# Incidence of the endophyte *Neotyphodium occultans* in annual ryegrass in southern New South Wales

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**Summary** Annual ryegrass (*Lolium rigidum* Gaudin) is a major weed of economic importance in winter crops in southern Australia. A shift in farming practices from cultivation to minimum or no-till systems with a heavy dependence on herbicides for weed control, has led to the evolution of herbicide resistance in many annual ryegrass populations. This has resulted in a loss of diversity in the chemical modes of action left available to control this weed.

Annual ryegrass is host to the fungal endophyte *Neotyphodium occultans* (Moon *et al*. 2000). Studies in other grass/*Neotyphodium* associations have determined significant effects on the morphology and physiology of the host plant. Studies by Vila-Aiub *et al*. (2003) have found that Italian ryegrass infected with *Neotyphodium* sp. may play a part in the evolution of herbicide resistance. This paper reports the incidence of *N. occultans* in populations of annual ryegrass across southern New South Wales. Results will provide a benchmark for further studies across Australia relating to herbicide resistance and endophyte infection. Endophyte was found in all populations studied but the level of infection varied with an average infestation of 50%.

**Keywords** Ryegrass, *Neotyphodium*, endophyte, herbicide resistance, symbiotic.

## INTRODUCTION

Annual ryegrass is native to the Mediterranean and introduced into Australia in the early 1900s following research in pasture improvement however it is now one of the worst weeds in the cereal producing areas of Australia. Changes in farming practices have led to the development of herbicide resistant populations (Heap and Knight 1982, Powles *et al*. 1997 and Broster and Pratley 2006).

There is abundant research on perennial ryegrass (*Lolium perenne* L.) and its symbiotic relationship with the endophyte *Neotyphodium lolii*, however little is known about the relationship of annual ryegrass and *N. occultans*. The incidence of *Neotyphodium lolii* in perennial ryegrass in Australia is well documented. Reed *et al*. (2000) reported the incidence of *N. lolii* in Victorian and Kangaroo Valley ecotypes in Australia and found the mean frequency of infection as 88% and 93% respectively. Guy (1992) surveyed Tasmania and found a mean infection frequency of 66%.

## MATERIALS AND METHODS

Seed samples collected by Charles Sturt University Herbicide Resistance Testing Service since 1991 have been stored in paper envelopes within plastic boxes at ambient temperature. From this collection 100 seeds of 36 populations were sampled from 13 postcodes within southern NSW to determine incidence status.

The percentage of seeds infected with endophyte from each sample was determined using the seed squash technique (Latch *et al*. 1987), although it does not distinguish between live and dead hyphae (Hume *et al*. 1993). The seeds were observed using a light compound microscope on 10X objective to locate the aleurone layer, before using 40X objective to distinguish hyphae.

Dry seeds were examined using scanning electron microscopy to observe hyphae in their natural dehydrated state. Seeds were fixed into sample holders with low temperature Tissue Tek and fractured under liquid nitrogen ($LN_2$). They were then transferred under $LN_2$ with the holder to the stage of a cryo-SEM (Cambridge 360). Warming the specimen to −90°C at low kV sublimed the surface frost. The specimen was moved to the preparation chamber where it was cooled to −160°C, sputter-coated with gold and returned to the cold stage in the microscope column for observation.

## RESULTS

The incidence of endophyte in the samples considered is provided in Table 1. Endophytes were present in all populations studied although incidence varied from 15% to 80% with most populations showing moderate infection between 50 and 69% (Table 2). Overall, the infection averaged 50% across the total number (i.e. 3600) of seeds screened.

The populations sampled covered a range of growing seasons as determined by rainfall and temperature throughout southern NSW. Frequency of infection varied both within and between postcodes.

Microscopic examination of seed squashes showed hyphae (1–2 μm) are abundant within the seed. The hyphae are highly convoluted, bunched and rarely branched consistent with the description given by Moon *et al*. (2000). Dry seeds examined with the scanning electron microscope showed the hyphae in their natural state (Figure 1).

**Table 1.** Incidence status of *N. occultans* in 100 seeds of 36 samples of annual ryegrass from southern NSW.

| Postcode | Town | Proportion | 95% Confidence | |
|---|---|---|---|---|
| | | | Lower | Upper |
| 2700 | Narrandera | 0.72 | 0.62 | 0.81 |
| | Narrandera | 0.30 | 0.21 | 0.40 |
| | Narrandera | 0.38 | 0.28 | 0.48 |
| 2877 | Condobolin | 0.26 | 0.18 | 0.36 |
| | Condobolin | 0.44 | 0.34 | 0.54 |
| | Condobolin | 0.43 | 0.33 | 0.53 |
| 2590 | Cootamundra | 0.31 | 0.22 | 0.41 |
| | Cootamundra | 0.29 | 0.20 | 0.39 |
| | Cootamundra | 0.47 | 0.37 | 0.57 |
| 2800 | Orange | 0.63 | 0.53 | 0.72 |
| | Orange | 0.71 | 0.61 | 0.80 |
| | Orange | 0.55 | 0.45 | 0.65 |
| 2650 | Wagga Wagga | 0.72 | 0.62 | 0.81 |
| | Wagga Wagga | 0.28 | 0.19 | 0.38 |
| | Wagga Wagga | 0.15 | 0.09 | 0.24 |
| 2671 | West Wyalong | 0.37 | 0.28 | 0.47 |
| | West Wyalong | 0.56 | 0.46 | 0.66 |
| | West Wyalong | 0.33 | 0.24 | 0.43 |
| 2871 | Forbes | 0.49 | 0.39 | 0.59 |
| | Forbes | 0.60 | 0.50 | 0.70 |
| | Forbes | 0.29 | 0.20 | 0.39 |
| 2710 | Deniliquin | 0.56 | 0.46 | 0.66 |
| | Deniliquin | 0.61 | 0.51 | 0.71 |
| | Deniliquin | 0.64 | 0.54 | 0.73 |
| 2711 | Hay | 0.63 | 0.53 | 0.72 |
| | Hay | 0.68 | 0.58 | 0.77 |
| 2680 | Griffith | 0.34 | 0.25 | 0.44 |
| | Griffith | 0.55 | 0.45 | 0.65 |
| | Griffith | 0.58 | 0.48 | 0.68 |
| 2582 | Yass | 0.63 | 0.53 | 0.72 |
| | Yass | 0.55 | 0.45 | 0.65 |
| | Yass | 0.31 | 0.22 | 0.41 |
| 2795 | Bathurst | 0.54 | 0.44 | 0.64 |
| 2640 | Albury | 0.57 | 0.47 | 0.67 |
| | Albury | 0.57 | 0.47 | 0.67 |
| | Albury | 0.80 | 0.71 | 0.87 |

## DISCUSSION

All populations surveyed were infected with the grass endophyte *N. occultans*. This research showed the presence of *N. occultans* in all populations sampled with variation in and between populations within southern NSW. *N. occultans* is an obligate endophyte and cannot survive without its host. If infection provided no selective advantage then natural selection would select for endophyte free plants, however the

**Table 2.** Infection frequencies of *N. occultans* in annual ryegrass seed populations from southern NSW.

| Infection % | Number of populations |
|---|---|
| 0 | 0 |
| 1–10 | 0 |
| 11–20 | 1 |
| 21–30 | 5 |
| 31–40 | 6 |
| 41–50 | 4 |
| 50–59 | 9 |
| 60–69 | 7 |
| 70–79 | 3 |
| 80–89 | 1 |
| 90–100 | 0 |

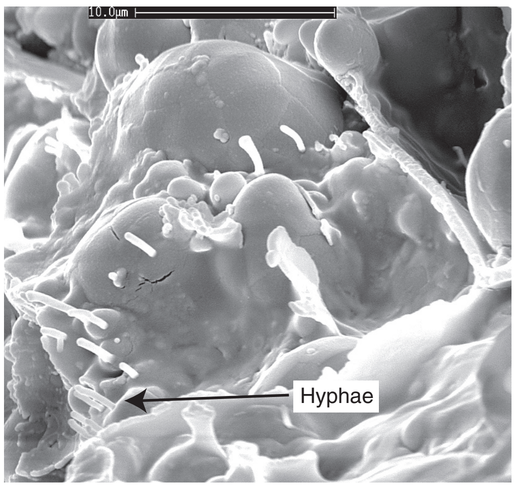


**Figure 1.** Dry seed observed using cryo-SEM (Cambridge 360) microscope to observe hyphae within the seed in their natural state of dehydration.

results of this survey show moderate infection ranging from 15–80%. This significant percentage of infection indicates this symbiotic relationship may offer benefits to the host. The next step is to find out what those benefits are. Observations from the cryo-SEM have raised further questions regarding the survival strategy of desiccated hyphae within the mature seed.

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