x2), PPM 0.2% (x2)

9.4 Endophyte presence as a cause of the seedling fungal infections.

Kikuyu's domination of mixed swards across a wide range of conditions is evident and allelopathic effects have been reported as at least part of the explanation (Lovett, 1986; Chou, 1989). This dominance may also reflect the presence of an endophyte, which would be transferred from one generation to the next via the seed. Endophytes in grasses causing death in cattle, horses, goats and sheep have been reviewed by Clay (1988), but he does not mention kikuyu. However, given the widespread usage of kikuyu as a grazing fodder, the possibility exists that the presence of endophytes may play a role in the kikuyu-related deaths of grazing ruminants commonly attributed to nitrite poisoning.

In the light of the demonstration in the previous section that the fungi associated with seedling mortality are endogenous and appear to be concentrated in the scutellum region of the embryo, the hypothesis is put forward that these fungi live endophytically in the shoot system of the kikuyu plants and achieve transmission from generation to generation via invasion of the scutellum region of the developing embryo within the seed. A further hypothesis is that during germination the fungal hyphae grow out from the scutellar region and re-infect the developing shoot system, weakening or killing many seedlings in the process. Although there was insufficient time to explore these hypotheses in detail, this section and the following section contain some preliminary observations.

9.4.1 Leaf staining

In an effort to demonstrate the presence of an endophyte, small sections of leaf of the cultivar 'Whittet' germinated from seed was incised perpendicularly before staining with fresh 0.5% Rose Bengal solution and viewed under a compound microscope at 400x, Figures 9.7 and 9.8.

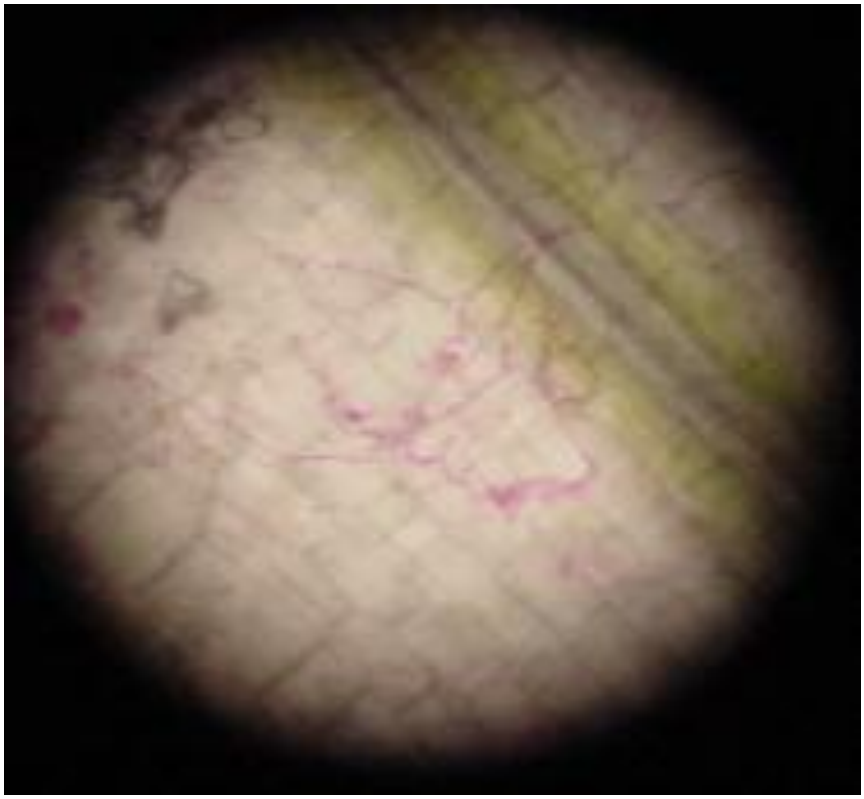


Figure 9.7. Hyphae observed in leaf of kikuyu grass cv. ‘Whittet’.

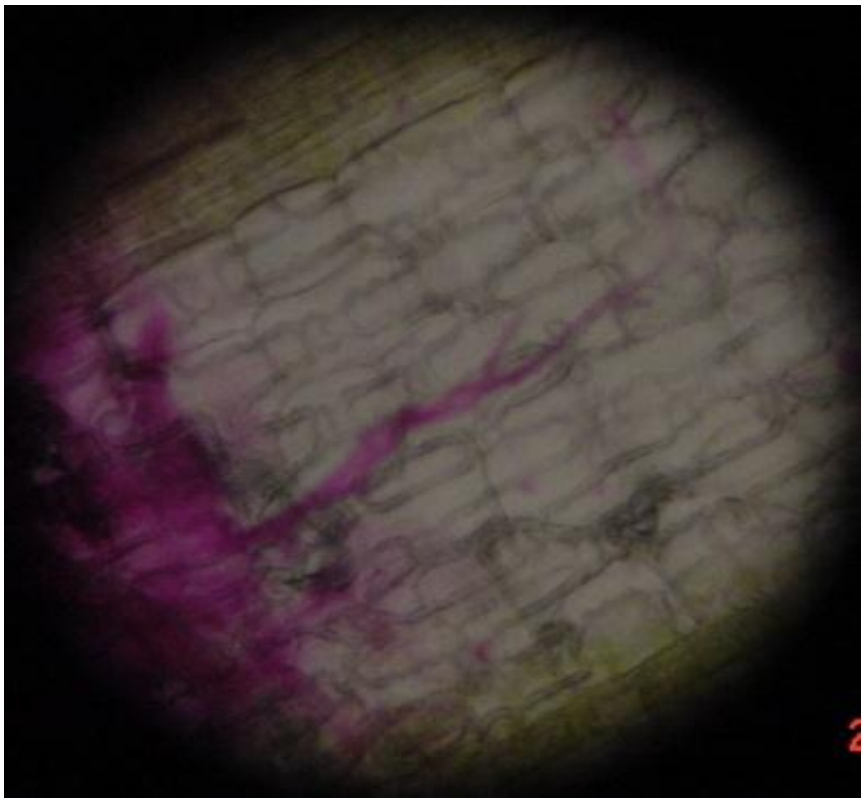


Figure 9.8. Hyphae observed in leaf of kikuyu grass cv. ‘Whittet’.

Hyphal growth through the inter-cellular spaces was evident, characteristic of an endophyte. This is the first time images of inter-cellular hyphal growth resembling that of an endophyte have been reported. What is most unusual about this observation of an endophyte presence is its being identified in a C_4 grass species. Endophytes have occasionally been reported in C_4 grasses, such as the *Myriogenospora* genus in *Paspalum* spp. (Clay, 1988), but the vast majority of endophytes found in turf and pasture settings are *Acremonium* spp. in C_3 grasses, especially as found and exploited by breeders in cultivars of the genera *Festuca* and *Lolium*.

9.5 Fungal identification

Fungi associated with infected seedlings were isolated and cultured on the same base agar mixture to that of the seeds with seven distinct types identified. Pure cultures of these were sent to the NSW Department of Primary Industries diagnostic laboratories for identification. Of the samples, two were identified as *Phoma* spp., two were identified as *Bipolaris* spp., one as *Nigrospora* spp., and one as *Alternaria* spp. The seventh sample was unable to be identified, remaining sterile even under UV black light conditions. Clay (1988) writes "*Acremonium* endophytes are closely related to the Balansiae, but are "anamorphic" or "imperfect" fungi, which are not known to have sexual reproduction. Their hyphae occur intercellularly in leaf and stem tissue, but unlike the ascospore-producing Balansiae fungi, they do not produce fruiting bodies on host plants. Instead, they are transmitted maternally by growth of hyphae into developing ovules and seeds of infected maternal plants." It is probable that hyphae of this sterile fungus were represented by some of the endophyte threads shown in Figures 9.7 and 9.8, but to what extent the other four genera were behaving as true endophytes remains unknown. This is not the first time that an undescribed sterile fungus has been isolated and cultured from kikuyu, Wong *et al*. (1987) having isolated two, but it is not possible to say whether the sterile fungus isolated in this study is the same as one of theirs, as the original cultures were not maintained following the study.

9.5.1 Possible role of kikuyu endophytes in ruminant toxicity

The toxic effects on grazing ruminants of *Acremonium* endophytes in the *Lolium, Festuca, Stipa* and *Melica* genera have been summarised by Clay (1988). Incidents of cattle poisoning after grazing on kikuyu have been reported in New Zealand (Busch *et al.,* 1969), South Africa (Wong *et al.,* 1987) and Australia (Gabbedy *et al.,* 1974). Initial observations by Cordes *et al.* (1969) noted that the deaths occurred after rainfall broke a relatively dry period and cattle grazed on the subsequent lush new growth. Analysis of the lush growth however showed that the levels of nitrate (<1%), cyanide (0%) and alkaloids (0%) were not the cause of death.

Further studies suggested that the fungus *Myrothecium* spp. may be responsible (Martinovich *et al.,* 1972), and that death could occur on closely grazed pastures, as well as pastures damaged by previous activity of army caterpillar, *Pseudaletia separate* (Smith and Martinovich, 1973). However in Western Australia, neither disease nor insect activity nor nitrates were observed in association with the deaths reported by Gabbedy *et al.* (1974). These observations were reinforced by Wong *et al.* (1987) who failed to find any occurrence of the *Myrothecium* spp. or of armyworm caterpillar in kikuyu which had caused cattle death, leading to the conclusion that a plant toxin may be involved. Therefore, it seems reasonable to suggest that under certain conditions the endophytic fungi reported above could be producing alkaloids or other toxic compounds in sufficient quantities to cause death of ruminants on kikuyu pastures.

9.6 Conclusions

In this chapter seed germination investigations reveal that:

- Rigorous surface sterilization has no effect on the fungal infection of kikuyu seedlings raised in otherwise aseptic conditions.
- Endogenous fungi emerging from the scutellum rapidly engulf the germinating seed causing a high proportion of seedling mortality.
- Additions of 0.1% Plant Preservative Mixture to agar bases reduces the incidence of mortality when germinating kikuyu seeds on agar slopes.
- Intercellular hyphae were observed growing in leaf tissue following staining with Rose Bengal.
- Images of endophyte-like hyphal growth have been produced for the first time.
- An embryo-transmitted sterile fungal species isolated in culture may well be the main endophyte in 'Whittet' kikuyu.
- The six other apparently embryo-transmitted fungi, belonging to the genera *Phoma*, *Bipolaris*, *Nigrospora* and *Alternaria*, are not normally considered to be endophytic fungi and may have reached the embryo via infection of the flowers.
- The question of systemic seedling infection and the possibility of true endophyte growth in kikuyu provide attractive lines of future research.