# BIOLOGY AND CONTROL OF THE ANGUINID NEMATODE ASSOCIATED WITH FLOOD PLAIN STAGGERS

by

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## Summary

Flood plain staggers is a poisoning of livestock similar to annual ryegrass toxicity (ARGT). In 1991, it resulted in the deaths of 1722 cattle, 2466 sheep and 11 horses in northern New South Wales (NSW) and over the past 30 years, has caused livestock losses in south-eastern South Australia (SA). The toxins involved are produced by the bacterium *Rathayibacter toxicus*, which is carried into the grasses *Polypogon monspeliensis* and *Agrostis avenacea* by nematodes belonging to the genus *Anguina.* Both hosts dominate in flood prone pastures, which may remain inundated for up to five months of the year. This study was initiated to examine the distribution, biology and ecology of the nematode and bacterium associated with flood plain staggers and to use this information to examine potential pasture and livestock management practices that could be employed to reduce the impact of flood plain staggers to the livestock industry.

Surveys conducted in SA and NSW showed that the incidence of the nematode and bacterium is greater than indicated by reported outbreaks of flood plain staggers. The organisms appear to be restricted to flood prone areas and pastures adjacent to water courses. While both *P. monspeliensis* and *A. avenacea* were found to be infested in NSW, infested *A. avenacea* has not been found in SA.

Host range studies demonstrated that the nematodes from both provenances can reproduce in either host. No galls were induced by the nematodes in closely related grasses that are known hosts of other species of *Anguina*, indicating that a new species may be involved. An examination of nematode populations from both provenances and hosts by allozyme electrophoresis showed that while the nematodes within each provenance were genetically uniform, there was some genetic variation between provenances. Morphological and

cytological evidence indicated that they are likely to be the same species but different from described species of *Anguina*. This was corroborated by sequencing the internal transcribed spacer regions of the nematodes from both provenances.

Monitoring of nematode populations from SA over two seasons revealed that the nematode can initiate galls in the vegetative shoot apical meristem in addition to ovary initials. Shoot galls can be initiated at any time of the year, which may play an important role in nematode survival during flooding. Although the nematodes are unable to invade host plants in flooded conditions, experiments also indicated that nematodes can survive for at least three months in flooded soil and are able to induce galls in both hosts once conditions become favourable. The nematodes only complete one generation within a gall but can have two or more generations per year by repeated gall formation.

Agronomic methods that have been effective in controlling other *Anguina* species, particularly *Anguina funesta*, were examined in the field. While significant reductions in seed galls were achieved by heavily grazing pastures in spring, it appears that shoot galls are not consumed and thus there will be a limited effect on nematode populations. Excellent control of the host grasses was achieved with herbicides. However, where pastures remained inundated for long periods of time, no pasture regenerated on the treated areas, reducing the productivity and increasing the risk of erosion and pasture degradation.

The plant pathogenic fungus *Dilophospora alopecuri* was found to be associated with *Anguina* sp. populations in *P. monspeliensis* and was assessed as a potential biocontrol agent, as it is already providing useful control of *Anguina funesta* in Western Australia. A strain was tested against other available Australian strains of the fungus for its

effectiveness in reducing nematode populations. The strains examined were neither host nor vector specific. The strain isolated from *P. monspeliensis* was the most aggressive strain tested and reduced nematode numbers significantly. An investigation of strains by allozyme electrophoresis showed that *D. alopecuri* is either highly variable or consists of several species.

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**Terry Bertozzi**

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# Chapter 1

## Introduction

Poisonous plants grow in most plant communities imposing some degree of risk for grazing livestock. In general, common forage grasses are relatively free from intrinsic toxins, especially in comparison to herbaceous plants, which are often rich in alkaloids, glycosides, toxic amino acids and phenolic compounds. However, the colonisation of important pasture grasses by fungi and bacteria and the subsequent production of toxic metabolites by these micro-organisms, results in direct and indirect economic losses similar to those caused by plants that are inherently toxic.

The toxic metabolites produced by the bacterium *Rathayibacter toxicus* (Riley and Ophel 1992) Sasaki, Chijimatsu & Suzuki 1998 are known as corynetoxins (Vogel *et al.*, 1981) and are closely related in molecular structure to the tunicamycin antibiotics (Edgar *et al.*, 1982). Ingestion of these toxins by livestock results in a neurological disorder characterised by convulsions and often death. Although *R. toxicus* is a plant pathogen, studies have shown that successful colonisation of host grasses by the bacterium appears to be dependent on nematode vectors of the genus *Anguina* (Vidaver, 1982; Riley and McKay, 1991). Other *Rathayibacter*/*Anguina* associations have been reported (Vidaver, 1982; Riley, 1992; Riley *et al.*, 2001) but only those involving *R. toxicus* are known to be toxigenic.

Annual ryegrass toxicity (ARGT) was the first toxicosis attributed to *R. toxicus*. The bacterium is carried into annual ryegrass, *Lolium rigidum* Gaudin, by the nematode *Anguina funesta* Price, Fisher & Kerr 1979 (Price *et al.*, 1979). First recorded near Black Springs in the mid-north of South Australia (SA) in 1956 (Fisher, 1977), ARGT has

become well established in other cropping regions of SA (McKay *et al.*, 1985) and Western Australia (Stynes and Wise, 1980; Pink, 1989) and has also been recorded in South Africa (Schneider, 1981).

During 1990/1991, livestock deaths occurred along the flood plains of a number of rivers in the Bourke and Moree districts in northern New South Wales (NSW). The clinical signs and pathology shown by affected livestock were identical to those reported for ARGT (Bryden *et al.*, 1991) and feeding trials confirmed that blown grass, *Agrostis avenacea* C. Gemelin, was the source of the toxin (Bryden *et al.*, 1991). Distorted inflorescences of *A. avenacea* encrusted with an "orange material", subsequently identified as *R. toxicus* (McKay *et al.*, 1993), were reported from toxic areas (Davis *et al.*, 1995) and examination of the inflorescences used in the feeding trials revealed the presence of nematode seed galls (McKay *et al.*, 1993). This disorder became known as Flood Plain Staggers (FPS).

This new *Rathayibacter/Anguina* association renewed interest in a neurological condition which had been recognised for nearly 20 years in southeastern SA between Naracoorte and Kingston (Finnie, 1991). Locally known as Stewart's Range Syndrome, subsequent investigation of toxic pastures revealed the presence of *R. toxicus* and a seed-gall forming nematode, thought to be the same species as the one involved in flood plain staggers but different from *A. funesta* (McKay *et al.*, 1993), in the inflorescences of annual beardgrass, *Polypogon monspeliensis* (L.) Desf. Furthermore, chemical, biochemical and toxicological evidence indicated that the corynetoxins involved were identical to those produced by *R. toxicus* in FPS and ARGT (Edgar *et al.*, 1994). For these reasons McKay *et al.* (1993) proposed that the term "Flood Plain Staggers" be used to describe livestock poisonings associated with both *P. monspeliensis* and *A. avenacea*. That convention will be followed here.

Ecological studies by Price (1973) and McKay *et al.* (1981) indicated that the nematode is the weakest link in the disease cycle. Thus, agronomic methods to control ARGT have focused primarily on disrupting the nematode life cycle (McKay *et al.*, 1982). There is also some evidence that the plant pathogenic fungus *Dilophospora alopecuri* (Fr.) Fr. has been responsible for the decline of ARGT in some areas (Riley, 1994).

While it seems likely that the methods used to manage ARGT may prove effective against FPS, pastures where the organisms associated with FPS are known to occur are prone to flooding during winter and spring. In southeastern SA, flooding is an annual event and pastures can remain inundated for up to five months. In contrast, flooding in northern NSW flooding is sporadic. Considering that most members of the genus *Anguina* are highly specialised parasites, it is likely that the nematode vector will be highly adapted to these environments, even though *R. toxicus* appears to be able to survive in a wide range of environments.

This thesis reports on investigations into flood plain staggers. The objectives of this study were to define the distribution of the organisms associated with FPS, to examine the biology and ecology of the nematode involved and to indicate potential pasture and livestock management practices that could be employed to reduce the impact of the disease. Since the initial outbreak of FPS in northern NSW, rainfall in the infested areas has been well below average and so much of the study has concentrated on southeastern SA.

# Chapter 2

# Review of Literature

## 2.1 Introduction

This review focuses on the bacterium *R. toxicus* and nematodes involved in annual ryegrass toxicity (ARGT) and flood plain staggers (FPS). After reviewing the literature concerning *R. toxicus*, the corynetoxins it produces and their effect on livestock, the systematics and biology of the nematode vectors will be discussed and management strategies currently in use and under investigation will be examined. Toxigenic *Rathayibacter/Anguina* associations have been reviewed by McKay and Ophel (1993), however, new information published since then necessitates a re-examination of some of the information presented in that review.

## 2.2 The Bacterium

### 2.2.1 Taxonomic status

Originally described as a species of *Corynebacterium* Lehmann and Neuman 1896 (Price *et al.*, 1979), the bacterium associated with ARGT was reclassified into a new genus, *Clavibacter* Davis, Gillaspie, Vidaver & Harris 1984, based on the presence of 2,4-diaminobutyric acid (DAB) in its cell walls (Riley, 1987). Further studies involving allozyme electrophoresis (Riley *et al.*, 1988a) and bacteriophage specificity (Riley and Gooden, 1991) confirmed the uniqueness of the bacterium which was subsequently described as *Clavibacter toxicus* (Riley and Ophel, 1992). McKay *et al.* (1993) found that the bacterium associated with FPS was indistinguishable from *C. toxicus,* based on colony morphology, serology and bacteriophage specificity. More recently, an examination of 52 isolates of *C. toxicus* from around Australia confirmed that they are closely related and

genetically distinct from other plant pathogenic species of *Clavibacter* (Johnston *et al.*, 1996).

Zgurskaya *et al.* (1993) proposed a further division of *Clavibacter*, with the formation of a new genus *Rathayibacter*. They suggested inclusion of *Clavibacter rathayi* (Smith 1913) Zgurskaya *et al.* 1993, *Clavibacter tritici* (Carlson and Vidaver 1982) Zgurskaya *et al.* 1993 and *Clavibacter iranicus* (Carlson and Vidaver 1982) Zgurskaya *et al.* 1993 within *Rathayibacter* based on the number of isoprene units of the major menaquinones, whole-cell sugar compositions, ability to utilise different carbon sources, cell sensitivity to lysing agents and DNA-DNA homology (Zgurskaya *et al.*, 1993). Although they studied one of the strains, *C. toxicus* was not included in the reclassification because the type strain was not examined. In contrast to this reclassification, Lee *et al.* (1997) suggested that *R. rathayi* and *R. tritici*/*R. iranicus* be subdivided into two separate genera distinct from *Clavibacter* on the basis of 16S ribosomal RNA analyses. Unfortunately, they also did not include *C. toxicus* in their analyses and so its status among the other species was not determined.

Altenburger *et al.* (1997) studied polyamine patterns in a number of actinomycetes containing group B peptidoglycans and found that while *Clavibacter* and *Rathayibacter* could be separated from the other bacteria studied on the basis of high polyamine content (predominantly spermidine and spermine), not all strains of *Rathayibacter* conformed to the characteristic features of the genus. There was also no suggestion that *C. toxicus* should be included into the genus *Rathayibacter*. However, further examination of group B peptidoglycan by Sasaki *et al.* (1998) suggested that *Clavibacter* and *Rathayibacter* can be differentiated, based on the proportion of D- and L- stereoisomers of DAB. They reported that *Rathayibacter* possessed L-DAB type peptidoglycan, while the peptidoglycan of

*Clavibacter* spp. had almost equal proportions of both stereoisomers. The type strain of *C. toxicus* contained L-DAB almost exclusively and in combination with menaquinone composition data, Sasaki *et al.* (1998) proposed the new combination *Rathayibacter toxicus*. As *R. toxicus* appears to have been accepted in the literature, I will follow this proposal in the remainder of this thesis.

### 2.2.2 The toxins and toxin production

*Rathayibacter toxicus* produces a unique mixture of individual toxins referred to as corynetoxins (Vogel *et al.*, 1981).These belong to a subclass of nucleoside antibiotics collectively known as tunicaminyuracils (TMUs; Bourke *et al.*, 1992). The other members of this subclass are produced by species of *Streptomyces* and include tunicamycin, streptovirudin and antibiotic MM19290 (Eckardt, 1983). While no poisonings attributable to *Streptomyces* spp. have been confirmed in Australia, a number of these bacteria were isolated from water damaged grain responsible for the deaths of 48 pigs in NSW (Cockrum *et al.*, 1987).

Structurally, the TMUs are composed of two main parts, a common uracil-tunicamine-*N*-acetylglucosamine moiety and a fatty acid linked via an amide bond to the tunicamine amino group (Edgar *et al.*, 1982). Each bacterial species produces a mixture of TMUs which can be characterised by the structure of the fatty acid component (Cockrum and Edgar, 1983). The corynetoxins differ from the other members of the group by having longer fatty acid chains, which can be hydroxylated in the β-position and either saturated or αβ-unsaturated, and antesio terminal branching in addition to the iso and normal branching forms (Edgar *et al.*, 1982). High-performance liquid chromatography (HPLC) was used to identify the seven individual corynetoxins associated with ARGT (Edgar *et al.*, 1982) based on their fatty acid components. More recently, Edgar *et al.* (1994) established

that flood plain staggers was caused by the same toxins responsible for ARGT by means of HPLC, thin layer chromatography (TLC) and toxicological testing.

Corynetoxin production by *R. toxicus* appears to be positively correlated with the presence of bacteriophage particles in the capsule of the bacterium (Ophel *et al.*, 1993). These particles were observed by Bird *et al.* (1980) from bacterially colonised ryegrass galls and subsequently isolated by Riley and Gooden (1991), who found that the bacteriophage was specific to the bacterial strains associated with ARGT. The same bacteriophage was isolated from bacterial galls on *P. monspeliensis* and *A. avenacea* (McKay *et al.*, 1993). The mechanism by which the bacteriophage affects toxin production is unknown, however, the bacteriophage DNA does not appear to be incorporated into the bacterial DNA and toxin producing cells are in a pseudolysogenic state (Ophel *et al.*, 1993). Ophel *et al.* (1993) postulated that the bacteriophage genome may encode a regulatory gene that "switches on" toxin production, encodes a final step in the corynetoxin biosynthetic pathway or possibly makes the cell more permeable to the export of toxin.

### 2.2.3 Symptoms of poisoning

Toxicity caused by the corynetoxins, and the tunicaminyuracils in general, stems from their inhibition of protein glycosylation (Jago *et al.*, 1983) and more specifically the depletion or impared synthesis of *N*-glycosylated glycoproteins (Culvenor and Jago, 1985). The toxins are cumulative and a lethal dose may be ingested in one dose or in smaller doses over a nine week period (Jago and Culvenor, 1987). The minimum oral lethal dose for sheep was determined to be 3-5mg $kg^{-1}$ (Jago and Culvenor, 1987) and there appears to be a latent period of approximately 60 hours between the ingestion of a lethal dose of toxin and the first development of clinical signs (Bourke, 1994).

The sublethal effects of corynetoxins have not been adequately investigated. Davies *et al.* (1996) found that wool growth and fibre diameter were significantly reduced by 10% and 7% respectively, in sheep exposed to extremely low levels of corynetoxins. Moreover, they recorded an increase in ovulation and, coupled with no embryonic or fetal losses, found a resultant 30-35% increase in the number of lambs weaned at the levels of toxin investigated. While this contradicts reports of abortions in ewes (Berry *et al.*, 1980; Schneider, 1981) and the interference with embryo development in mice (Atienza-Samols *et al.*, 1980) and sea urchins (Schneider *et al.*, 1978) caused by tunicamycin, it is possible that a threshold level of toxin accumulation exists for the manifestation of these symptoms.

Although the clinical expression of poisoning emanates from the central nervous system, it appears that this is due to vascular dysfunction in the brain (Bourke *et al.*, 1992). Affected animals can develop impairment to vascular integrity without nervous signs being apparent but additional stress factors such as forced exercise or high ambient temperatures can precipitate severe nervous signs (Bourke, 1994).

Davis *et al.* (1995) gave a detailed description of the clinical manifestation of flood plain staggers which, not suprisingly, mirrored those of ARGT (Bryden *et al.*, 1991). They found that sheep displayed similar signs to cattle but that stress factors had less impact on the course of the symptoms. Briefly, affected animals show disturbed equilibrium, often typified by a wide-based stance and hypermetria of the forelimbs, ataxia and muscle tremors which progressively increase before the onset of convulsions. Convulsive episodes can last for minutes or can be continuous. These usually commence with opisthonotous, extension of thoracic limbs and flexion of the shoulder joints (Davis *et al.*, 1995) and eventually end in sternal or lateral recumbency. While recumbent, tetanic spasms, paddling of the forelimbs, head weaving and faciculation of skeletal muscle are exhibited. Death is

usually preceded by severe or prolonged convulsive activity (Bryden *et al.*, 1994). However, between convulsive episodes, animals often exhibit normal behaviour, despite some degree of cerebellar dysfunction (Davis *et al.*, 1995) and if quietly removed to safe pasture in the early stages of poisoning, provided with shade, good quality feed and water, often some will recover.

Despite the prominent clinical symptoms, the macroscopic and microscopic pathology of corynetoxin poisoning has been described by several authors as neither specific nor consistent (Schneider, 1981; Bourke *et al.*, 1992; Davis *et al.*, 1995). Necropsy findings include pale swollen friable livers (McIntosh *et al.*, 1967; Davis *et al.*, 1995), haemorrhage, oedema and congestion in the brain (Berry *et al.*, 1980; Schneider, 1981; Bourke *et al.*, 1992; Davis *et al.*, 1995) and congestion in the lungs, kidneys and various mucosa (McIntosh *et al.*, 1967; Davis *et al.*, 1995). According to Bourke (1994), successful diagnosis of corynetoxin poisoning can be made on the basis of the nervous signs displayed and the neuropathology present, eliminating other neurotoxicities such as phalaris staggers, perennial ryegrass staggers, enterotoxaemia, polioencephalomalacia, tremorgenic mycotoxicoses and ergotism. Nevertheless, FPS remained undiagnosed in southeastern of SA for longer than 20 years and was not confirmed until the responsible organisms were identified.

### 2.2.4 Association with nematodes

*Rathayibacter rathayi*, *R. tritici*, *R. iranicus* and *R. toxicus* can be considered as unusual phytopathogenic bacteria in that nematode transmission is required for characteristic disease development (Price, 1973; Vidaver, 1982). The bacteria adhere to the cuticle of juvenile nematodes (Bird, 1985) and are passively carried into their hosts by the nematodes. *Anguina tritici* (Steinbuch 1799) Chitwood 1935 is known to carry *R. tritici*

and *R. iranicus* into *Triticum* spp. (Sabet, 1954; Bradbury,1973; Riley and Reardon, 1995) and has been shown to be a potential vector of *R. toxicus* (Riley, 1992). An unknown species of *Anguina* carries *R. rathayi* into *Dactylis glomerata* L. (Bradbury, 1973a), *A. funesta* can carry *R. toxicus* into a number of species of *Lolium* (Bird and Stynes, 1977; Price *et al.*, 1979; McKay *et al.*, 1982) and *Vulpia myuros* (L.) C.C. Gemlin (Riley and McKay, 1991) and *Anguina australis* Steiner1940 can carry *R. toxicus* into *Ehrharta longiflora* Smith (Riley *et al.*, 2001). Examination of distorted inflorescences of *P. monspeliensis* and *A. avenacea* in FPS prone areas also revealed the presence of seed gall producing *Anguina* associated with the *R. toxicus* infection (McKay *et al.*, 1993).

## 2.3 Nematodes of the genus *Anguina*

### 2.3.1 Taxonomy and systematics

Members of the genus *Anguina* Scopoli, 1777 were the first plant parasitic nematodes discovered (Needham,1743). Unfortunately most of the original descriptions are inadequate and, for some, type specimens are non existent (Southey *et al.*, 1990). Riley *et al.* (1988) summed up the status of the genus *Anguina* as being composed of species that are taxonomically well recognised, populations whose taxonomy is subject to debate and populations that have not been identified or described. *Anguina tritici*, *Anguina graminis* (Hardy 1850) Filipjev 1936, *Anguina agropyri* Kirjanova 1955, *Anguina agrostis* (Steinbuch 1979) Filipjev 1936, *A. australis* and *Anguina microlaenae* (Fawcett 1938) Steiner 1940 have all been recognised as taxonomically distinct in a number of reviews (Brzeski, 1981; Fortuner and Maggenti, 1987; Chizov and Subbotin, 1990; Ebsary, 1991). Other species such as *Anguina agropyronifloris* Norton 1965, *Anguina amsinckiae* (Steiner and Scott 1935) Thorne 1961, *Anguina balsamophila* (Thorne 1926) Filipjev 1936, *Anguina caricis* Solovyeva and Krall 1982, *Anguina cecidoplastes* (Goody 1934) Filipjev

1936, *A. funesta*, *Anguina danthoniae* Maggenti, Hart & Paxman 1974, *Anguina hyparrheniae* Corbett 1966, *Anguina tumefasciens* (Cobb 1932) Filipjev and Schuurmans Stekhoven 1941, *Anguina pustulicola* (Thorne 1934) Goodey 1951 and *Anguina spermophaga* Steiner 1937 have been in a state of taxonomic flux. These species are part of the genus *Anguina*, synonymised with other genera or reclassified into new genera depending on the revision.

The separation of taxa within the genus *Anguina* has been based predominantly on morphological differences. However, Brzeski (1981) stated that "Adults of different species of *Anguina* are morphologically indistinguishable, although they develop on different hosts in widely separated geographical areas". This similarity is likely to be the predominant reason for the taxonomic confusion that surrounds *Anguina*. In an effort to overcome this problem, Chizhov and Subbotin (1985) proposed a system for the classification of the entire family based on non-morphological criteria that they perceived to be of important adaptive significance. These criteria included ontogenetic peculiarities and trophic specialisation (Chizhov and Subbotin, 1985) and were later amended to include host specialisation (Chizhov and Subbotin, 1990).While Fortuner and Maggenti (1987) agreed that biological considerations could be useful, they were critical of the revision proposed by Chizov and Subbotin (1985) on the basis that "biology alone does not differentiate a genus, when there are no morphological differences". Ebsary (1991) followed Fortuner and Maggenti's (1987) conservative classification while Krall (1991) favoured the proposal of Chizhov and Subbotin (1985) because he felt that it reflected some evolutionary trends within the group.

Cytogenetic features have been of some use in delineation of the *Anguina*. *Anguina graminis* and *A. agropyri* have a haploid chromosome number of $n = 9$ as does

*Heteroanguina graminophila* (T. Goodey 1933) Chizhov 1980 which apparently represents the basic chromosome number of these genera (Krall and Aomets, 1973; Solov'eva and Gruzdeva, 1977). *Anguina tritici* has $n = 19$ (Triantaphyllou and Hirschmann, 1966), *A. funesta* has $n = 22$ (Stynes and Bird, 1980) and *A. agrostis* has been reported to have haploid complements of $n = 6$ (Krall and Aomets, 1973) and $n = 18$ (Solov'eva and Gruzdeva, 1977). Triantaphyllou and Hirschmann (1980) suggest that species of *Anguina* may be polypoloid or aneuploid, derived from forms with $n = 9$.

The advent of molecular genetic techniques has provided alternate methods of resolving species boundaries (Baverstock, 1988), particularly when more classical approaches have proved less than satisfactory. The value of biochemical approaches to nematode identification and phylogenetic studies have been discussed at length (Fox and Atkinson, 1986; Curran, 1991; Williamson, 1991; Caswell-Chen *et al.*, 1993; Hyman, 1996; Powers *et al.*, 1997). Riley *et al.* (1988) used allozyme electrophoresis to resolve species boundaries in *Anguina* from Australia and New Zealand. Their study clearly showed that *A. funesta* was different from *A. agrostis,* with which it had been synonymised (Stynes and Bird, 1980), and that undescribed species were present infesting *Holcus lanatus* L. and *Stipa scabra* ssp. *falcata* Vickery, Jacobs & Everett. More recently, McKay *et al.* (1993) used the same technique to differentiate two undescribed *Anguina* nematodes from *P. monspeliensis* and *A. avenacea* from other Australian and New Zealand *Anguina* species. McKay *et al.* (1993) also suggested that the nematodes from *P. monspeliensis* and *A. avenacea* were likely to be the same species. However, pooling the two populations collected from each host did not enable them to assess the amount of variation within nematode populations from the same hosts.

For the purposes of focusing this review and stimulating further taxonomic discussion, this thesis will follow the classification proposed by Chizhov and Subbotin (1985) because of the holistic approach they took in their delineation of taxa. However, their classification will be modified to include information presented by Riley *et al.* (1988) and McKay *et al.* (1993). Thus, *A. tritici*, *A. graminis*, *A. agropyri*, *A. australis*, *A. microlaenae*, *A. funesta*, *A. spermophaga*, *A. hyparrheniae*, *A. tumefaciens*, *A. pustulicola* and *A. agrostis* will be regarded as distinct species in this thesis. Undescribed species of *Anguina* will be cited as "*Anguina* sp. ex "Genus/Species", where Genus/Species refers to the host plant in which it is found.

### 2.3.2 Life cycle

Species of *Anguina* are highly evolved and specialised plant parasites (Singh and Sitaramaiah, 1994). They parasitise monocotyledonous plants of the family Poaceae, including commercially important species, and show marked host specificity, generally at the host-genus level or between botanically related genera (Southey *et al.*, 1990). All are gall forming, although there are differences in the structure, size and shape of the galls and in the plant organs in which they are produced. Galls are formed in either reproductive tissue (seed galls) or in vegetative tissue (leaf and stem galls). Southey (1969) reported that an unidentified species of *Anguina* ex *Dactylis glomerata* was able to form galls in both vegetative and reproductive tissue. However, leaf galls were only observed in pot tests where very high inoculum levels had been applied and not at field sites. Thus, it is unclear whether the observations are an experimental artefact. McKay *et al.* (1993) found stem galls on field collected *P. mospeliensis*, which appeared to have originated in branch primordia on the rachilla, in addition to seed galls. However, only seed galls were observed

in *A. avenacea* samples even though the nematodes infesting both hosts are thought to be the same species (McKay *et al.*, 1993).

Gall formation is essential for the survival of the nematodes. Adult *Anguina* are only found inside galls. The number of adult nematodes per gall varies from one to seven for *A. funesta* (Price, 1973), one to 12 for *A. microlaenae* (Fawcett, 1938) and up to 40 for *A. tritici* (Krall, 1991). Parthenogenesis has not been recorded in the *Anguina* and so both male and female nematodes must be present for successful reproduction. After copulation the female lays a large number of eggs. Fawcett (1938) counted as many as 2,000 eggs from a single *A. microlaenae* leaf gall, although when only one adult of each sex was present there were between 150 and 400 eggs laid. Price (1973) recorded between five and 2, 110 eggs per seed gall for *A. funesta* but further studies recorded up to 3,560 (Riley and McKay, 1991). McKay *et al.* (1993) found that seed galls on *P. monspeliensis* contained up to 2,871 second stage juveniles (J2s) and those on *A. avenacea* 2,427 J2s.

Embryogenic and life cycle biology has been well studied in few species of *Anguina*, probably due to the fact that most are not commercially important pathogens. The following discussion is based primarily on the life cycle of *A. funesta* which, because of its association with *R. toxicus*, has been well documented. The life cycle of *A. funesta* is generally similar to that of *A. agrostis* in *Agrostis capillaris* L. (Courtney and Howell, 1952; Jensen *et al.*, 1958) and *A. tritici* in *Triticum aestivum* L. (Southey, 1973). However, unlike *A. tritici*, the first stage juveniles moult in the eggs and hatch as J2s. After hatching, the J2s feed on the gall and develop into infective second stage larvae (Bird and Stynes, 1981). This change is accompanied by the accumulation of lipid storage granules and a thickening of the cuticle (Bird and Stynes, 1981) and marks the transition from feeding to survival stage. As the host senesces and the galls dry, the J2s enter an anhydrobiotic state

(Bird and Buttrose, 1974) and oversummer inside the dry gall, which acts as a further barrier against water loss and serves to protect the J2s. All other nematode stages in the gall except the J2s die (Price, 1973). According to Price *et al.* (1979), the J2s rehydrate and emerge from the gall after 2 to 6 weeks of moist conditions in the following autumn, following partial decomposition of the gall. They then invade developing host plants by climbing up the outside of the plant when moisture conditions are suitable and move between the leaf sheaths towards the apical meristem. The J2s congregate around the apical meristem until the initiation of the floral primoidia. During this period the J2s do not feed. However, ectoparasitism between leaf sheaths has been reported for *A. tritici* (Marcinowski, 1909; Byars, 1920; Leukel, 1957) and *A. agrostis* (Goodey, 1959; Norton and Sass, 1966). The J2s attack the developing ovary after the differentiation of the palea and lemma and stimulate the production of a gall (Price *et al.*, 1979). Galls can also be formed in stamens, glumes and the rachis (Stynes and Bird, 1982). The nematodes feed inside the gall and quickly pass through three moults to become adults. *Anguina funesta* has one generation per year. Price et al. (1979) reported that J2s could survive the summer outside the galls but were less efficient at producing galls than those oversummering within galls. In contrast, Courtney and Howell (1952) found that *A. agrostis* could not survive for twelve months without a host.

## 2.4 Management

Mortality rates of livestock affected by corynetoxin poisoning can exceed 90% (McKay and Riley, 1993). However, losses can usually be minimised if livestock are inspected daily and are immediately moved to an uninfected pasture at the first signs of poisoning (McKay and Ophel, 1993). While this strategy may be effective, it is laborious, costly and does nothing to improve the infected pasture. A number of possible approaches for the

management of R. *toxicus* related syndromes have been considered that minimise the risk of poisoning and seek to improve infected pastures so they can be fully utilised.

### 2.4.1 Identification

Diagnosis of corynetoxin poisoning as the cause of livestock mortality can be difficult (Schneider, 1981; McKay *et al.*, 1993) and often relies on the subsequent identification of *R. toxicus* and *Anguina* sp. from the suspected pasture. Detecting the organisms in pasture can be difficult (Bird and Stynes, 1977; Riley and McKay, 1991) and ideally, detection should occur before livestock ingest a lethal dose of toxins. Two tests have been developed to address this issue (Riley, 1992a; McKay and Riley, 1993). The pre-flowering test, based on an enzyme linked immunosorbent assay, is used to detect *R. toxicus* in emerging inflorescences when toxin concentrations are low. The post-flowering test determines the number of nematode and bacterial galls in the seed. Early detection ensures that there is sufficient time to either utilise the pasture or apply agronomic treatments to minimise toxin production (McKay and Riley, 1993).

### 2.4.2 Agronomic methods

Studies of the life cycle of *A. funesta* and etiology of ARGT have shown that the nematode is the weak link in the disease cycle (Price, 1973). The nematode is vulnerable to desiccation for a short period each season before the J2s enter anhydrobiosis and total gall production is dependent on the density of *L. rigidum* (McKay *et al.*, 1986). Therefore, management has centred on disrupting the nematode's life cycle and manipulation of *L. rigidum*.

McKay *et al.* (1981) showed that large reductions in nematode gall number could be achieved by mowing or grazing the pasture after the ryegrass had become reproductive.

However, the timing is critical and must be after the nematodes complete their second moult but before the first moult in the eggs of the new generation (McKay *et al.*, 1981). As an alternative to mechanical topping, McKay *et al.* (1982) found that desiccating the ryegrass with the herbicide paraquat also effectively controlled the nematode, particularly when followed by grazing to remove any untreated inflorescences. This procedure also has the advantage of stopping toxin production, providing the pasture remains dry (McKay and Ophel, 1993). A similar strategy was used to reduce gall production by *A. agrostis* from 70% to 2% in Colonial and Seaside bentgrass (Apt *et al.*, 1960).

Stubble burning has also been used to control *A. agrostis* in Seaside bentgrass (*Agrostis stolonifera* L.) and Chewing's fescue (*Festuca nigrescens* Lam.) in the United States but its success has been limited where nematode numbers are very high (Jensen, 1961). Although it is now discouraged in Australia, Price (1973) found that burning ryegrass pastures in early summer effectively destroyed nematode galls still retained in the ryegrass inflorescence but did not destroy those at the soil surface. It is not surprising therefore, that Stynes and Wise (1980) achieved little control of nematode numbers by burning in autumn as most galls would have been dislodged from the inflorescence by this time. However, they did find that the number of galls that were colonised by bacteria decreased significantly after burning.

### 2.4.3 Fungal Antagonists

A number of potential fungal antagonists have been investigated as alternative methods of managing ARGT. A nematode parasitic fungus, *Verticillium* sp., was found by McKay *et al.* (1982) in association with *A. funesta* during ecological studies. However, field trials showed that it was ineffective in reducing nematode populations (McKay *et al.*, 1982). A recent novel approach focussed on the metabolism of the corynetoxins by fungal species.

Payne *et al.* (1994) reported that *Alternaria alternata* (Fr.) Keissler was able to hydroxylate, saturate and desaturate the fatty acid tail of the corynetoxin molecule. These changes to molecular structure significantly decreased but did not eliminate toxicity. While this is a possible pathway by which corynetoxins are detoxified in the soil, it seems unlikely that such a strategy would be effective in plant inflorescences as many strains of *A. alternata* are saprophytic or weak opportunistic pathogens.

The plant parasitic fungus *D. alopecuri* is the most promising of the fungal antagonists that have been investigated and has been implicated in the decline of ARGT from some areas in Western Australia (Riley, 1994). *Dilophospora alopecuri* is a monotypic species (Walker and Sutton, 1974) causing twist disease in a number of the Poaceae (Atanasoff, 1925; Sprague,1950). Atanasoff (1925) and other researchers (Protsenko, 1957; Riley, 1994) suggested that *Anguina* nematodes appear necessary for successful colonisation by *D. alopecuri* of its hosts. Atanasoff (1925) showed that mechanical inoculation of conidial suspensions onto the apical meristem produced no disease symptoms in the absence of nematodes. Other reports indicate that the fungus can be transmitted by mycelial growth from diseased grain (Schaffnit and Weiben, 1928), air currents and insects (Rainio, 1936) or by splash dispersed air-borne conidia (Gibson and Sutton, 1976) and that nematodes are not required. However, it is worth noting that species of *Anguina* are known to parasitise almost all the host genera of *D. alopecuri* presented by Atanasoff (1925) and Walker and Sutton (1974). In addition, *A. funesta* juveniles have been observed to invade non host plants (Riley and McKay, 1991).

The conidia of *D. alopecuri* have finger-like setulose appendages that attach to the cuticle of J2 nematodes (Bird and McKay, 1987), mediated possibly by lectin-hapten binding (Spiegel and McClure, 1991). McKay *et al.* ( 1981) observed that movement of *A. funesta*

J2s could be restricted by large numbers of adhering conidia, which were carried from the soil to the apical meristem. As the conidia are able to divide and form secondary spores (Atanasoff, 1925), they are able to attach to other J2s which enter the plant free of adhering conidia (McKay *et al.*, 1981). Conidia are carried in to the developing gall where they often form a dense hyphal mat which destroys the gall and prevents the nematode from reproducing (McKay *et al.*, 1981). McKay *et al.* ( 1981) also reported that *D. alopecuri* could spread to the ryegrass spikelet and rachis causing part of the inflorescence to die and even prevent inflorescence emergence. *Dilophospora alopecuri* has also been found in southeastern SA in association with an unknown species of *Anguina* ex *Holcus lanatus* (Riley, 1996) but its involvement in FPS, if any, is unknown.

### 2.4.4 Other strategies

The advent of herbicide resistance in *L. rigidum* populations in Australia (Heap and Knight, 1986) has sparked fears that ARGT will become more common (Gill *et al.*, 1993). This was reflected in a survey of *L. rigidum* from Western Australia where significantly higher numbers of *A. funesta* galls were found in samples with high levels of herbicide resistance (Riley and Gill, 1994). A cultivar of *L. rigidum,* called "Guard", has been developed which is susceptible to selective herbicides and also shows resistance to *A. funesta*. "Guard" averaged 3 galls per 1000 florets compared to an average 620 galls in a susceptible line (McKay and Ophel, 1993). However, the success of this strategy and long term control of ARGT will depend on the stability of the resistance in the field, particularly as *L. rigidum* is outcrossing.

In studies with the non-toxigenic *R. rathayi*, *R. tritici* and *R. iranicus*, the bacteria adhered to some populations of *A. funesta* (Riley and McKay, 1990) and were less fastidious and faster growing in culture than *R. toxicus* (Riley and Ophel, 1992). Although they require

nematode vectors, nematode galls colonised by these bacteria have not been reported. Riley (1994b) suggested that gall initiation was probably inhibited by the fast growth rate of these bacteria and that they may be able to displace *R. toxicus*, providing control of ARGT. However, only *R. tritici* is found in Australia and it is uncertain whether approval will be given to evaluate the exotic species in field experiments. Also, it is unlikely that the non-toxigenic *Rathayibacter* species would be visually indistinct from *R. toxicus* in the field (Riley, 1994) thereby causing confusion and undue concern.

An antidote against ARGT corynetoxins has been developed by the CSIRO Division of Animal Health. It works by binding strongly to the toxins and has been designed to be used when poisoning symptoms first appear in livestock (May *et al.*, 1995). Trials in 1994/1995 showed an average reduction in livestock losses from 34% in untreated animals to 9% if the antidote was applied (May *et al.*, 1995). Whether the antidote neutralises the toxins, stimulates an immune response or aids in excretion of the toxins is unclear. Prophylactic vaccines against corynetoxins have been suggested, however issues regarding immunological clearance from the animal, the accumulation of natural toxicant residues in animal products and manufacturing costs and market size have not been suitably resolved (Edgar, 1994).

## 2.5 Conclusions

As shown in this review, there are a number of strategies available for the management of ARGT. These strategies are based on knowledge of the biology and ecology of *R. toxicus* and its nematode vector, *A. funesta*. Studies have found that aspects of the nematode life cycle can be exploited and have correlated these with development of the host plant, providing a visual cue for the correct timing of treatments.

It is possible that management strategies proven successful in controlling ARGT will also prove effective for FPS. Both toxicoses are caused by *R. toxicus* and involve an *Anguina* nematode vector that produces galls in the host inflorescence. However, little is known about the nematode and how it interacts with its hosts and environment. South-eastern SA and northern NSW are very different environments from the wheat/sheep belts where *A. funesta* occurs and the nematodes are likely to be well adapted to the peculiarities of these habitats. Thus, an understanding of the biology and ecology of the nematode is critical in developing successful management options for FPS.

# Chapter 3

# General Methods

## 3.1 Field sites

Field sites were selected in paddocks where flood plain staggers had been a problem in previous seasons. Although good trial sites were available at the commencement of the project, the unseasonably dry weather during the remainder of the project forced the selection of new trial sites. A total of seven trial sites were used (Table 3.1).

**Table 3.1** Site reference numbers, location details (locality, state, grid reference) and soil type of field sites used throughout the study.

| Site No. | Location | Soil Type[a] |
|---|---|---|
| 1 | Lucindale, S.A. (37°00′ S, 140° 21′ E) | Ug5.11 |
| 2 | Lucindale, S.A. (37°01′ S, 140° 20′ E) | Dy5.43 |
| 3 | Stewart's Range, S.A. (37°00′ S, 140° 39′ E) | Dy5.43 |
| 4 | Biscuit Flat, S.A. (37° 05′ S, 140° 02′ E) | Dy5.43 |
| 5 | Biscuit Flat, S.A. (37° 00′ S, 139° 59′ E) | Dy5.43 |
| 6 | Cape Jaffa, S.A. (36° 57′ S, 139° 46′ E) | Dy5.43 |
| 7 | Reedy Creek, S.A. (36° 58′ S, 139° 57′ E | Dy5.43 |

[a]Description of soil types are from (Northcote *et al.*, 1975).

Dy5.43 - Sandy pedal mottled-yellow duplex soils. Sandy surface over mottled grey clayey subsoil. Carbonate segregation is a feature of the deeper subsoil. The soil has an alkaline pH and is also referred to as solodized solonetz and solodic soil (Stace *et al.*, 1968).

Ug5.11 - Black self mulching cracking clays. Dark to very dark medium to heavy clays underlain by a highly calcareous layer. Also referred to as Rendzinas (Stace *et al.*, 1968).

## 3.2 Collection and storage of *Polypogon monspeliensis* and *Agrostis avenacea* seed

Inflorescences of *P. monspeliensis* and *A. avenacea* were collected from mature senesced plants in the field. The seed was removed by inserting the inflorescences into a 100 mm section of bicycle inner tube, placing the tube on a flat surface and rolling it with the finger tips while applying pressure. This method also strips the palea and lemma from the seed, which speeds up germination and makes surface sterilisation easier. The seed was separated from other plant material by gently blowing air across the sample. All seed collected was stored in screw topped polycarbonate containers at 5°C until required.

## 3.3 Surface sterilisation and germination of seed

Seeds for pot experiments or *in vitro* studies were surface sterilised in a laminar flow hood by exposure to 70% ethanol for 30 seconds before transferring it to a sodium hypochlorite solution (1% available chlorine) for 7 min, with occasional agitation. The seed was then rinsed three times in sterile distilled water and allowed to dry for easier handling. For pot experiments, seed was germinated on 0.8% distilled water agar (DWA) in 90 mm petri dishes. Sterile filter paper was originally used in place of the agar, however, it was difficult to remove the seedlings without damaging the very fine roots. For *in vitro* studies, seeds were sown directly into 30 ml polycarbonate tubes (one seed per tube), which had been previously autoclaved and contained 10 ml of sterile 0.8% DWA. The petri dishes/tubes were placed in a growth cabinet at 20°C with a 16 hour photoperiod. Light was supplied by three 30W fluorescent cool white tubes. Germination took place in about 36 hours.

## 3.4 Collection and storage of nematode galls

Mature senesced *P. monspeliensis* inflorescences and tillers containing nematode and bacterial galls were collected from field sites (Table 3.1). Nematode seed galls were detached from the rachilla with fine forceps (Dumont Inox No. 3) and the palea and lemma removed if present. Shoot meristem galls (see Chapter 7.3.3) were removed by dissecting them from tillers with fine forceps. Bacterial galls were kept separately from nematode galls. Galls were stored in seed envelopes in a cool, dry location until required.

## 3.5 Extraction of juvenile nematodes from galls

Dry seed or shoot meristem nematode galls were soaked in distilled water overnight and the infective second stage juvenile nematodes (J2s) released by carefully breaking the galls using fine forceps. All gall material was removed and discarded.

## 3.6 Counting nematodes

Nematodes were counted in a modified Doncaster dish (Doncaster, 1962) with four concentric rings using a dissecting microscope at 40x magnification.

## 3.7 Pot experiments

Three days post sowing on agar as described in 3.2, seedlings were transplanted with fine forceps into black, plastic, propagation tubes (50 mm x 50 mm x 120 mm) containing moist soil. Two types of soil were used throughout the study; a sand/peat/loam mix with added nitrogen, phosphorous and potassium and with a pH of approximately 6.5, subsequently referred to as "potting compost", and "flood plain soil", collected from

Biscuit Flat (site 4; see section 3.6). Both were steam pasteurised at 100°C for 45 min and allowed to cool before use.

The tubes were placed into mesh crates (Plate 3.1) that were bedded in solarised sand beds so that the base of each tube was in contact with the sand. This method allowed the plant roots to grow into the sand bed and enabled the small tubes to support large plants. The arrangement of tubes within the crates varied for each experiment.

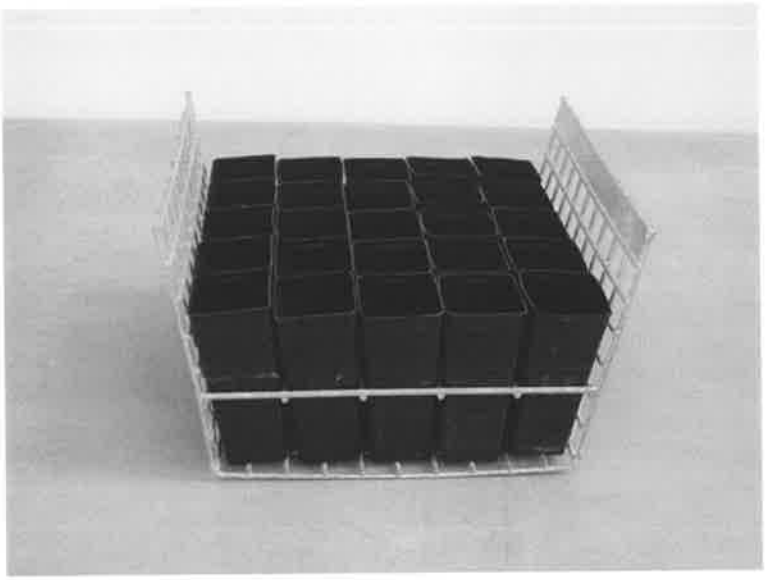


**Plate 3.1** Propagation tubes used for pot experiments arranged in a mesh crate

# Chapter 4

# Distribution of Flood Plain Staggers

## 4.1 Introduction

The distribution of pastures where livestock are at risk from FPS in northern NSW and southeastern SA is currently based solely on reports of livestock deaths. Livestock deaths in northern NSW have been reported from properties along the flood plains of the Bogan, Barwon and Darling Rivers in the Bourke, Brewarrina, Gongolgon, Mungindi and Moree districts (Bryden *et al.,* 1991; Bourke *et al.,* 1992; Davis *et al.,* 1995). Veterinary records from southeastern SA indicate that livestock losses now attributed to FPS, have occurred in a number of areas over 20 years but that these have been localised (C. Trengove pers. comm.).

Experience with annual ryegrass toxicity has shown that distributions based on livestock deaths underestimate the number of pastures where the causal organisms occur (Fisher *et al.*, 1979). Low populations of nematodes and bacteria are often difficult to detect and while they may not pose an immediate threat to grazing livestock, both organisms may proliferate under suitable conditions. Therefore, a knowledge of potentially high risk pastures would enable producers to commence management strategies to minimise the effects of FPS.

This chapter reports the results of surveys to determine the incidence of the nematode *Anguina* sp. and the bacterium *R. toxicus*, associated with FPS, in SA and in northern NSW. Surveys were confined to areas either surrounding pastures with a reported history of FPS or with good populations of the grass hosts. While FPS has not been reported from

Murray River, the Murray (via the Coorong in SA) links the river systems in northern NSW to the flood plains in southeastern SA, warranting investigation of grass populations along its flood plains. The results of the surveys in South Australia have been published (Bertozzi and McKay, 1995).

## 4.2 Materials and Methods

Inflorescences of *A. avenacea* and *P. monspeliensis* were examined in the field for the nematode seed galls and evidence of bacterial colonisation described by McKay *et al.* (1993). The proportion of each grass in the pasture was rated visually on a scale of 0-3 at each site, where 0 = no *P. monspeliensis* or *A. avenacea* and 1, 2, 3 corresponded to 1-33%, 34-66% and greater than 67% of *P. monspeliensis* or *A. avenacea* respectively. The levels of nematode and bacterial colonisation were rated on the same scale, where 0 = no colonisation visible and 1, 2, 3 corresponded to 1-33%, 34-66% and greater than 67% of inflorescences colonised respectively. Nematode seed galls were collected from a number of sites for further analyses. The latitude and longitude of each site were recorded using a Trans*pak II*® (Trimble Navigation CA, USA) global positioning system.

### 4.2.1 Survey of Murray River flood plains

Flood plains along the Murray River (Figure 4.1) were surveyed in October 1991. A preliminary survey indicated that good stands of *A. avenacea* occurred between Lock 3 (Overland Corner) and the SA border and thus the majority of time was spent in this area. Downstream from Lock 3, suitable areas for the host grasses has decreased due to intensive farming along the floodplains. At least 30 min was allocated to thoroughly search each site.

### 4.2.2 Survey of southeastern South Australia

The survey of southeastern SA was conducted between November 1991 and February 1992 and confined to areas prone to winter flooding (Figure 4.1). Sites were chosen as close as possible to a 5 km grid and 15 min were allocated to search each site. Most sites were pasture paddocks but some roadsides and swamp surrounds were also sampled. The northern areas were surveyed first and the survey extended south as grasses matured. Twenty-four producers encountered during the survey were interviewed to ascertain both their knowledge and their experiences with flood plain staggers. Where possible, the history of outbreaks in particular paddocks was recorded.

### 4.2.3 Surveys of northern New South Wales

In September 1993, the flood plains of the numerous rivers in the Darling Basin along the western slopes of the Great Dividing Range were surveyed to determine the incidence of the nematode and bacterium (Figure 4.1). Sites were chosen about 20 km apart so that the greatest possible area could be covered in the time available. At least 15 min were allocated to search each site.

Following heavy winter rains in 1995, pastures in the Brewarrina area were investigated after confirmation of the presence of *A. avenacea* by Dr Greg Curran, Senior Field Veterinarian, Cobar and Mr Danny Norris, District Agronomist, Bourke. At least 30 min were allocated to search each site.

Further rainfall in 1996 promoted the growth of *A. avenacea*, predominantly around the Bogan and Darling Rivers. Samples of *A. avenacea*, collected by Dr Greg Curran, were forwarded to Adelaide where they were examined for the presence of nematode galls and

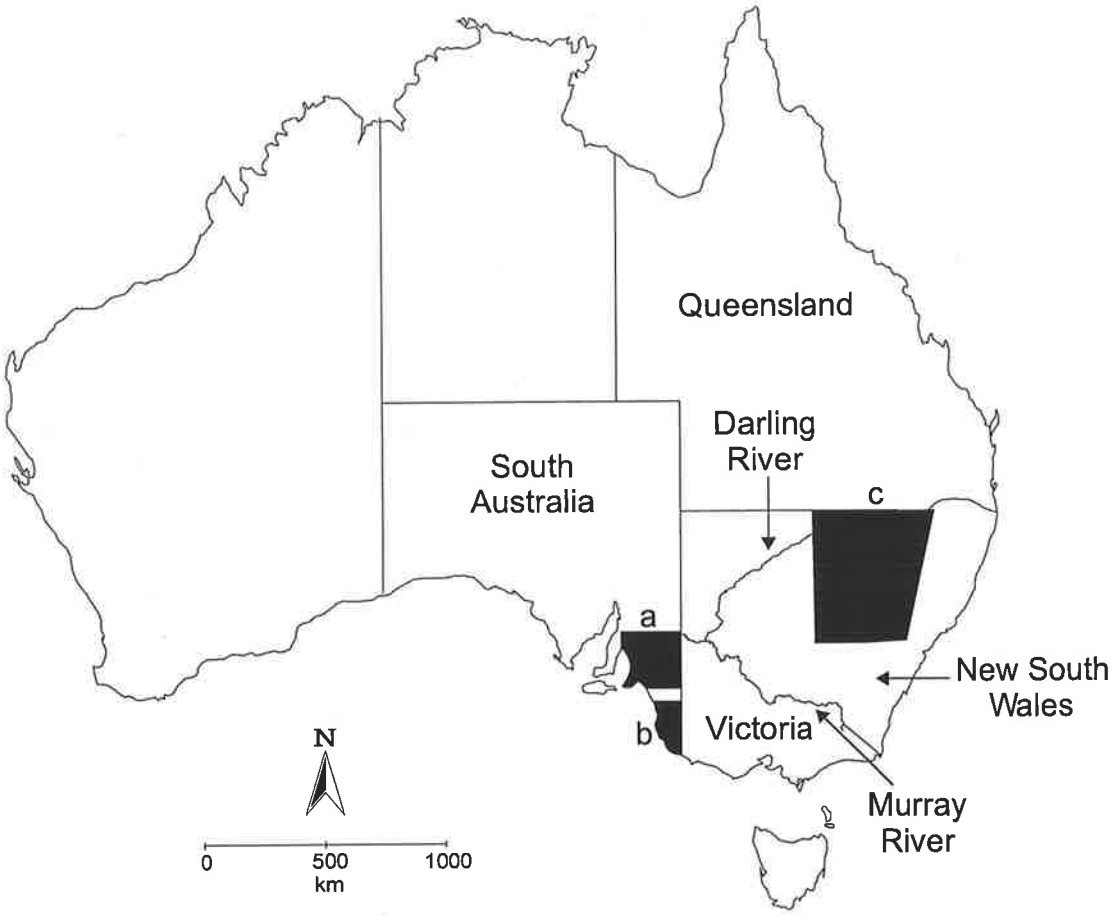



**Figure 4.1** Areas examined during surveys conducted in South Australia and New South Wales. a, b, c refer to surveys of the Murray River flood plains, southeastern South Australia and northern New South Wales respectively.

bacterial colonisation. Where nematode galls were found in the sample, an enzyme-linked immunosorbent assay (ELISA; see Chapter 7.2.3) was performed to determine if *R. toxicus* was present.

## 4.3 Results

### 4.3.1 Survey of Murray River flood plains

*Agrostis avenacea* is a common component of the flood plain vegetation along the upper reaches of the Murray River in SA. It occurs on grey cracking clays in areas flooded during winter. Densities ranged from isolated patches to the dominant grass species. While neither *Anguina* sp. nor *R. toxicus* was detected in *A. avenacea* at any of the 27 sites surveyed, a population of *A. funesta* was found infesting *L. rigidum* near Lake Alexandrina (Figure 4.2). Some of the *L. rigidum* inflorescences were colonised by *R. toxicus*.

### 4.3.2 Survey of southeastern South Australia

*Polypogon monspeliensis* was observed at 265 of the 336 sites examined (Figure 4.3a). Densities ranged from a minor component of the pasture to the dominant pasture species. Its abundance was greatest in areas that are inundated during winter. Within individual pastures, *P. monspeliensis* was more prevalent in low lying areas, such as drainage channels and depressions. Nematode seed galls were observed at 96 (36%) of the sites where *P. monspeliensis* occurred and *R. toxicus* was found at 78 (81%) of these sites (Figure 4.3b). Up to 90% of the inflorescences examined contained nematode seed galls in the most heavily colonised pastures, with the number of seed galls present in each inflorescence ranging between 1 and 58.

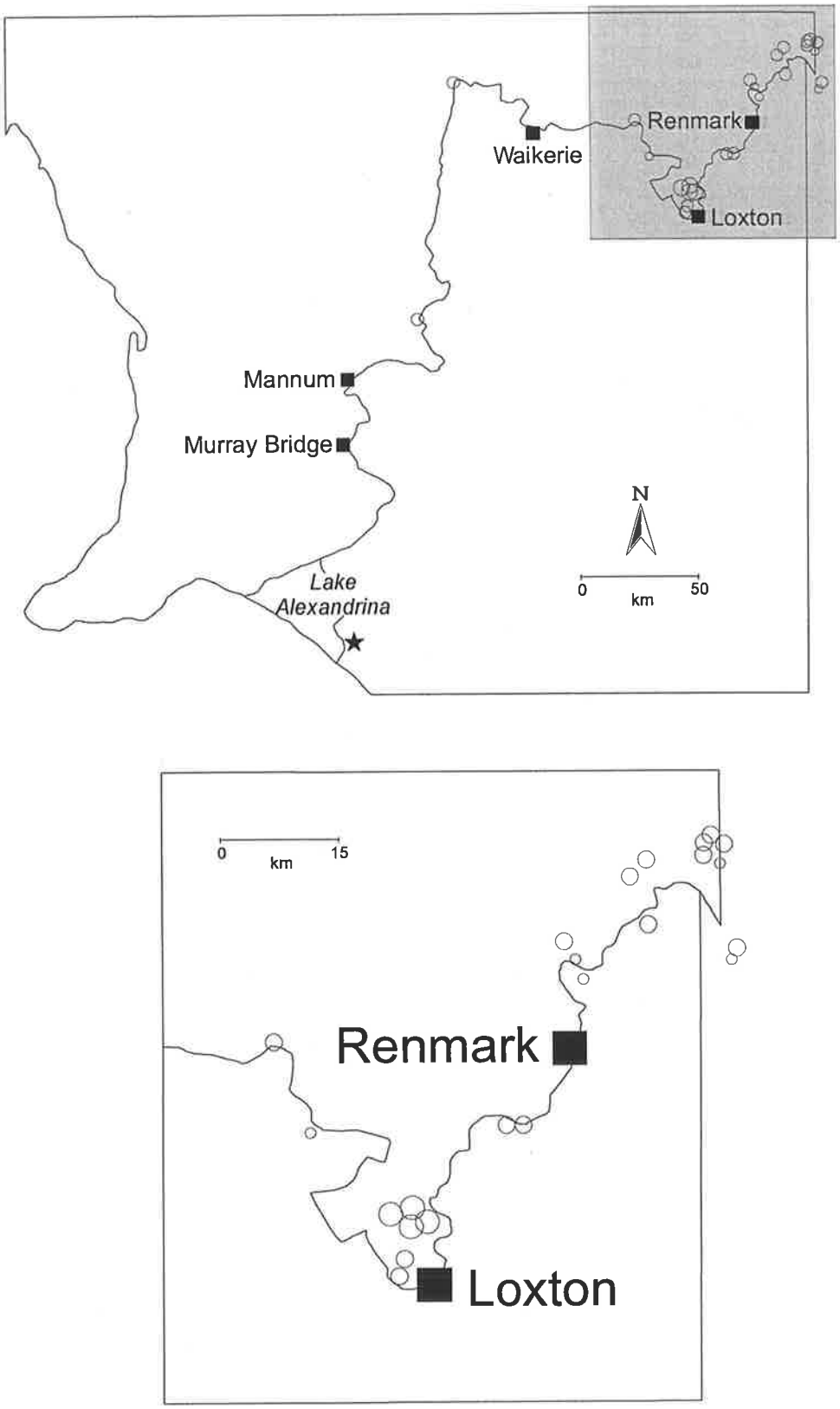



**Figure 4.2** Incidence of *Agrostis avenacea* along the Murray River flood plains. The density of *A. avenacea* at each site is represented by ◦1-33%, ○ 34-66%, ○ 67-100%. The site where *Anguina funesta* and *Rathayibacter toxicus* was found is designated by ★ .

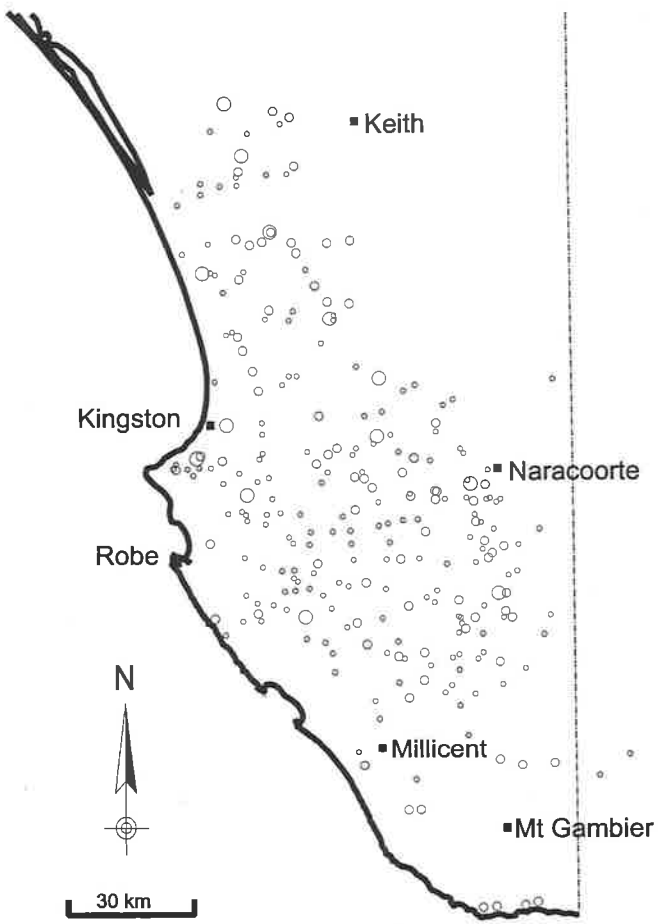



**Figure 4.3a** Incidence of *Polypogon monspeliensis* in southeastern South Australia. The proportion of the pasture occupied by *P. monspeliensis* at each site is represented by ◦1-33%, ○ 34-66%, ○ 67-100%.

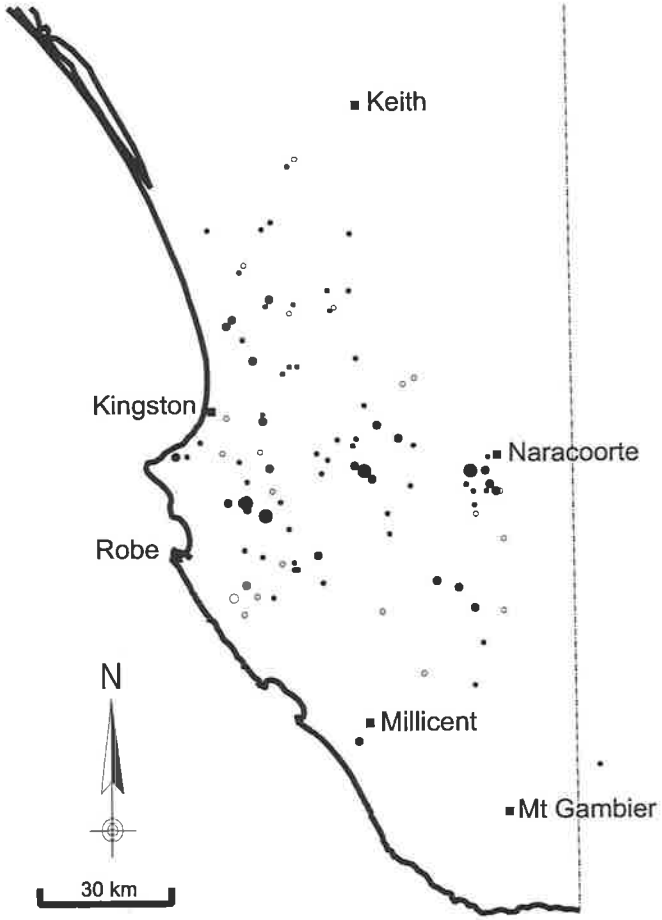



**Figure 4.3b** Incidence of *Anguina* sp. and *Rathayibacter toxicus* in southeastern South Australia. ○ *Anguina* sp. Only; ● *Anguina* sp. and *R. toxicus*. The percentage of *Polpogon monspeliensis* inflorescences colonised by *R. toxicus* at each site is represented by •1-33%, •34-66%, ● 67-100%.

*Agrostis avenacea*, *Agrostis billardieri* var. *filifolia* R. Br. (coast blowngrass) and an unidentified *Agrostis* species were found at 44 sites in the survey of southeastern SA. No nematode or bacterial colonisation of these grasses was observed, even when they occurred amongst infected *P. monspeliensis*.

The 24 producers interviewed had lost a total of 186 cattle and 609 sheep between 1989 and 1992. The worst individual outbreaks involved 200 sheep and 56 cattle. Also, 50% of the producers indicated that lambing and calving percentages were between 10% and 35% lower than was expected in paddocks that had a history of livestock losses.

### 4.3.3 Surveys of northern New South Wales

The density of *A. avenacea* in 1993 ranged from a few plants per square metre to the dominant pasture species and its abundance was greatest in areas that became inundated during the winter. As with *P. monspeliensis* in southeastern SA, *A. avenacea* was more prevalent in depressions several centimetres deep. Nematode seed galls were observed at 11 sites where *A. avenacea* occurred (Figure 4.4), even in areas with less than 10 plants per square metre. Shoot meristem galls (see Chapter 7.3.3) were also found in some plants. *Rathayibacter toxicus* was not found at any of these sites. *Polypogon monspeliensis* was found at two sites in the northern most part of the survey area but was not the dominant species. However, at one of these sites, plants were heavily colonised by *R. toxicus* and contained both seed and shoot meristem nematode galls (Figure 4.4).

In 1995, the greatest densities of *A. avenacea* in the Brewarrina area were found in table drains, gilgai's and cattle hoof prints. No nematode galls or bacterial colonisation were found in any of the pastures examined. *Agrostis avenacea* was also found growing along

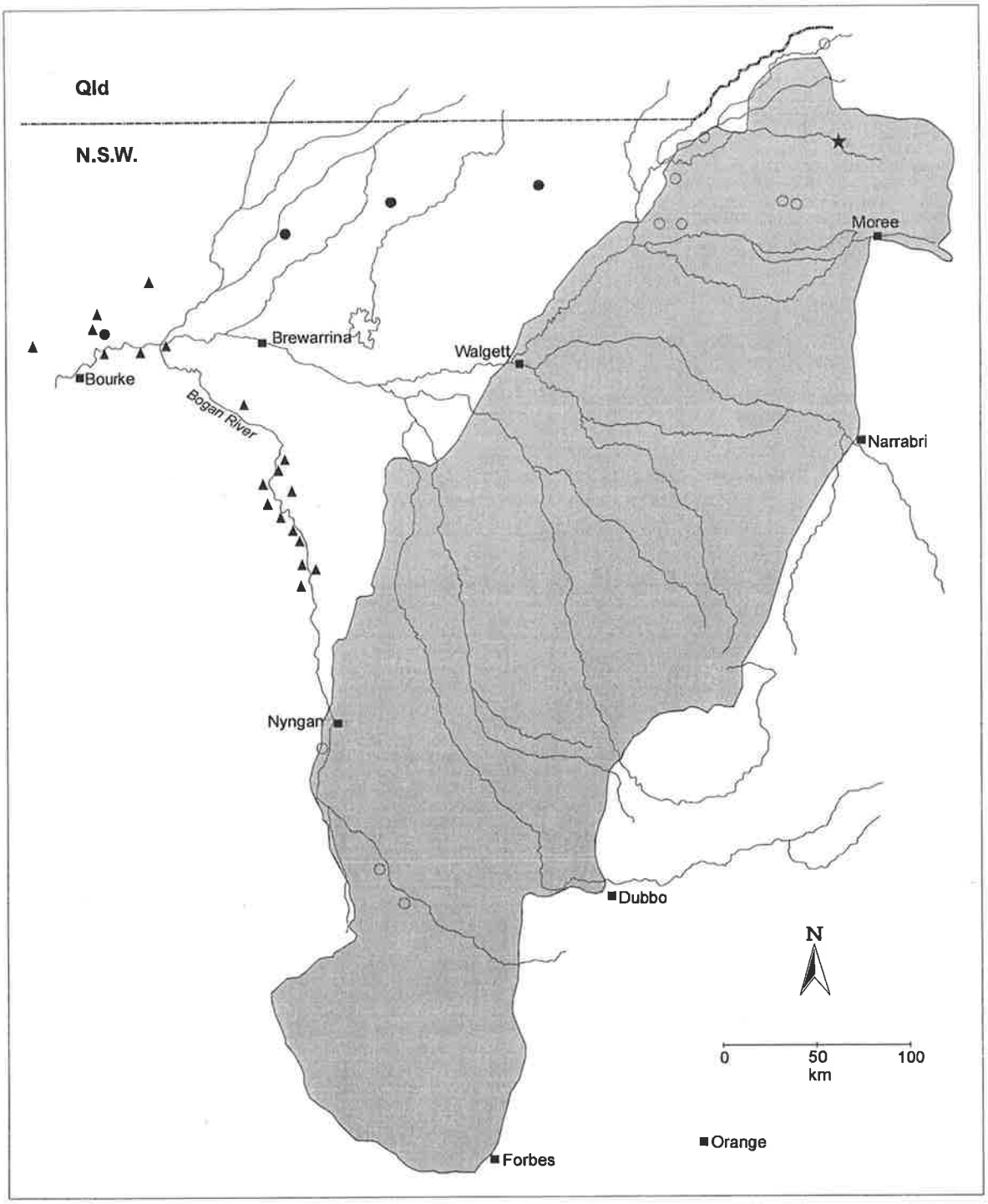



**Figure 4.4** Incidence of *Anguina* sp. and *Rathayibacter toxicus* in northern New South Wales. The shaded region represents the area covered by the survey in 1993. Open circles, *Anguina* sp. only; solid circles *Anguina* sp. and *R. toxicus*. The solid star is where infected *P. monspeliensis* was found. Solid triangles represent recorded outbreaks of flood plain staggers in 1990/91.

the Bogan River in areas that had been inundated for several months. According to Dr Curran, the grass had germinated after the flood waters had receded.

A total of 39 samples representing 12 districts were examined in 1996. Nematode galls were found in samples from three of the districts. While bacterial colonisation was clearly evident in the samples from one district, ELISA confirmed that *R. toxicus* was present in all three. An outbreak of FPS was later reported from one of these districts.

## 4.4 Discussion

*Polypogon monspeliensis* and *A. avenacea* are widespread in southeastern SA and particularly abundant in the area bordered by Kingston and Naracoorte in the north and Millicent in the south. This area is subject to annual winter flooding, which appears to favour the growth of both grasses over other species such as barley grass (*Hordeum leporinum* Link). Further south, increased drainage and alternate land use, such as cropping and forestry, appear to have reduced the area where conditions favour these grasses. In northern NSW, *A. avenacea* was widespread but tended to be more abundant in pastures close to rivers and watercourses. This indicates that the distribution of *A. avenacea* is highly dependent on winter rains, which are somewhat unreliable in this area. In fact, the area surveyed had not received substantial winter rainfall for three years. The distribution of *A. avenacea* appears to be limited to the east by the foothills of the Great Dividing Range. Further east, a change in topography precludes inundation and the rainfall pattern tends to be summer dominant.

The incidence of *Anguina* sp. and *R. toxicus* in both northern NSW and southeastern SA is greater than suggested by reported outbreaks of FPS. Both organisms occur throughout the

flood-prone areas in southeastern SA and extend into western Victoria. This was the first record of *R. toxicus* in Victoria (Bertozzi and McKay, 1995). Populations of *A. funesta* have been recorded in several areas in Victoria, but *R. toxicus* and outbreaks of ARGT have not been reported (Brown, 1987). The most heavily infected pastures correspond to areas where the first outbreaks of FPS were observed more than 20 years ago (P.R. Giesecke pers. comm.) and outbreaks continue to be reported from these pastures. This is in contrast to ARGT where the incidence of outbreaks has declined markedly in the original areas (Roberts and Bucat, 1992). In northern NSW, both the nematode and bacterium were found well removed from known 'toxic' areas even though the distribution of *A. avenacea* has been severely reduced by the low rainfall in this area since the large livestock losses in 1990/91. This withstanding, localised outbreaks of FPS have occurred each year (G. Curran pers. comm.) indicating that the nematode and bacterium can reproduce in areas that receive an average annual rainfall of 300 – 400 mm. The nematode and bacterium may also occur in southern Queensland, as some of the rivers have their origins in that State. In addition, the southwestern-most extent of nematode and bacterial colonisation along the Darling River needs to be determined. Further investigation of these areas is required when conditions are favourable for *A. avenacea*.

The nematode populations in southeastern SA and northern NSW appear to be isolated from each other. The lack of nematode populations along the Murray River is noteworthy given that the river forms a natural link between both populations and is essential for the continued persistence of the host grasses. Thus, it is unclear at present whether populations in southeastern SA and northern NSW are fragments of a larger distribution, the result of separate introductions or indicate a limitation in the survey methodology that could be resolved by more intensive sampling. Interpreting the distribution is difficult given that it is

not known if the nematode is endemic or has been introduced since European settlement. Of its known hosts, *A. avenacea* is native while *P. monspeliensis* is an introduced species(Cunningham, 1992).

The absence of nematode galls and bacteria in *A. avenacea* in southeastern SA is surprising considering that a preliminary study of the nematodes from both NSW and SA indicated that they are probably the same species, even though some genetic differences do exist between them (McKay *et al.*, 1993). The existence of host races of *Anguina*, as present in the genus *Ditylenchus* (Seinhorst, 1959; Sturhan, 1971; Janssen, 1994), a close relative of *Anguina*, could explain this observation. However, host range studies have shown that the nematode from *P. monspeliensis* in southeastern SA will produce galls in *A. avenacea* (see Chapter 5 ). This suggests that the ecology of *A. avenacea* in this area may be unsuitable for nematode invasion. In contrast, conditions in northern NSW appear to be suitable for invasion of both hosts.

Bacterial slime was observed on *P. monspeliensis* inflorescences at all sites where the bacterium was recorded in southeastern SA and has been recorded in northern NSW (McKay *et al.,* 1993), although it was not observed during the surveys. The high levels of bacteria present in southeastern SA create the potential for large livestock losses to occur but this has not been substantiated by the number of livestock deaths reported by producers. While observations have confirmed that livestock consume bacterially colonised *P. monspeliensis*, it is possible that they prefer to graze other pasture species, thereby reducing the amount of infected *P. monspeliensis* ingested.

The effects of consuming sub-lethal levels of corynetoxins, particularly the impact on animal production, have not been adequately investigated, although there are reports of

abortions (McIntosh *et al.*, 1967; Schneider, 1981) and severe organ damage (Berry and Wise, 1975; Schneider, 1981). Half of the producers encountered during the survey of southeastern SA reported that lambing and calving percentages in paddocks with a history of flood plain staggers were about 10-35% below normal. While this is in contrast to the 30-35% increase in the number of weaned lambs reported by Davies *et al.* (1996) after administration of low levels of corynetoxin, paddocks with a history of FPS outbreaks are likely to be heavily contaminated with *R. toxicus,* and hence, grazing livestock will accumulate a higher level of toxin than was administered by Davies *et al.* (1996).

These surveys are likely to underestimate the number of potential risk paddocks because low densities of nematodes are difficult to detect and because the nematode's ability to produce shoot meristem galls in the absence of seed galls was not discovered until the SA surveys had been completed. However, they provide data to monitor changes in the distribution of host, nematode and bacterium.

# Chapter 5

# Host range of *Anguina* sp.

## 5.1 Introduction

Species of nematodes in the genus *Anguina* parasitise a narrow range of closely related plant species, a characteristic which has been used to delineate species boundaries within the genus (Chizov and Subbotin, 1985; 1990). *Anguina funesta*, for example, reproduces on members of the tribe Poeae, *viz.* five species of *Lolium*, tall fescue *Festuca arundinacea* Schreber and rats-tail fescue *V. myuros*, although the latter is a poor host (Riley and McKay, 1991; Riley, 1995). While *A. funesta* is also able to invade non-host grasses (Price, 1973; Riley and McKay, 1991) and even transmit *R. toxicus* to these (Chatel *et al.*, 1979), the non-host grasses are always found in heavily infected stands of the natural host (Chatel *et al.*, 1979; Chatel, 1992).

*Polypogon monspeliensis* and *A. avenacea* are the only known hosts for nematodes associated with FPS. However, the possibility exists that other closely related grasses may also be hosts. Also, the observation described in the previous chapter that *A. avenacea* remained uninfested in heavily infested *P. monspeliensis* stands in southeastern SA but that both hosts were found infested in northern NSW is unexpected, given that preliminary analysis of the nematodes from both areas indicates that they are likely to be conspecific (McKay *et al.*, 1993).

This chapter reports on experiments examining the host range of the nematodes from southeastern SA and northern NSW. The aim of the work was to determine if there are other potential hosts for the nematodes in question, which would allow a better estimation

of the risk from FPS, and to provide further information on the relationship between the nematode populations.

## 5.2 Materials and Methods

### 5.2.1 Pot experiments

The grasses selected for the host range trial (Table 5.1) were restricted to members of the subtribe Agrostidinae (Simon, 1990) that have been recorded in the areas where flood plain staggers occurs (Black, 1986). Seed of *Deyeuxia* spp., *Dichelachne* spp., *Echinopogon* spp., *Gastridium* spp. and *Pentapogon* spp. could not be obtained and hence were not tested. *A. capillaris* (syn. *Agrostis tenuis* Sibth.) and *Agrostis stolonifera* L. (syn. *Agrostis palustris* Huds.) were included as they are hosts for the nematode *A. agrostis sensu stricto* (Southey, 1973) and are also naturalised members of the Agrostidinae in Australia.

**Table 5.1** Species, cultivar and source of grasses included in host range experiments.

| Grass | Source |
|---|---|
| *Agrostis avenacea* (SA) | Willalooka, SA, (36°24′ S, 140°18′ E) |
| *Agrostis avenacea* (NSW) | Gil Gil Creek, NSW (29°14′ S, 148°53′ E) |
| *Agrostis capillaris* | Seed Bank No.12599, Royal Botanic Gardens, Kew |
| *Agrostis capillaris* cv. SR1020 | Munn's Seeds, SA |
| *Agrostis capillaris* cv. Highland | Adelaide Seed Company Pty Ltd, SA |
| *Agrostis stolonifera* cv. Penncross | Adelaide Seed Company Pty Ltd, SA |
| *Agrostis stolonifera* cv. Seaside | Adelaide Seed Company Pty Ltd, SA |
| *Alopecurus geniculatus* | Seed Bank No.69825, Royal Botanic Gardens, Kew |
| *Phleum pratense* | Seed Bank No.51998, Royal Botanic Gardens, Kew |
| *Polypogon monspeliensis* | Lucindale, SA (36°59′ S, 140°18′ E) |

All seed was surfaced sterilised and germinated as described in Chapter 3.2 except for *A. capillaris*, *Alopecurus geniculatus* L. and *Phleum pratense* L., which were germinated following instructions supplied with the seed. Thirty seedlings of each grass were prepared and transplanted into propagation tubes containing potting compost as described in Chapter 3.7. The tubes were arranged in mesh crates in three randomised blocks, with each block containing 10 tubes of each grass, and the crates arranged in solarised sand beds.

After allowing the plants to acclimatise to field conditions for nine days, each tube in a single block was either inoculated with two dry nematode seed galls collected from either *P. monspeliensis* (Biscuit Flat, SA, 37°01′ S, 139°58′ E) or *A. avenacea* (Gil Gil Creek, NSW, 29°14′ S, 148°53′ E) or was left uninoculated. The blocks were kept separate to avoid cross contamination of nematodes.

As the plants began to senesce, they were removed from the tubes, rinsed free of soil and the tillers separated. The number of tillers per plant, shoot meristem galls (see Chapter 7.3.3) and seed galls present on each tiller were recorded. For tiller counts, treatment means were compared using nested analysis of variance to compensate for the non-random experimental design (Zar, 1996), used to avoid cross-contamination of nematode inocula. Where significant differences were detected between the means, the Least Significant Difference (LSD) method was used to examine the differences between all possible pairs of means.

### 5.2.2 *In vitro* experiments

Given that the above experiment indicated that *A. avenacea* was a poor host, compared to field observations in northern NSW, both ecotypes of *A. avenacea* were retested using the following *in vitro* methodology. Second-stage juvenile nematodes (J2s) were extracted

from a sub-sample of the *P. monspeliensis* and *A. avenacea* seed galls used in the pot experiments described above according to the methods in Chapter 3.4 and each suspension was diluted with distilled water so that 10 µl contained approximately 20 J2s.

Thirty seeds of *P. monspeliensis* and both ecotypes of *A. avenacea* were sterilised and germinated in 30 ml polycarbonate tubes, as described in Chapter 3.2. As soon as the seed germinated, 10 tubes of each grass were each inoculated with 10 µl of each nematode suspension, which was applied directly onto the germinated seed. Ten microlitres of distilled water were applied to the germinated seeds in the remaining 10 tubes of each grass. The tubes were arranged in a completely randomised block design and placed in a growth cabinet at 20°C with 16 hour photoperiod supplied by 30W fluorescent cool white tubes.

Plants were assessed after six weeks for the production of shoot meristem galls (SMG; see Chapter 7.3.3) by removing each plant from the tube and carefully removing successive leaf sheaths with fine forceps.

## 5.3 Results

### 5.3.1 Pot experiments

The mean number of tillers per plant produced by each of the grasses tested is presented in Table 5.2. There was no statistically significant difference between the number of tillers produced as a result of inoculation with nematodes from either *P. monspeliensis* or *A. avenacea* for any of the grasses. However, *P. monspeliensis*, both ecotypes of *A. avenacea*, both cultivars of *A. stolonifera* and *A. capillaris* cv. SR1020, all produced significantly more tillers ($P < 0.001$) when inoculated with nematodes than the uninoculated controls.

Nematode galls were recorded only in *P. monspeliensis* and both ecotypes of *A. avenacea* (Table 5.3). There was no difference in number of SMG or seed galls produced by the two nematode populations in each species of grass. However, both nematode populations produced more galls in *P. monspeliensis* than in *A. avenacea*. *Agrostis capillaris* cv. SR1020 and both cultivars of *A. stolonifera* did not flower and thus could not be assessed for seed gall production.

**Table 5.2** Mean number of tillers per plant produced on seven grasses in response to inoculation with two populations of *Anguina* sp. Superscripts indicate significance of cell means at the 0.05 level.

| Grass Species | *Anguina* ex *Polypogon* | *Anguina* ex *Agrostis* | Uninoculated control |
|---|---|---|---|
| *Agrostis avenacea* (NSW) | 22.5 [a] | 22.6 [a] | 11.3 [b] |
| *Agrostis avenacea* (SA) | 28.3 [a] | 29.2 [a] | 13.6 [b] |
| *Agrostis capillaris* | 15.4 | 22.3 | 19.1 |
| *Agrostis capillaris* (SR1020) | 37.7 [a] | 37.0 [a] | 19.3 [b] |
| *Agrostis capillaris* (Highland) | 26.0 | 22.4 | 27.1 |
| *Agrostis stolonifera* (Pencross) | 34.3 [a] | 31.8 [a] | 20.1 [b] |
| *Agrostis stolonifera* (Seaside) | 30.8 [a] | 29.9 [a] | 20.2 [b] |
| *Alopecurus geniculatus* | 27.1 | 26.2 | 25 |
| *Phleum pratense* | 8.3 | 9.9 | 7.9 |
| *Polypogon monspeliensis* | 27.3 [a] | 20.2 [a] | 11.0 [b] |

LSD for comparison of treatments means = 7.259

**Table 5.3** Mean number of shoot meristem galls and seed galls per plant produced on seven grasses in response to inoculation with two populations of *Anguina* sp. Data are means ± standard deviations.

| Grass Species | Shoot Meristem Galls (no. / plant) | | | Seed Galls (no. / plant) | | |
|---|---|---|---|---|---|---|
| | *Anguina* ex *Polypogon* | *Anguina* ex *Agrostis* | Uninoculated controls | *Anguina* ex *Polypogon* | *Anguina* ex *Agrostis* | Uninoculated controls |
| *Agrostis avenacea* (NSW) | 0.1 ± 0.3 | 0.8 ± 2.0 | 0 | 0 | 0.2 ± 0.4 | 0 |
| *Agrostis avenacea* (SA) | 0.3 ± 0.7 | 1.5 ± 3.9 | 0 | 0 | 0 | 0 |
| *Agrostis capillaris* | 0 | 0 | 0 | 0 | 0 | 0 |
| *Agrostis capillaris* (SR1020) | 0 | 0 | 0 | * | * | * |
| *Agrostis capillaris* (Highland) | 0 | 0 | 0 | 0 | 0 | 0 |
| *Agrostis stolonifera* (Pencross) | 0 | 0 | 0 | * | * | * |
| *Agrostis stolonifera* (Seaside) | 0 | 0 | 0 | * | * | * |
| *Alopecurus geniculatus* | 0 | 0 | 0 | 0 | 0 | 0 |
| *Phleum pratense* | 0 | 0 | 0 | 0 | 0 | 0 |
| *Polypogon monspeliensis* | 16.1 ± 7.3 | 9.9 ± 7.7 | 0 | 21.0 ± 22.7 | 14.6 ± 11.1 | 0 |

* = Did not flower

### 5.3.2 *In vitro* experiments

By the end of the six week period, all plants consisted of a single tiller, irrespective of inoculation with nematodes. Swelling was observed at the base of the tillers after nine days in plants inoculated with nematodes, which increased in size over the duration of the experiment. A single SMG was found within each tiller in each tube inoculated with nematodes, regardless of host or inoculum used. No swelling was observed in, or galls recovered from, tubes where only distilled water was applied.

## 5.4 Discussion

By their very nature, host range experiments can be difficult to interpret and may prove inconclusive. They can be influenced by a large number of factors including environmental variables at the time of testing (Wallace, 1966), variation in the genotype of the parasite and host (Briggs and Johal, 1994), differences in viability of inocula (Wallace, 1966), inoculation failures (Thorne, 1961) and the use of resistant cultivars of a particular plant species (Whitehead, 1998). The results of the pot experiment indicate that while both populations of nematodes are able to successfully reproduce in *P. monspeliensis* and *A. avenacea*, the former appears to be a superior host for both populations. However, this was not supported by the *in vitro* results, which indicated that *P. monspeliensis* and *A. avenacea* were equally good hosts for both nematode populations. This indicates that either the experimental conditions under which the pot experiment was conducted were not optimal for invasion of *A. avenacea* or that gall production was affected in some manner following successful invasion. The invasion model proposed by Price *et al.* (1979) for *A. funesta*, where J2s enter the outer leaf sheath by climbing up the outside of the plant, depends on the existence of a water film on the outer surface of the plant, the thickness of which is

crucial for successful upward movement of the nematodes (Wallace, 1959). It is likely that different grass species require different moisture regimes for successful maintenance of water films due to differences in gross anatomy and the interaction of various anatomical structures with rainfall, dews and humidity in the maintenance of water films warrants further investigation.

The increase in the number of tillers produced by *P. monspeliensis* and *A. avenacea* as a result of successful nematode invasion, is probably to compensate for the loss of tiller function resulting from SMG initiation (See Chapter 7.3.3). The fact that an increase in tiller number also occurred in several of the other grasses in the absence of gall production is noteworthy. It appears that the nematodes are able to elicit a plant response, possibly as they attempt to feed on the shoot apical mersitem, but cannot induce galls. A natural hybrid between *A. stolonifera* and *P. monspeliensis* known as *x Agropogon littoralis* (Sm.) C.E.Hubb has been recorded (Weiller *et al.*, 1995; Rúgolo de Agrasar and Molina, 1997), indicating that they may share similar features that are attractive to the nematodes. It would be interesting to test this hybrid along with *A. stolonifera* (cv. Seaside and Pencross) and *A. capillaris* cv. SR1020 further using the in vitro system, to determine if an increase in tillering can be attributed to nematode activity around the shoot meristem. It is also interesting that the grasses that showed an increase in tiller number with nematode infestation, excluding *P. monspeliensis* and *A. avenacea*, did not flower. However, the lack of flowering was not attributable to the nematode treatments and is likely to be a function of environmental conditions.

In contrast to the differences in gall production between *P. monspeliensis* and *A. avenacea*, both nematode populations produced similar numbers of SMG and seed galls per plant in each host. Thus, it is not possible to differentiate the two nematode populations on the

basis of host range or relative performance on a particular host, unlike other nematode genera such as *Meloidogyne* (Taylor and Sasser, 1978). Given the narrow host ranges of other species of *Anguina*, it is likely that the nematode populations in NSW and SA are not separate species. The only other species of *Anguina* that is recorded as reproducing in grasses in the subtribe Agrostidinae is *A. agrostis sensu stricto,* whose type host is *A. capillaris* and which has been reported to reproduce in *A. stolonifera*, *Agrostis gigantea* Roth (syn *Agrostis alba* Auct.), *Agrostis canina* L. and *Agrostis exarata* Trin. (Courtney and Howell, 1952). It has also been reported to reproduce in species of *Apera*, *Arctagrostis*, *Calamagrostis*, *Dactylis*, *Festuca*, *Hordeum*, *Koeleria*, *Phalaris*, *Phleum*, *Poa*, *Puccinellia*, *Sporobolus* and *Trisetum* (Southey, 1973). However, recent phylogenetic studies have found that *A. agrostis s. s.* and two other undescribed species, which includes the nematodes under investigation in this thesis, reproduce in *Agrostis* species and that there probably are at least nine different species infesting the other grass genera (Subbotin *et al.*, 2002).

As *A. avenacea* is endemic to Australia and *P. monspeliensis* is introduced, it is unclear which is the original host. Given that endemic anguinid species occur in Australia, it is possible that *A. avenacea* is the original host. However, endemic anguinids have thus far only been found infesting grasses in the tribes Ehrharteae, Stipeae and Pommereullinae. Anguinid nematodes infesting grasses in the Poeae and Aveneae, which includes the subtribe Agrostidinae, appear to be introductions. This points to a European origin for *Anguina* sp. being more likely.

# Chapter 6

# Characteristics and Taxonomy of *Anguina* Nematodes Forming Galls on *Agrostis avenacea* and *Polypogon monspeliensis*

## 6.1 Introduction

McKay *et al.* (1993) used allozyme electrophoresis to show that the nematodes infesting *A. avenacea* and *P. monspeliensis* were different from other described seed-gall forming *Anguina* species known from Australia and New Zealand. However, they did not speculate on whether the nematodes from *A. avenacea* and *P. monspeliensis* were the same species, possibly due to the limited number of populations they examined, even though the genetic variation they found between populations was within the variation expected for conspecific allopatric populations (Richardson *et al.*, 1986).

This chapter describes studies aimed at resolving the taxonomic status of the nematodes from *A. avenacea* and *P. monspeliensis* using methods previously proven useful in the delineation of *Anguina* species. It includes a re-examination of the nematodes from both hosts by allozyme electrophoresis, including populations from throughout the known distribution in NSW and SA; a morphological study aimed at determining whether any consistent morphological or morphometric differences are present in the populations and a study to describe the karyotype of nematodes collected from *P. monspeliensis*. While the data presented in this chapter indicate that the nematodes constitute a new species, the description will be formally published elsewhere according to the Code of Zoological Nomenclature, to avoid the creation of a *nomen nudem*.

## 6.2 Materials and Methods

### 6.2.1 Allozyme electrophoresis

Thirteen populations of *Anguina* from *P. monspeliensis* and *A. avenacea* were collected from southeastern SA and northern NSW during the survey work reported in Chapter 4 (Table 6.1). Two populations of *A. funesta* used in previous studies (Riley *et al.* 1988; McKay *et al.* 1993), were included as reference populations. Fifty seed galls from each population were soaked individually in sterile 0.02M phosphate buffered saline (PBS) at room temperature for three hours, then ruptured using fine forceps to release the juvenile nematodes from the galls. Galls were soaked individually to identify any contaminated by *R. toxicus*, which were then discarded. Nematodes within each population were combined and filtered overnight using a modified Baerman funnel to remove any plant material. They were then transferred to centrifuge tubes and spun at 12000 g for 5 min. After removing the supernatant, the pellets were washed three times by centrifugation at 12000 g for 5 min and resuspended in sterile PBS. After the final centrifugation, excess liquid was removed and an equal volume of buffered lysing solution (0.02M Tris-HCl, pH 8.0, containing 0.2% β-mercaptoethanol and 0.02% NADP) was added to the pellet and the mixture sonicated for 15 s in three bursts of 5 s. The homogenates were centrifuged at 4°C at 8000 g for 5 min and 20 µl aliquots of each supernatant were drawn into glass capillary tubes which were stored at -20°C until used for electrophoresis.

Allozymes were designated alphabetically and multiple loci, where present, were designated numerically, both in order of increasing electrophoretic mobility. As the electrophoretic analysis was conducted on pooled individuals within populations, measures of genetic distance such as Nei D ( Nei, 1972; 1978) or Rogers S (Rogers, 1972) could not

**Table 6.1** Population reference code, host species and location details (locality, state and geodetic position) of nematode populations used for allozyme electrophoresis.

| Ref. No. | Host | Location |
|---|---|---|
| PM1 | *Polypogon monspeliensis* | Lucindale, SA (37°00′ S, 140°22′ E) |
| PM2 | | Biscuit Flat, SA (37°01′ S, 139°58′ E) |
| PM3 | | Water Valley, SA (36°34′ S, 140°08′ E) |
| PM4 | | Lucindale, SA (36°59 S, 140°18′ E) |
| PM5 | | Willalooka, SA (36°24′ S, 140°18′ E) |
| PM6 | | Naracoorte, SA (37°02′ S, 140°44′ E) |
| PM7 | | Canunda, SA (37°38′ S, 140°18′ E) |
| PM8 | | 20 km S of Lake Mundi, Vic (37°43′ S, 141°02′ E) |
| PM9 | | Garah, NSW (29°04′ S, 149°39′ E) |
| AA1 | *Agrostis avenacea* | Collarenebri, NSW (29°23′ S, 148°49′ E) |
| AA2 | | Dungle Ridge Bore, NSW (37°43′ S, 141°02′ E) |
| AA3 | | Gil Gil Creek, NSW (29°14′ S, 148°53′ E) |
| AA4 | | Nyngan, NSW (31°42′ S, 147°10′ E) |
| LR1 | *Lolium rigidum* | Lochiel, SA (33°55′ S, 138°09′ E) |
| LR2 | | Geranium, SA (35°22′ S, 140°09′ E) |

be applied. Therefore, allelic profiles were converted into a matrix of pairwise comparisons between populations based on the percentage of loci showing fixed differences (Richardson *et al.*, 1986). From this matrix, a phenogram showing relationships between populations was constructed using the average linkage cluster method (Sneath & Sokal 1973).

### 6.2.2 Morphological examination

Nematodes were obtained from immature seed and shoot meristem galls from pastures of *P. monspeliensis* at Lucindale, SA, to supplement specimens from *P. monspeliensis* and *A. avenacea* already held in the Waite Insect and Nematode Collection (WINC), Adelaide. Galls were dissected in tap water and the nematodes collected, relaxed and fixed in hot FA (formalin – glacial acetic acid, 4:1) before being processed to anhydrous glycerol, following Davies and Lloyd (1996). Processed nematodes were mounted in anhydrous glycerol on glass slides and examined using interference microscopy.

Drawings and measurements were made using a camera lucida. Body width was measured at the greatest width. The De Man ratios determined are as follows: a = body length divided by greatest body width, b = body length divided by distance from anterior end to base of oesophageal glands, c = body length divided by tail length, T = length of testis from cloaca to flexure as a percentage of body length, V = anterior end to vulva as a percentage of body length. Comparisons were made with described species using published descriptions and specimens held in the WINC.

### 6.2.3 Cytology

Adult female and male nematodes from *P. monspeliensis* seed-galls were transferred to clean microscope slides using a sharpened feather shaft and most of the water surrounding

them was allowed to evaporate. The reproductive tracts were smeared onto the slides by removing the head of the nematode using the tip of a hypodermic syringe needle and simultaneously drawing the body across the slide by the tail using fine forceps. The resultant smears were hydrolysed, fixed and stained using the acetic-orcein method of Triantaphyllou & Hirschmann (1966). Following the removal of excess stain, the reproductive tracts were mounted in 45% acetic acid, squashed under a cover slip and examined using an Axiophot-T compound microscope. The ovaries and testes were scanned for regions where the oogonia and spermatogonia respectively, were at pro-metaphase and the stained chromosomes counted. Specimens from *A. avenacea* seed-galls were not available for comparison.

## 6.3 Results

### 6.3.1 Allozyme electrophoresis

In total, 24 presumptive loci were able to be scored from the 21 enzymes examined. Of these, the following six loci were invariant in all 15 *Anguina* populations: *Ald-2*, *Ap*, *Enol*, *Ga3pd*, *G6pd* and *Mdh-1*. The allelic profiles for the remaining 18 loci in each population are presented in Table 6.2. The phenogram constructed by average linkage cluster of the matrix of pairwise comparisons between populations, based on the percentage of loci differing between populations, is shown in Figure 6.1.

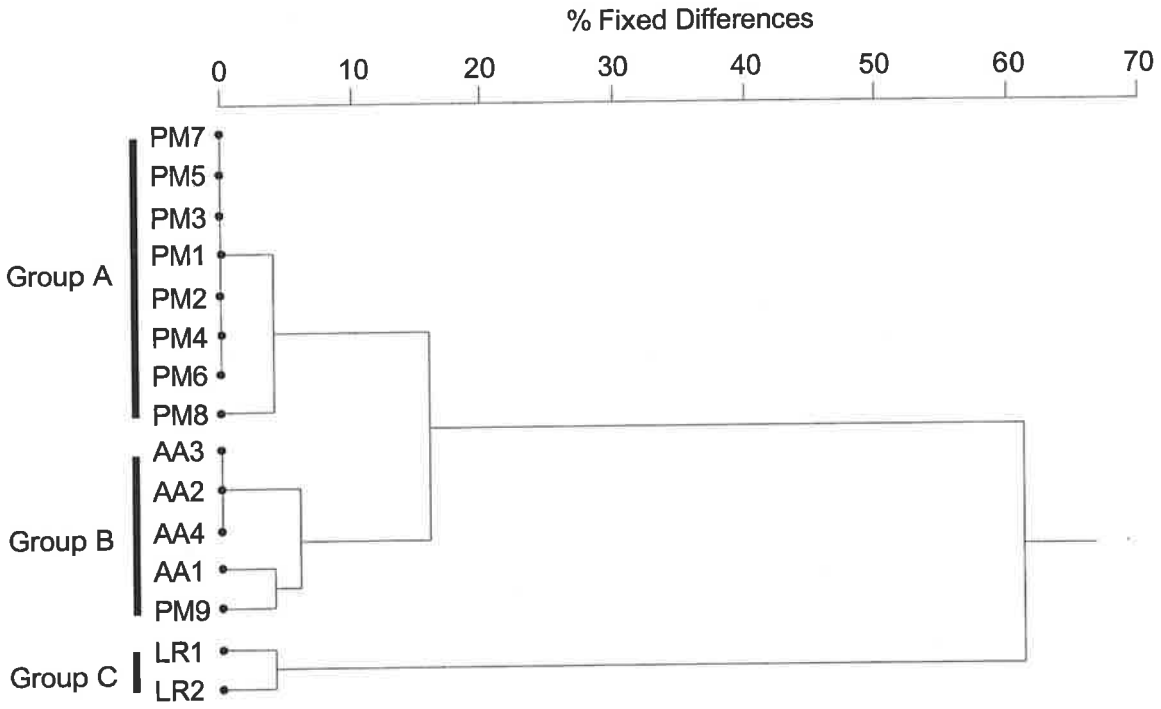
The phenogram shows the occurrence of three major electrophoretic groups. Group A comprised eight populations collected from *P. monspeliensis* from southeastern SA (PM1 – PM7) and western Victoria (PM8). The Victorian population differed from the SA population only at locus *PepB*. Group B was made up of the four populations collected from *A. avenacea* (AA1 – AA4) and a population collected from *P. monspeliensis* (PM9),

**Table 6.2** Allelic profiles of the fifteen *Anguina* populations examined from *Polypogon monspeliensis*, *Agrostis avenacea* and *Lolium rigidum*. Population reference codes are analogous to those in Table 6.1.

| Locus | *Polypogon mospeliensis* | | | | | | | | | *Agrostis avenacea* | | | | *Lolium rigidum* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PM1 | PM2 | PM3 | PM4 | PM5 | PM6 | PM7 | PM8 | PM9 | AA1 | AA2 | AA3 | AA4 | LR1 | LR2 |
| *Acon* | bc | - | - | - | - | - | - | - | a | c | c | c | c | b | b |
| *Ald-1* | b | b | b | b | b | b | b | ab | a | a | a | a | a | b | b |
| *Est* | b | b | b | b | b | b | b | b | b | ab | b | ab | b | c | c |
| *Fum* | a | a | a | a | a | a | a | a | a | a | ab | a | a | b | b |
| *Got* | c | c | c | c | c | c | c | c | c | c | ac | c | c | b | b |
| *Gpd* | b | ab | b | b | b | b | b | b | b | - | - | - | - | a | a |
| *Gpi* | b | b | b | b | b | b | b | b | a | a | b | b | b | a | a |
| *Idh* | b | b | b | b | b | b | b | b | b | b | ab | b | b | a | a |
| *Mdh-2* | a | a | a | a | a | a | a | a | b | b | b | ab[1] | ab[1] | c | c |
| *Mpi* | d | d | cd | d | d | d | d | d | d | d | d | d | d | a | b |
| *PepA* | a | a | a | a | a | a | a | a | a | a | ab | a | a | b | b |
| *PepB* | b | b | b | b | b | b | b | c | c | c | c | c | c | ab | ab |
| *Pgam* | b | b | b | ab | b | b | b | b | b | b | b | b | b | a | a |
| *6pgd* | a | a | a | a | a | a | a | a | - | a | a | a | a | b | b |
| *Pgk* | c | c | c | c | c | c | c | c | a | a | a | a | a | a | a |
| *Pgm* | b | b | b | b | b | b | b | b | b | b | b | b | b | a | a |
| *Pk* | b | b | b | b | b | b | b | b | b | b | b | b | b | a | a |
| *Tpi* | b | b | b | b | b | b | b | b | b | b | b | b | b | c | c |

[1] Ratio of allele frequencies for *Mdh-2*; a:b = 1:1 and 8:2 respectively

**Figure 6.1** Phenogram of *Anguina* populations based on average linkage cluster analysis of the percentage fixed differences among populations. Population reference codes are analogous to those in Table 6.1.



all from northern NSW. Populations PM9 and AA1 also differed at a single locus (*Acon*) as did these populations from AA2 – AA4 (*Gpi*). While Group B as a whole differed from Group A at an average of 16%, *Pgk* is the only fully diagnostic locus that separates the groups. Loci *Ald-1*, *Mdh-2* and *PepB* are partially diagnostic. Group C consisted of two populations of *A. funesta* which differed from the other groups at 62% of their loci and each other at a single locus.

### 6.3.2 Morphological examination

No consistent differences in gross morphology were observed between nematodes from *P. monspeliensis* or *A. avenacea*, regardless of the nematode stage examined or the gall type from which they were extracted (Tables 6.3, 6.4, 6.5). Therefore, the following morphological observations encompass the range of variation observed for each of the stages examined.

*Male* (Table 6.3; Figures 6.2 a, 6.3 c-d)

Body cylindrical, slender, curved into a "C" shape with dorsal surface innermost when heat relaxed. Head much narrower than body, offset. Anterior cephalids not observed, posterior cephalids seven to nine annules behind the head at level of dorsal oesophageal gland orrifice. Excretory pore distinct, immediately behind hemizonid. Lateral field not observed. Cephalic sclerotization weak, stylet delicate, basal knobs small and rounded; prorhabdions half stylet length. Procorpus wide, metacorpus oval with refractive thickenings slightly anterior to middle, isthmus shorter and narrower than procorpus, constricted where encircled by nerve ring. Stricture evident where isthmus joins oesophageal glands, forming so-called storage organ or accessory bulb (Thorne, 1949). This bulb is partially enclosed by the anterior of the oesophageal glands which form a single lobe abutting the intestine.

**Table 6.3** Measurements (μm) of males of *Anguina* from different gall types produced in *Polypogon monspeliensis* and *Agrostis avenacea*. The number of individuals measured is shown in parentheses below the mean ± standard deviation. Superscripts indicate significance of cell means at the 5% level.

| | *Polypogon monspeliensis* | | | | *Agrostis avenacea* | | |
|---|---|---|---|---|---|---|---|
| | Seed gall | | Shoot meristem gall | | Seed gall | | |
| | Mean ± S.D. | Range | Mean ± S.D. | Range | Mean ± S.D. | Range | |
| Body length | 1972.2 ± 278.2 (20) | 1593.8 - 2478.1 | 1979.6 ± 285.6 (20) | 1500.0 - 2486.8 | 2056.1 ± 164.5 (16) | 1613.4 - 2280.1 | n.s. |
| Max. Body width | 71.8 ± 15.7 (20) | 50.0 – 100.0 | 70.2 ± 12.4 (20) | 53.3 – 100.0 | 78.6 ± 6.4 (16) | 66.7 - 86.7 | n.s. |
| Oesophagous length | 187.9 ± 20.1$^{ab}$ (20) | 147.6 - 221.1 | 175.1 ± 19.4$^{a}$ (20) | 140.0 - 226.7 | 191.5 ± 16.2$^{b}$ (16) | 158.1 – 225.0 | * |
| Tail length | 80.3 ± 6.9$^{a}$ (20) | 66.3 - 91.6 | 77.1 ± 8.4$^{ab}$ (19) | 60.0 - 98.4 | 72.7 ± 6.6$^{b}$ (16) | 58.3 - 83.4 | * |
| T % | 82.6 ± 3.0$^{a}$ (20) | 77.5 - 87.3 | 83.7 ± 4.0$^{ab}$ (20) | 74.3 - 88.6 | 85.8 ± 2.1$^{b}$ (16) | 81.0 – 89.0 | * |
| Spicule length | 32.4 ± 2.7$^{ab}$ (20) | 28.1 - 38.1 | 33.8 ± 1.9$^{a}$ (20) | 30.3 - 37.3 | 30.8 ± 1.9$^{b}$ (16) | 27.3 - 33.4 | ** |
| Gubernaculum length | 9.8 ± 1.6 (20) | 6.9 - 12.5 | 10.3 ± 1.3 (20) | 7.7 - 12.7 | 10.1 ± 1.7 (14) | 7.3 – 11.3 | n.s. |
| Stylet length | 9.8 ± 0.6$^{ab}$ (20) | 8.1 - 10.6 | 10.3 ± 1.4$^{a}$ (19) | 7.7 - 13.3 | 9.6 ± 0.9$^{b}$ (15) | 8.0 - 11.3 | * |
| De Man's ratio a | 28.1 ± 4.0 (20) | 18.3 - 37.3 | 28.5 ± 3.0 (20) | 23.5 - 33.6 | 26.2 ± 1.9 (16) | 23.0 - 29.7 | n.s. |
| De Man's ratio b | 10.5 ± 1.3 (20) | 9.1 - 13.6 | 11.4 ± 1.4 (20) | 8.9 - 14.4 | 10.8 ± 1.0 (16) | 9.0 - 12.4 | n.s. |
| De Man's ratio c | 24.6 ± 3.0$^{a}$ (20) | 18.0 – 32.0 | 25.7 ± 2.4$^{a}$ (20) | 20.2 - 29.8 | 28.4 ± 2.2$^{b}$ (16) | 25.9 - 35.3 | *** |

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. not significant.

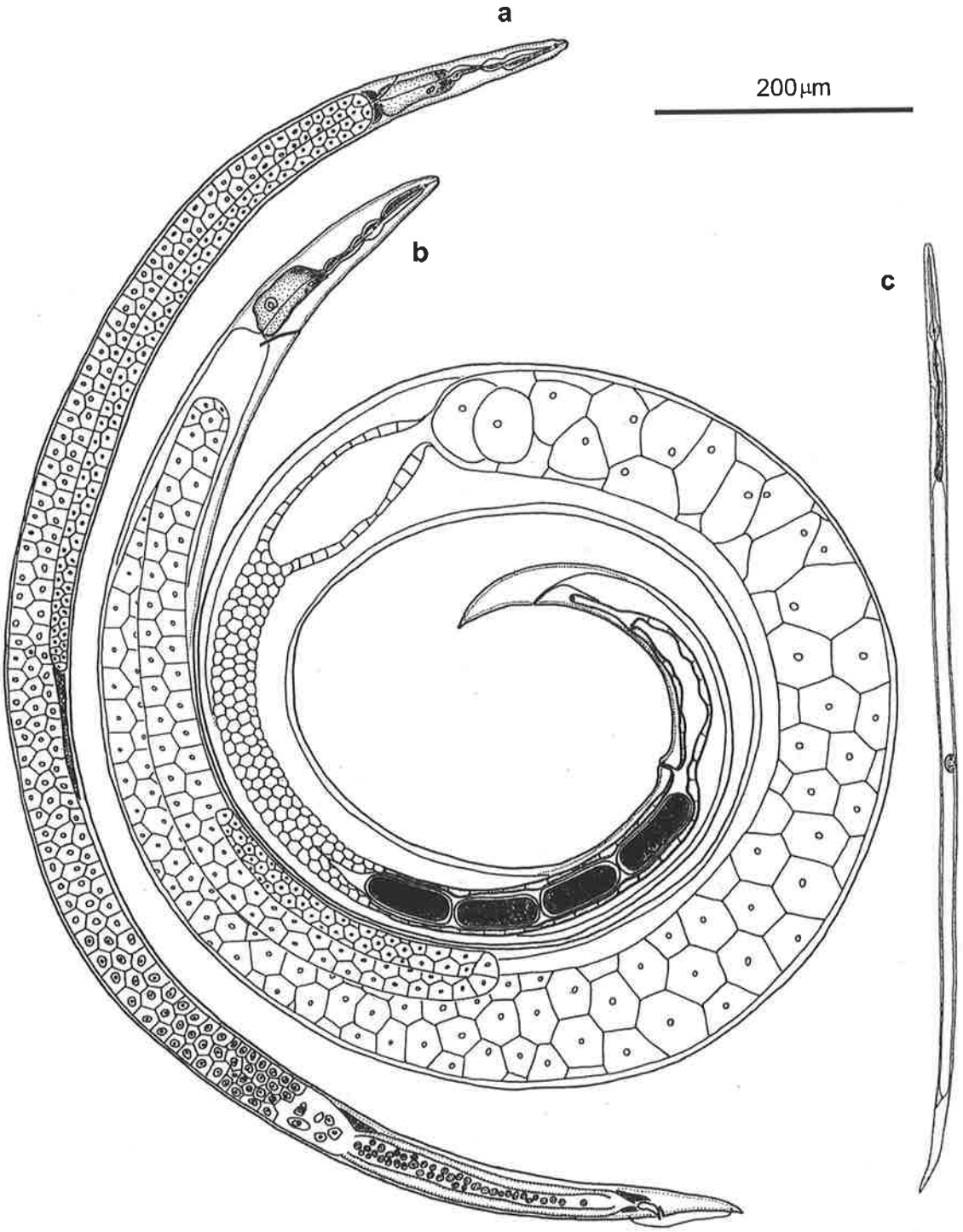



**Figure 6.2** *Anguina* sp.ex *Polypogon* (a) Male, (b) Female, (c) Infective juvenile

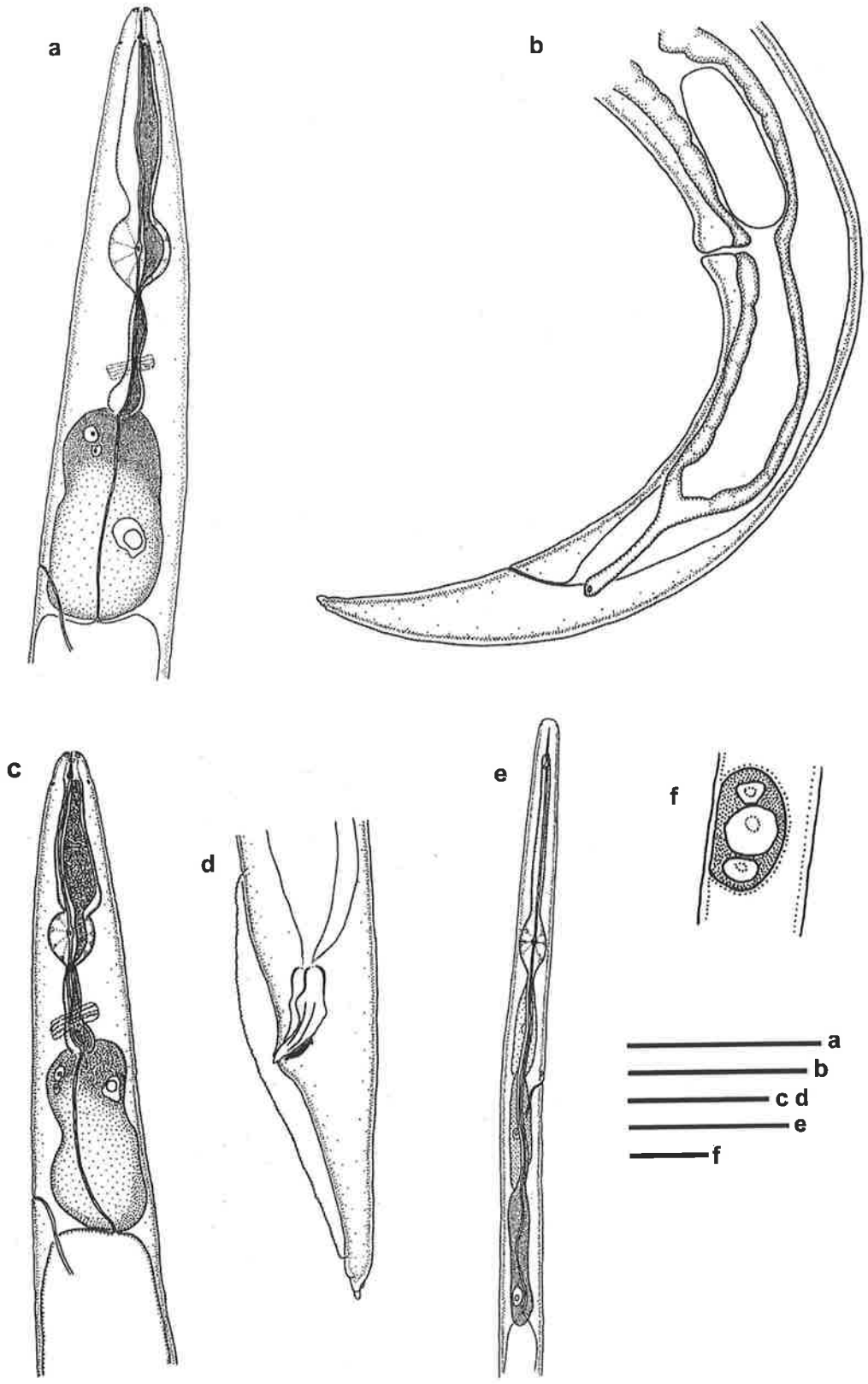



**Figure 6.3** *Anguina* sp.ex *Polypogon* (a) Oesophageal region of female, (b) Posterior region of female, (c) Oesophageal region of male, (d) Posterior region of male, (e) Oesophageal region of second stage juvenile, (f) Genital primordium of second stage juvenile (lateral view). Scale bars represent 50 μm.

Dorsal gland densely packed with granular material from stricture to short duct behind base of stylet. Dorsal gland nucleus larger than subventral nuclei. Junction of oesophagus and intestine at base of dorsal gland.

Testis single, reflexed once. Spermatocytes arranged around a rachis, terminating in a prominent cap cell. Chromosomes in pro-metaphase before spermatid formation haploid, n = 18. Spicules paired, arcuate with two ridges running length of shaft. Gubernaculum tapering ventrally, dorsal end hooked. Caudal alae crenate, extending from about 20 - 77μm in front of cloaca to about 9.5 – 27 μm before tail tip. Tail conical with irregular and variable terminus, appears lobed in some specimens.

Measurements of the males from *P. monspeliensis* and *A. avenacea* overlapped considerably (Table 6.3) but there were some small but statistically significant differences between the means of some characters, *viz.* oesophagous, tail, spicule and stylet lengths along with ratios derived from testis length and tail length. None of these differences occurred between specimens from the different gall types examined from *P. monspeliensis*. Only for ratio c, based on tail length, were the males from both gall types from *P. monspeliensis* different from those from *A. avenacea*.

*Female* (Table 6.4; Figures 6.2 b, 6.3 a-b)

Mature females obese, coiled with ventral surface innermost. Head and oesophagous as in male.

Female reproductive tract usually reflexed twice, first fold 45.5–331.3 μm (mean 183.1 μm) below junction of oesophageal gland and intestine. In some specimens, the ovary extends past the oesophageal glands forcing the oesophagous to one side. Oocytes in

**Table 6.4** Measurements (μm) of females of *Anguina* from different gall types produced in *Polypogon monspeliensis* and *Agrostis avenacea*. The number of individuals measured is shown in parentheses below the mean ± standard deviation. Superscripts indicate significance of cell means at the 5% level.

| | *Polypogon monspeliensis* | | | | *Agrostis avenacea* | | |
|---|---|---|---|---|---|---|---|
| | Seed gall | | Shoot meristem gall | | Seed gall | | |
| | Mean ± S.D. | Range | Mean ± S.D. | Range | Mean ± S.D. | Range | |
| Body length | 3063.3 ± 759.4 (20) | 1511.5 - 4268.8 | 3188.7 ± 618.4 (20) | 1903.4 - 4320.2 | 3491.8 ± 399.3 (11) | 3.0 - 4.4 | n.s. |
| Max. Body width | 121.0 ± 40.4$^{a}$ (20) | 56.3 - 193.8 | 129.5 ± 33.9$^{ab}$ (20) | 66.7 - 186.7 | 160.4 ± 23.1$^{b}$ (11) | 131.3 - 220 | * |
| Oesophagous length | 198.9 ± 27.9 (20) | 137.4 – 240.0 | 201.2 ± 21.9 (20) | 160 - 229.2 | 220.2 ± 18.2 (10) | 184.7 - 253.4 | n.s. |
| Tail length | 90.5 ± 10.0 (20) | 69.2 - 105.8 | 88.0 ± 10.6 (20) | 70 - 116.7 | 82.0 ± 9.8 (11) | 66.7 – 100.0 | n.s. |
| V % | 89.5 ± 2.6$^{a}$ (20) | 81.1 - 93.6 | 90.4 ± 1.6$^{ab}$ (20) | 87.2 - 92.9 | 91.7 ± 0.8$^{b}$ (11) | 90.6 - 92.8 | * |
| Post uterine sac length | 144.4 ± 29.2$^{a}$ (20) | 99.5 - 203.7 | 132.2 ± 25.3$^{a}$ (20) | 91.7 - 164.2 | 101.2 ± 10.2$^{b}$ (11) | 83.4 – 115.0 | *** |
| Post uterine sac as % of vulva to anus distance | 68.1 ± 13.4$^{a}$ (20) | 46.7 – 90.2 | 63.2 ± 10.5$^{a}$ (20) | 42.9 – 84.6 | 27.9 ± 8.4$^{b}$ (11) | 20.9 – 51.8 | *** |
| Post uterine sac knob length | 35.0 ± 21.2 (14) | 6.3 – 75.0 | 34.6 ± 18.3 (12) | 10.7 - 79.3 | 16.7 ± 11.7 (7) | 3.3 – 34.0 | n.s. |
| Stylet length | 10.1 ± 0.7$^{a}$ (19) | 8.8 - 11.3 | 11.2 ± 0.7$^{b}$ (18) | 10.0 - 12.7 | 9.9 ± 0.5$^{a}$ (9) | 9.3 - 10.7 | *** |
| De Man's ratio a | 26.1 ± 3.2$^{a}$ (20) | 21.1 - 33.2 | 25.2 ± 2.8$^{a}$ (20) | 20.1 – 31.0 | 21.9 ± 2.0$^{b}$ (11) | 18.8 - 24.2 | * |
| De Man's ratio b | 15.5 ± 3.4 (20) | 8.5 - 22.1 | 15.9 ± 2.6 (20) | 11.0 - 20.7 | 15.6 ± 1.8 (10) | 11.9 - 17.8 | n.s. |
| De Man's ratio c | 33.5 ± 6.3$^{a}$ (20) | 21.8 - 45.5 | 36.1 ± 4.9$^{a}$ (20) | 27.2 - 46.3 | 42.8 ± 4.5$^{b}$ (11) | 38.5 – 54.0 | *** |

$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. not significant.

mutiple rows arranged around a rachis, terminating in a prominent cap cell. Spermatheca oval to elongate, separated from ovary by oviduct. Crustaformaria comprising ten rows of helically arranged cells. Uterus separated from crustaformaria by a constriction. Crustaformaria and uterus with up to 9 eggs. Vaginal length about 28% body width at vagina; vulval lips prominent. Postvulval uterine sac similar in length to uterus, sometimes with a process of varying length extending distally, reflexed several times if long and sometimes with a cell distally (Plate 6.1). Tail conical, terminus irregular and variable, similar to that of the male.

The range of measurements of females from both hosts and gall type overlapped (Table 6.4) but there were significant differences between the means of some characters including maximum body width, V%, post uterine sac length, stylet length and ratios based on width, post uterine sac and tail length. Females from seed and shoot meristem galls from *P. monspeliensis* differed only in stylet length. However, they differed from females from *A. avenacea* by virtue of their significantly larger post uterine sacs and in the ratios based on tail length and maximum body width. The mean length of the process on the post uterine sac in *A. avenacea* females was only half that in specimens from *P. monspeliensis*, but was very variable and as such not statistically different.

*Infective juvenile* (Table 6.5; Figure 6.2 c, 6.3 e-f)

Body cylindrical, tapering gradually to posterior from 80% of length. Head narrower than body, not offset, flattened in front and lacking distinct lip annules. Cephalids not visible. Excretory pore immediately behind hemizonid which extends over three annules. Lateral field occupies slightly less than one third of body width with five incisures. Cephalic framework weakly sclerotized, stylet with small basal knobs sloping slightly backwards,

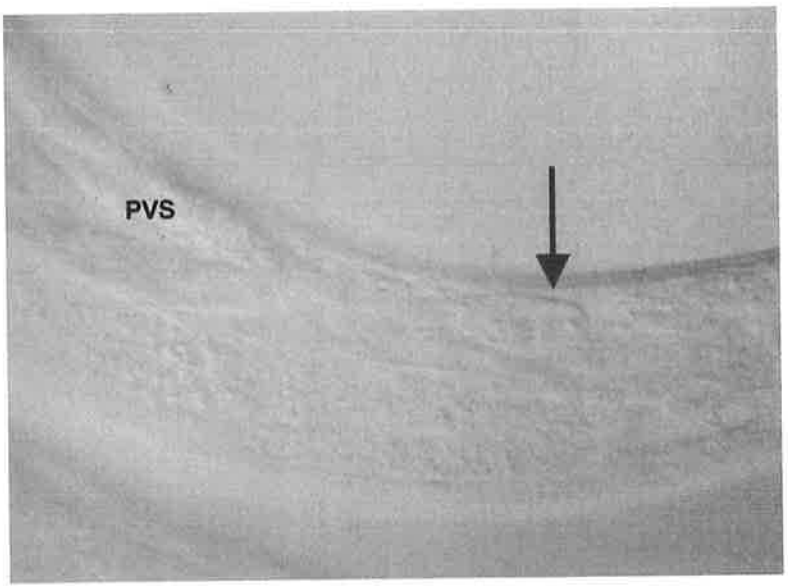



**Plate 6.1** Postvulval uterine sac with a process extending distally. The cell at the distal end of the process is indicated by the arrow. PVS designates the lumen of the postvulval uterine sac

**Table 6.5** Measurements (μm) of infective juveniles of *Anguina* from different gall types produced in *Polypogon monspeliensis* and *Agrostis avenacea*. The number of individuals measured is shown in parentheses below the mean ± standard deviation. Superscripts indicate significance of cell means at the 5% level.

| | *Polypogon monspeliensis* | | | | *Agrostis avenacea* | | |
|---|---|---|---|---|---|---|---|
| | Seed gall | | Shoot meristem gall | | Seed gall | | |
| | Mean ± S.D. | Range | Mean ± S.D. | Range | Mean ± S.D. | Range | |
| Body length | 663.7 ± 29.5$^{a}$ (20) | 604.2 – 714.6 | 689.1 ± 36.2$^{ab}$ (20) | 603.8 - 755.7 | 707.4 ± 44.8$^{b}$ (21) | 633.4 - 770.9 | ** |
| Max. Body width | 12.4 ± 0.5 (20) | 11.3 – 13.3 | 12.4 ± 0.5 (20) | 11.3 - 13.1 | 12.6 ± 0.5 (21) | 11.4 - 13.3 | n.s. |
| Oesophagous length | 168.4 ± 11.6$^{a}$ (20) | 144.0 – 186.8 | 191.3 ± 6.2$^{b}$ (20) | 175.0 - 204.2 | 160.2 ± 10.0$^{c}$ (21) | 141.7 - 180.9 | *** |
| Tail length | 59.2 ± 5.8 (19) | 47.0 – 70.0 | 61.6 ± 4.5 (20) | 55.6 - 74.1 | 61.3 ± 5.7 (19) | 49.0 – 70.0 | n.s. |
| Length from anterior end to hemizonid | 97.2 ± 8.4$^{a}$ (12) | 77.1 – 114.8 | 110 ± 5.2$^{b}$ (13) | 102.5 - 121.7 | 101.1 ± 5.4$^{a}$ (12) | 96.7 – 115.0 | *** |
| Length from anterior end to excretory pore | 102.8 ± 6.4$^{a}$ (10) | 96.8 – 120.0 | 117.9 ± 8.3$^{b}$ (10) | 110.0 - 136.7 | 103.5 ± 6.2$^{a}$ (12) | 98.4 - 118.4 | *** |
| Genital primordium length | 14.4 ± 1.5$^{ab}$ (19) | 9.7 – 17.3 | 13.6 ± 1.2$^{a}$ (20) | 11.9 - 15.9 | 14.6 ± 1.2$^{b}$ (21) | 12.7 - 17.3 | * |
| Length from genital primordium end to tail end | 309.7 ± 31.8$^{a}$ (19) | 252.1 – 370.9 | 312 ± 28.2$^{b}$ (20) | 265.4 - 357.7 | 328.5 ± 37.9$^{b}$ (20) | 254.2 - 412.5 | ** |
| Stylet length | 9.8 ± 0.4 (12) | 9.3 – 10.7 | 9.6 ± 0.4 (20) | 8.8 - 10.3 | 9.7 ± 0.4 (19) | 8.7 - 10.3 | n.s. |
| De Man's ratio a | 53.2 ± 2.5$^{a}$ (20) | 49.8 – 59.5 | 55.6 ± 3$^{b}$ (20) | 49.2 - 60.5 | 56.3 ± 3.2$^{b}$ (21) | 49.0 - 60.8 | ** |
| De Man's ratio b′ | 4.0 ± 0.3$^{a}$ (20) | 3.6 – 4.5 | 3.6 ± 0.2$^{b}$ (20) | 3.3 - 4.2 | 4.4 ± 0.4$^{c}$ (21) | 3.7 - 5.1 | *** |
| De Man's ratio c | 11.3 ± 1.0 (19) | 9.9 – 13.3 | 11.2 ± 0.5 (20) | 10.0 – 12.0 | 11.6 ± 1.2 (19) | 10.1 - 15.2 | n.s. |

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. not significant.

prorhabdions half stylet length. Oesophagus with long narrow procorpus, metacorpus oval with refractive plates of valve slightly anterior to middle, long narrow isthmus to the dorsal gland, overlying and partly obscuring the two subventral glands. Nerve ring encircling isthmus near middle. Oesophageal glands lobed with prominent nuclei. Dorsal gland posterior to subventrals, slightly overlapping junction of oesophagus with intestine. Intestine packed with fat reserves. Genital primordium distinct, situated dorsally about half way along body with one central nucleus and two smaller nuclei. Tail tapering gradually to a variable non-mucronate tip.

Measurements of the infective second stage juveniles from each gall type are shown in Table 6.5. While the range of measurements overlapped for each character, some small statistically significant differences existed between some means. In contrast to the adults, the juveniles from *P. monspeliensis* seed and shoot meristem galls differed in a number of characters, *viz.* oesophagous length, length from anterior end to hemizonid and excretory pore, length from genital primordium end to tail end and the ratios based on body width and oesophagous length, but in combination did not differ in any measurements from those from *A. avenacea*. With respect to oesophagous length and the ratio based on it, differences between the different gall types from *P. monspeliensis* were as great as those between *P. monspeliensis* and *A. avenacea*.

### 6.3.3 Karyotype

Cells at pro-metaphase were observed in the upper part of both the ovary and testis. In both sexes, many cells in the same region were at the same stage of division (males illustrated in Plate 6.2). Chromosomes were small but easier to count in male specimens. At pro-

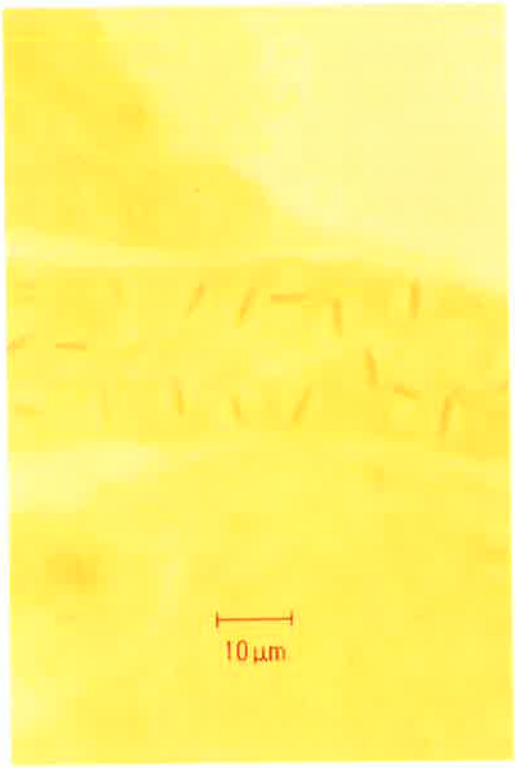



**Plate 6.2** Cells at pro-metaphase in the "germinal zone" (*sensu* Triantaphyllou and Hirschman, 1966) of the testis of *Anguina* sp. parasitic on *Polypogon monspeliensis*

metaphase in both males and females there appeared to be 2n=36 somatic chromosomes. Meiotic divisions in the female spermatotheca were not observed, however, at

pro-metaphase before spermatid formation, there was a haploid chromosome complement of n = 18.

## 6.4 Discussion

### 6.4.1 Allozyme electrophoresis

The separation of the nematode populations into three major electrophoretic groups is consistent with the results obtained by McKay *et al.* (1993). *Anguina funesta* is clearly different from the nematodes associated with *P. monspeliensis* and *A. avenacea*, which are resolved into two groups. However, this study shows that the basis for the separation of the two groups is geographical rather than based on host as suggested by McKay *et al.* (1993). Population PM9, which was collected from northern NSW, had an allozyme profile similar to other populations from NSW even though it was collected from *P. monspeliensis*. This is significant because it demonstrates that, at least in NSW, the nematodes are not host specific. Therefore, for the purposes of this discussion, the populations forming Groups A and B will be referred to as the "southern" and "northern" provenances respectively to reflect their geographical localities.

Estimates of genetic similarity between the electrophoretic groups, based on percentage fixed differences, are almost identical to those found by McKay *et al.* (1993), even though their analysis included only one population from each group. This can be attributed to the marked genetic similarity between populations from each provenance. Thus, a single population is very representative of the genetic variation from the whole provenance.

High intraspecific similarity has already been demonstrated for *A. agrostis* and an undescribed species of *Anguina* from *H. lanatus* (Riley *et al.*, 1988). While only a few populations were examined, they were from a wide geographic range. The same study found that 14 populations of *A. funesta* showed average inter-population differences of only 5%, even though these were collected from throughout its range in Western Australia, South Australia and Victoria. Moreover, fixed allelic differences of up to 11 % between these populations resulted entirely from two loci, only one of which was fixed (Riley *et al.*, 1988). Similarly, the differences between the northern and southern provenances examined here are based on only a few loci, with *Pgk* being the only fully diagnostic locus. Population PM8 collected from western Victoria shares alleles with both provenances even though its allelic profile resembles the southern provenance more closely.

### 6.4.2 Morphological examination

The nematodes examined from *A. avenacea* and *P. monspeliensis* represent the northern and southern provenances respectively, as outlined in the allozyme analysis. The lack of morphological differences between these populations is not surprising given the morphological similarity that occurs within the genus *Anguina*. Brzeski (1981) noted that "Adults of different species of *Anguina* are morphologically indistinguishable although they develop on different hosts in widely separated geographical areas" and other studies have also commented on this similarity (Stynes and Bird, 1980; Southey *et al.*, 1990).

In a review on the "value" of characters claimed to be useful in delineating species and a critical review of the morphological distinctness of some *Anguina* species, Southey *et al.* (1990) used canonical variates analysis to determine if a combination of character measurements could support putative species. They found that length, tail length and De Man's ratio c (length/tail length) had a large influence on the separation of the species they

examined. The present study also found that De Man's ratio c was statistically significantly different between both males and females from the northern and southern provenances.In addition, width, length of the post uterine sac and the ratio of the post uterine sac length to the vulva to anus distance (PUS/Van) also differed between females of both populations regardless of gall type. Interestingly, Southey *et al.* (1990) found that the PUS/Van ratio was one of only two characters that was not significant in the separation of the *Anguina* species they examined and Stynes and Bird (1980) considered this measurement unreliable for *A. funesta* due to the large range of the character.

It has been suggested by the authors of several studies that body measurements and proportions are of limited taxonomic value in the *Anguina*, with dimensions varying according to the method of killing and fixation (Stynes and Bird, 1980a), nutritional differences between hosts (Southey *et al.*, 1990) and state of development (Fisher *et al.*, 1984). Fisher *et al.* (1984) found that the length and width of adult females of *A. funesta* varied by factors of two and three respectively while in the gall. Males appear to be more stable in size and proportions and Southey *et al.* (1990) suggested that more attention should be paid to male characters. The males examined in this study differ from described species in a number of characteristics. The males are dorsally curved (dorsal surface inwards) when heat relaxed, which contrasts to those of *A. agrostis*, *A. australis* and *A. hyparrheniae*, which curve ventrally. The testis is reflexed once, whereas in *A. microlaenae* and *A. danthoniae* the testis is outstretched with no flexures. The spicules of *A. graminis* are longer (40-42 μm compared with 28.3-37.1 μm) and shaped differently from those of the males examined in this study. In *A. spermophaga*, the copulatory bursa expand into two lobes which overlap the tail tip, while those in the present study end about 9.5 – 27 μm from the tail tip. The tail tip of the both sexes are variable in shape but appear

to be lobed, resembling those of *A. tritici*. *Anguina agropyri* and *A. tumefaciens* have acutely pointed tail tips, while that of *A. funesta* is conically pointed.

The process extending distally from the post uterine sac appears to be the remnant of the posterior ovary, in some specimens complete with cap cell. In species of *Anguina* where such a structure has been reported (Southey, 1973; Southey *et al.*, 1990), it consists of a small knob of tissue. The primitive condition in the Tylenchida is generally considered to be two opposed gonads (Allen and Sher, 1967) and the loss of the posterior gonad is a step-wise process with a reduction in size of the posterior ovary followed by complete degeneration leaving the post uterine sac (Triantaphyllou and Hirschmann, 1980). This may indicate that the nematodes investigated in this study are more primitive than other members of the group.

### 6.4.3 Karyotype

The observation that many cells in the upper region of the ovary and testis were undergoing simultaneous development is consistent with the findings of Triantaphyllou and Hirschmann (1966) for *A. tritici*. They describe this as the "germinal zone" and it is the location of mitotic oogonial and spermatogonial division. The meiotic divisions that they describe in the spermatheca of females were not observed but those in the vas deferens of males were. Chromosomes were much easier to count in younger specimens, particularly males, and so the females examined may have been too mature. Males and females appear to have the same chromosome number which, in the absence of any easily recognisable sex chromosomes, indicates that if specialised sex chromosomes exist, they are probably of the XX-XY type (Triantaphyllou, 1971) as suggested for *A. tritici* (Triantaphyllou and Hirschmann, 1980).

The haploid chromosome complement of n = 18 is consistent with the postulation of Triantaphyllou and Hirschmann (1980) that n = 9 is the basic haploid chromosome number for the genus *Anguina* and that other species of *Anguina* may be polyploid or aneuploid forms derived from the basic number. Thus, under their proposal, the nematodes examined from *P. monspeliensis* would be tetraploids. *Anguina agropyri* and *A. graminis* have a haploid chromosome number of n = 9 (Krall and Aomets, 1973; Solov'eva and Gruzdeva, 1977), *A. tritici* has n = 19 (Triantaphyllou and Hirschmann, 1966) and *A. funesta* has n = 22 (Stynes and Bird, 1980). *Anguina agrostis* has been reported to have two different haploid chromosome complements (n = 6 and n = 18) (Krall and Aomets, 1973; Solov'eva and Gruzdeva, 1977) but due to the taxonomic uncertainty that exists for nematodes that are considered to be *A. agrostis*, it is impossible to determine which, if either, is correct for *A. agrostis sensu stricto.*

Closely related genera within the Anguinidae also appear to conform to the proposal of Triantaphyllou and Hirschmann (1980) with *H. graminophila* having a haploid chromosome number of n = 9 while *Heteroanguina caracis* (Solovyova and Krall 1982) Chizhov and Subbotin 1985 has n = 18-19 (Krall and Aomets, 1973). *Mesoanguina millefolii* (Löw 1874) Chizhov and Subbotin 1985 has n = 9-10 and *Mesoanguina plantaginis* (Hirschmann 1977) Chizhov and Subbotin 1985 has n = 27 (Triantaphyllou and Hirschmann,1980). *Subanguina radicola* (Greef 1872) Paramonov 1967 is thought to have n = 9-12 (Triantaphyllou and Hirschmann,1980).

## 6.5 Conclusions

The morphological and allozyme data indicate that the southern and northern provenances are likely to be the same species and thus differences observed in the material studied must simply reflect the variability that occurs between and within populations. Further support

for their conspecificity comes from recent genetic analysis of the first and second rDNA internal transcribed spacer regions. Powers *et al.* (2001) found that nematodes from the two provenances differed by only a single nucleotide substitution in the 553 bases of ITS-1 they examined. Another study looking at both ITS regions including the 5.8S gene also indicated very few character state changes (Subbotin *et al.*, 2002) between the provenances. Furthermore, evidence provided in this chapter and in related publications (Riley *et al.*, 1988; McKay *et al.*, 1993; Powers *et al.*, 2001) shows a clear separation of this nematode from currently described species of *Anguina*.

# Chapter 7

## Biology and Ecology of *Anguina* sp. in *Polypogon monspeliensis*

### 7.1 Introduction

McKay *et al.* (1982) showed that agronomic management techniques could effectively reduce populations of *A. funesta* but only if applied while the nematode was at a particular stage of development. Treatments applied at other times had no effect and some even increased total gall production. These studies were based on knowledge of the biology and ecology of *A. funesta*, both in terms of its life cycle (Price, 1973) and correlations between nematode and host development under natural conditions (McKay *et al.*, 1981). While parallels can be drawn with *A. funesta*, the nematode associated with flood plain staggers has not been studied in detail and occurs in an environment very different from the wheat/sheep belt where *A. funesta* is found. Therefore, an understanding of the biology and ecology of the nematode associated with flood plain staggers (FPS) is crucial to the development of successful management strategies.

This chapter describes the development of natural populations of *Anguina* sp. in *P. monspeliensis* in different seasons and sites in SA. Although a parallel study of *Anguina* sp. in *A. avenacea* in northern NSW would have been useful, it was not possible because of drought conditions over its known geographic range. During the study, the timing of nematode invasion could not be accurately determined and therefore an experiment investigating invasion was carried out. In addition, the impact of the newly discovered shoot meristem galls on both the distribution and level of *R. toxicus* in *P. monspeliensis*

plants at the end of the growing season was investigated using a quantitative enzyme linked immunosorbent assay.

## 7.2 Materials and Methods

### 7.2.1 Ecological field studies

The ecology of *Anguina* sp. ex *P. monspeliensis* was studied by monitoring naturally occurring populations in paddocks with a history of FPS. Observations were made during 1992 and 1993 at Stewart's Range (Site 3) and Cape Jaffa (Site 6: see Chapter 3.6 for site details). Stewart's Range is the area where FPS, locally known as Stewart's Range Syndrome, was first known to occur. Both sites had low lying areas that became inundated during winter and sampling sites were chosen that had high nematode and *P. monspeliensis* populations in the previous year. Sampling began as soon as *P. monspeliensis* seedlings could be identified, about four to six weeks after the opening rains. In 1992, the sites were sampled on a weekly basis, timed from the first week in April (week 1), for ease of comparison with studies of *A. funesta* (McKay *et al.*, 1981), until the pastures became inundated, after which they were sampled once every two weeks. Once the water receded, sites were sampled again at weekly intervals for the remainder of the growing season. In 1993, the sites were sampled at weekly intervals for the entire growing season. The reasons for these sampling strategies will be discussed later in this chapter.

Twenty plants were collected from both sites at each sampling time. For each plant, the number of surviving tillers was counted and the developmental stage of each tiller was assigned to one of the categories listed in Table 7.1. During stage D3, the developmental stage of the inflorescence was categorised according to Table 7.2.

**Table 7.1** List of developmental stages used to categorise the development of *Polypogon monspeliensis* tillers.

| Developmental Stage | Beginning of stage | End of stage |
|---|---|---|
| D1 | vegetative growth | internode elongation |
| D2 | internode elongation | ovary initiation |
| D3 | ovary initiation | inflorescence just emerged from leaf sheaths |
| D4 | inflorescence just emerged from leaf sheaths | inflorescence fully emerged |
| D5 | inflorescence fully emerged | anthesis |
| D6 | anthesis | seed set |
| D7 | post seed set | |

**Table 7.2** List of developmental stages used to categorise the development of *Polypogon monspeliensis* inflorescences. These stages occur during developmental stage D3 (Table 7.1).

| Developmental Stage | Beginning of stage | End of stage |
|---|---|---|
| I1 | stem apex elongation | double ridge stage |
| I2 | double ridge stage | appearance of branch primordia |
| I3 | appearance of branch primordia | appearance of spikelet primordia |
| I4 | appearance of spikelet primordia | appearance of floral primordia |

The development of the nematode was initially assessed by shredding individual tillers, following removal of leaves and roots, using a bank of fine needles (McKay *et al.,* 1981) and placing them in a modified Baermann funnel for 24 hours. Nematodes were then collected and counted (see Chapter 3.5). However, it became necessary to modify the assessment procedure after the discovery of galls in the apical meristem of the plant. To avoid rupturing the galls, tillers were dissected with fine forceps in a modified Doncaster counting dish containing a small amount of water. Successive leaf sheaths were removed and any nematodes found between them carefully eased into the counting dish. The same procedure was used to assess gall formation in the developing inflorescences. All galls were carefully detached and opened in a separate counting dish to determine the number of nematodes initiating each gall, the stage of nematode development and the number of each sex, if able to be determined. The observed frequencies of males and females were averaged for each gall type for the various site/year combinations and then tested for goodness of fit to an hypothesised 1:1 ratio using the chi-square statistic, incorporating Yates correction for continuity (Yates, 1934). Where expected frequencies were less than 5.0, the binomial test was employed for goodness of fit testing to avoid bias (Zar, 1996).

### 7.2.2 Nematode invasion and movement within the plant

Invasion of *P. monspeliensis* by the nematode was studied in more detail in a pot experiment conducted under field conditions at the Waite Campus of the University of Adelaide. One hundred and sixty seedlings were prepared and transplanted into tubes containing either potting compost or flood plain soil (see Chapter 3.7), 80 into each soil type. After allowing the plants to acclimatise to field conditions for five days, two dry nematode seed galls were placed at the base of each plant, just under the soil surface to ensure they would not be dislodged by wind or rain.

Ten plants were randomly collected from each soil type at 1, 3, 5, 7, 11 and 18 days after inoculation. At each sampling time, the numbers of surviving tillers of each plant were counted and each tiller was dissected with fine forceps as described above. The number of nematodes between each leaf sheath was recorded. Five plants from each soil type were collected 25 days after inoculation and the number of galls formed in apical meristems (see below) were counted.

The proportion of each tiller that had been penetrated by the nematodes (number of leaf sheaths penetrated / total leaf sheaths present) were transformed into empirical logits to better satisfy the assumptions of normality and constant variance required by the residual maximum likelihood method of analysis (Long, 1997). This analysis was required to compensate for the lack of balance in the data, *i.e.* different numbers of tillers on different plants at different times. The logit of a proportion r/n (where r = number of leaf sheaths penetrated, and n = total number of sheaths) is defined as

$$\text{logit} = \log \frac{r}{n-r}$$

Proportions equal to 0.5 correspond to an empirical logit of 0 while proportions of 0 or 1 will approach -∞ and +∞ respectively. However, clearly this can not be calculated when r = n or r = 0. In observed data, where r = 0 or r = n have a finite probability, an empirical logit can be obtained by adding 0.5 to both numerator and denominator,

$$\text{empirical logit} = \log \frac{r+0.5}{n-r+0.5}$$

Thus proportions equal to 0.5 still correspond to an empirical logit of 0, but proportions equal to 0 or 1 will be transformed to negative or positive values respectively, dependant on the value of n; the larger n, the larger will be the empirical logit.

Trends between different tillers over time were investigated by fitting smoothed spline models (Wang, 1998). This was only possible for the first three tillers as the others were either present only at the final sampling time or had too few time points to fit splines. The models can be described symbolically as

Empirical logit = Soil + linear(Time) + spline(Time) + Time + Plant + random error

where Time, Plant and random error are all random effects.

Logistic regression (Hosmer and Lemeshow, 1989) was used to test for differences in the number of tillers produced per plant and the number of leaf sheaths produced per tiller between the two soil types.

### 7.2.3 Quantification and distribution of *Rathayibacter toxicus*

#### *Collection and preparation of test samples*

To determine the distribution and level of *R. toxicus* colonisation in *P. monspeliensis* at the end of the growing season, 25 plants were collected randomly from Stewart's Range using a 250 x 500 mm quadrat. For each plant, the number of surviving tillers was counted and the length, weight and developmental stage recorded for each tiller (as above). Each tiller was visually assessed for the presence or absence of *R. toxicus* before being placed into a small plastic bag and crushed with a roller to release bacteria in galls or between leaf sheaths. After crushing, 3 ml of cold phosphate buffered saline containing 0.05% Tween 20

(PBS-T; Clark and Adams, 1977) was added to each bag. The bags were heat sealed and the contents mixed thoroughly, then stood at room temperature for 30 min with occasional mixing. The bags were then suspended from one corner for 10 min to allow the plant debris to settle before a 100 µl subsample of the supernatent was taken. The subsample was diluted with 700 µl of PBS-T and frozen.

***Preparation and assessment of standards***

A commercial quantitative double antibody sandwich enzyme linked immunosorbent assay (ELISA) for screening *L. rigidum* samples for *R. toxicus* is available from the South Australian Research and Development Institute (SARDI). The standards used in their assay are prepared by combining a known number of bacterially colonised *A. funesta* seed galls with the extract from a known weight of *L. rigidum* leaf tissue, which is in effect a control for the plant tissue present in the test sample. However, I was concerned that these standards were not truly representative of the *P. monspeliensis* samples to be tested, both in terms of possible plant-specific reactions and also in the volume of plant extract and buffer used in the preparation of the standards, which was derived from the weight of test sample to be assayed. Therefore, I prepared fresh standards using extracts from *P. monspeliensis* according to the following method.

Dried bacterial seed galls collected from *P. monspeliensis* and *L. rigidum*, of similar size and free of plant material, were placed into small plastic bags so that each bag contained either 1, 4, 16, 64, 256 or 1, 4, 16, 64, 256, 1024 galls respectively. After adding 1 ml of PBS-T to each bag and incubating overnight at 4°C, a roller was used to crush the galls within the bags. The resultant slurry was allowed to stand for 30 min at room temperature before 0.25 g of crushed *P. monspeliensis* leaf and seed head, the mean weight of the

samples to be tested, was added to each bag and the volume of each made up to 3 ml. After heat sealing and mixing the contents thoroughly, the bags were allowed to stand for 30 min at room temperature. A control containing no *R. toxicus* was prepared by adding 3 ml of PBS-T to 0.25g crushed *P. monspeliensis,* grown in the absence of both the nematode and bacterium. The bags were then suspended for 10 min before a 100 µl subsample was removed and diluted with 700 µl of PBS-T in a 1ml tube. The tubes were arranged in ascending order of bacterial concentration, with the control as the first tube. All tubes were capped and stored at -20°C, as was the remainder of the sample. Standards prepared using *L. rigidum* galls and *P. monspeliensis* galls will be referred to as the L-standards and P-standards respectively.

The optimum dilution for the standards, and hence samples, to allow quantification over the range of bacterial concentrations under study was determined by a series of preliminary assays. An eight-fold dilution series of each bacterial concentration for both the L-standards and P-standards was prepared in PBS-T, from 1:25 to $1:6.6x10^6$. These values were selected to incorporate the dilution factors used by the SARDI ARGT testing service. The serial dilutions were evaluated following the protocol outlined below.

***ELISA methodolgy***

The following protocol, a modification of the methods of Clark and Adams (1977), uses polyclonal antibodies (Immunoglobulin G fraction) raised in rabbits against CS14, the type strain of *R. toxicus* .

Polystyrene microtitre plate (Nunc Maxisorp, F96) wells were coated with trapping antibody by incubating 100 µl of antibody (1mg / ml), which had been diluted 1:5000 in

carbonate coating buffer (Clark and Adams, 1977), overnight at 4°C in a humid chamber. Nonadsorbed antibody was removed by washing the wells three times with cold PBS-T, allowing them to soak for 5 min each time. At this and each subsequent washing step, the plate was inverted and tapped firmly onto a folded cloth after the last wash, to completely empty the wells.

One hundred microlitres of each test sample, which had been diluted 1:1600 (see 7.3.6) in PBS-T, was pipetted into a single well in columns 2, 3, 4 or 5 (32 samples per plate). After further diluting the samples with PBS-T to 1: $1x10^5$, 100 µl was pipetted into single wells in columns 8, 9, 10 or 11 in the same orientation as the 1:1600 dilutions. Similarly, 100 µl of either the P-standards or L-standards, diluted 1:1600 and 1: $1x10^5$ in PBS-T, were loaded into columns 6 and 7 respectively in order of increasing bacterial concentration from row B onwards. One hundred microlitres of PBS-T was pipetted into the first well of both column 6 and 7.

After incubating at 25°C for 4 hr in a humid chamber, plates were washed as described above, taking care not to cross contaminate wells with antigen from adjoining wells. One hundred microlitres of antibody, conjugated with alkaline phosphatase and diluted 1:1000 in PBS-T, was added to each well and the plates incubated at 4°C overnight in a humid container. The conjugate was prepared with the same immunoglobulin fraction used to coat the wells, using the gluteraldehyde method of Clark and Adams (1977), except that unconjugated protein was removed after the final dialysis by running on a Sephacryl S300 column (Pharmacia).

After washing the plates once more, 100 µl of 1mg / ml p-nitrophenyl phosphate, prepared by dissolving phosphatase substrate tablets (Sigma, 104 -105) in substrate buffer (Clark

and Adams, 1977), was added to each well and incubated at room temperature. Absorbance was measured at 405 nm using a Titertek Plus Multiskan 2 plate reader (ICN Flow Laboratories). Plates were considered fully developed when the optical density ($OD_{405}$) of the well with the highest concentration of bacteria in column 7, had reached 1.6. To stop the reaction, 50 µl of 3M NaOH was added to each well. The $OD_{405}$ readings for each plate were downloaded from the plate reader for further analysis.

***Estimation of the level of bacterial contamination in field samples***

Standard curves were plotted for each plate using the $OD_{405}$ values for both the 1:1600 and 1: $1x10^5$ dilutions of the standards (See Chapter 7.3.6). The amount of *R. toxicus* in each test sample was quantified in terms of "bacterial gall equivalents" by linear interpolation of the 1: $1x10^5$ standard curve (Riley and McKay, 1991a) and adjusted relative to the initial mass of the sample. The process was automated using a Microsoft Excel macro which I developed from a program written by Dr Ian Riley. It was necessary to modify the original program to include the additional standard used. The commented macro listing is included in Appendix 2.

## 7.3 Results

### 7.3.1 Climatic conditions and host dynamics

In 1992, the opening rains occurred in week 1 and were followed by average rainfall until the end of the season, when rainfall was very much above average (Australian Bureau of Meterology). At both Stewart's Range (Figure 7.1) and Cape Jaffa (Figure 7.2), flooding was first observed in week 14. Water depth at Stewart's Range reached a maximum of 450 mm from week 22 to week 28 and the level had not dropped appreciably by the last

**Figure 7.1** Population dynamics of *Polypogon monspeliensis* and *Anguina* sp. in relation to rainfall and flooding events at Stewart's Range in 1992. ■ water depth, ▲ tillers/plant, ● juvenile nematodes/tiller, ■ shoot meristem galls/tiller and ▼ seed galls/tiller.

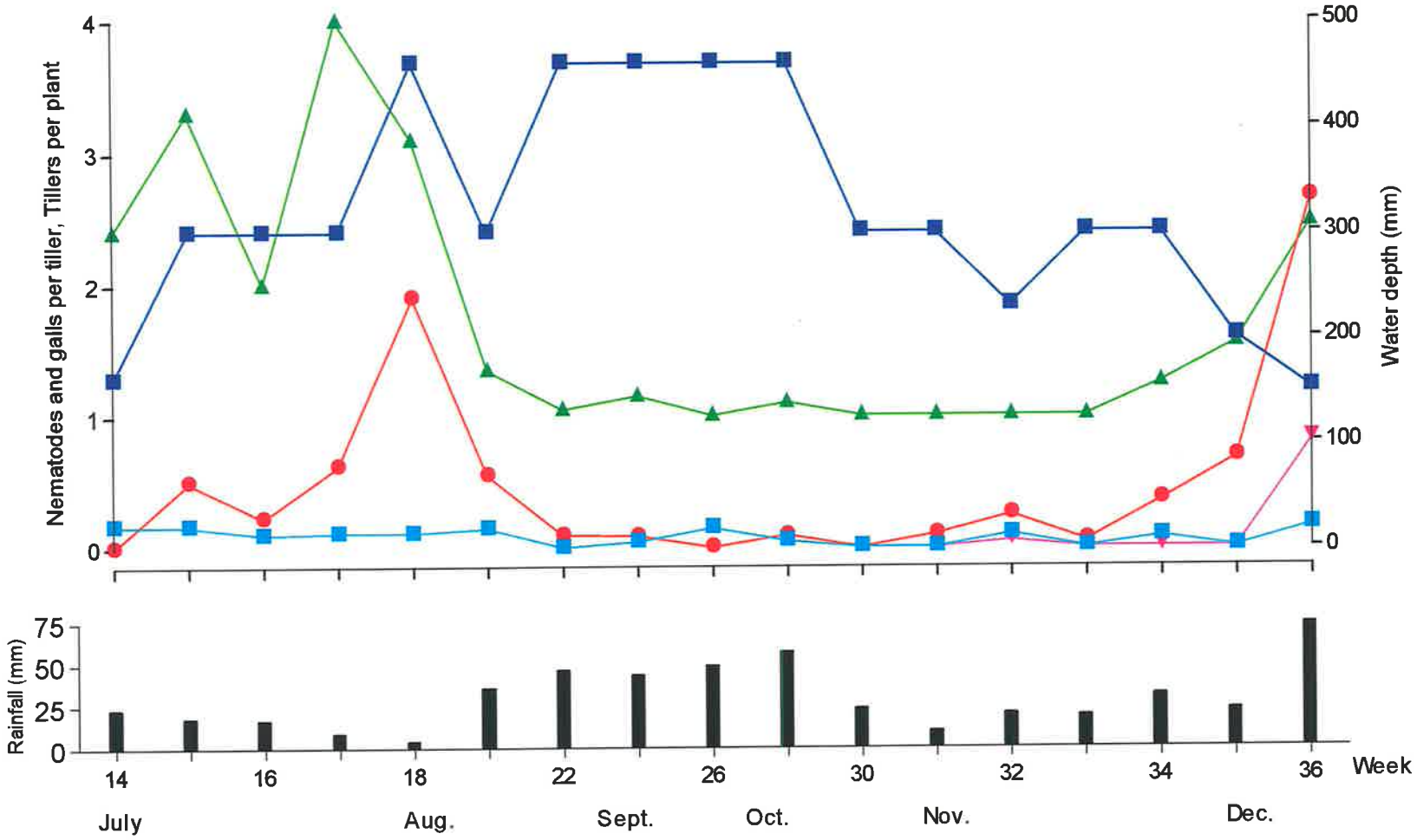
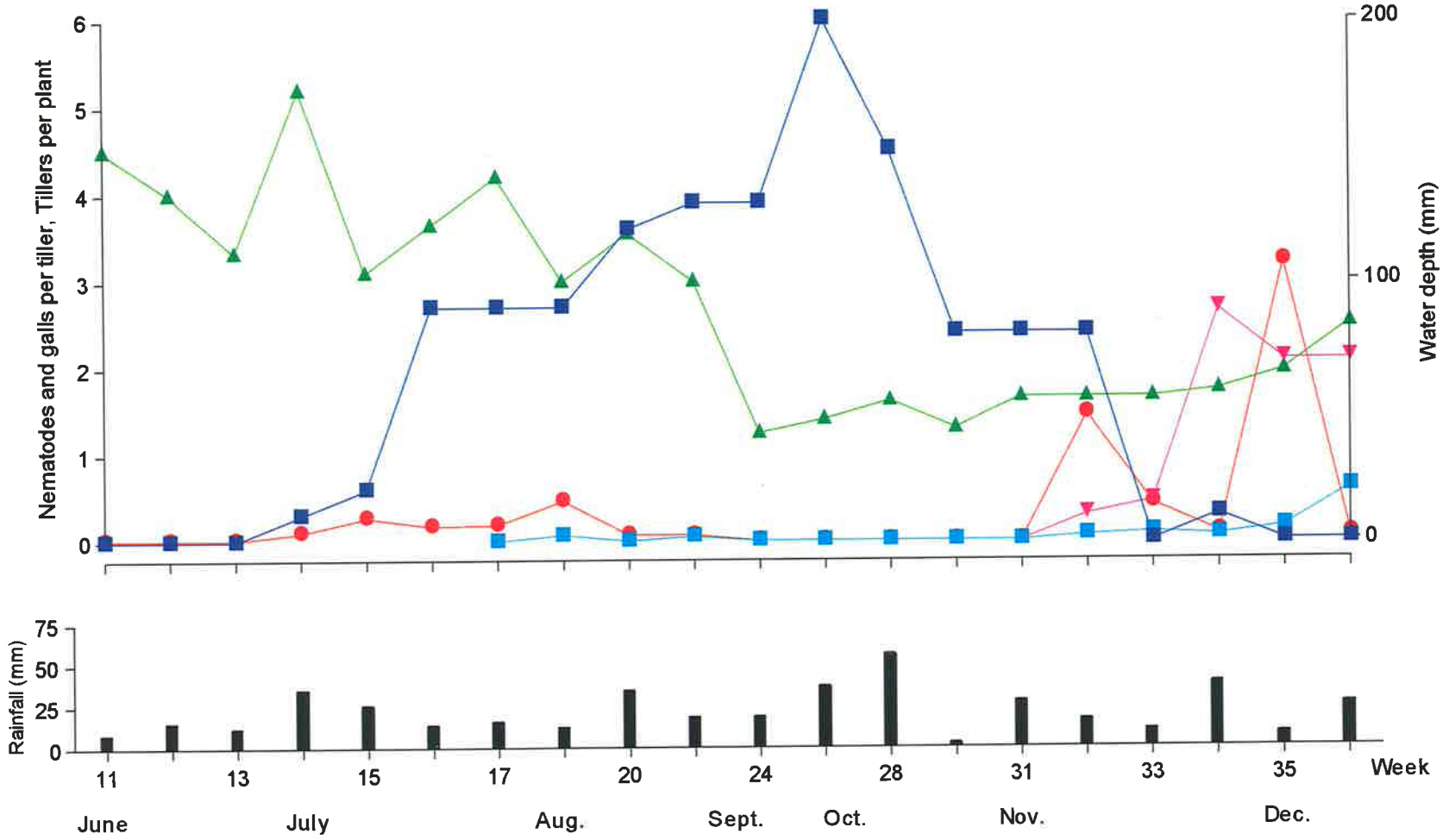


**Figure 7.2** Population dynamics of *Polypogon monspeliensis* and *Anguina* sp. in relation to rainfall and flooding events at Cape Jaffa in 1992. ■ water depth, ▲ tillers/plant, ● juvenile nematodes/tiller, ■ shoot meristem galls/tiller and ▼ seed galls/tiller.



sampling date (week 36). The average number of tillers per plant increased to 4 at week 17, then declined to 1 at week 22 before begining to increase again at week 34. At Cape Jaffa, the maximum water depth of 200 mm occurred at week 26 but in contrast to the Stewart's Range site, there was no surface water by week 34. The average number of tillers per plant increased to 5.2 at week 14, declined to 1.3 at week 24 and slowly began to increase once more.

In 1993, the opening rains were delayed until week 11. Rainfall for the rest of the season was below average to average and inconsistent (Australian Bureau of Meterology). At each site, there were two separate flooding events, the first between weeks 19 and 22 at both sites and the second between weeks 24 and 30 at Stewart's Range (Figure 7.3) and between weeks 24 and 28 at Cape Jaffa (Figure 7.4). At Stewart's Range, water depth peaked at 260 mm in week 25 but was greater than 80 mm only at three sampling dates. The average number of tillers per plant decreased from 3 in week 17 to 1.1 in week 22. For the next seven weeks, average tiller number fluctuated between 1 and 1.9 before slowly increasing to 2 at the end of the season (Figure 7.3). At Cape Jaffa, water depth also peaked in week 25 at 120 mm. The average number of tillers per plant decreased from 2.2 to 1 at week 22, fluctuated between 1 and 1.3 until week 28 and then began to increase (Figure 7.4).

When growing at the maximum water levels indicated above, *P. monspeliensis* plants generally consisted of a single, very thin tiller which was unable to support its weight when removed from the water. In the water, the tiller was supported by a single leaf which floated on the water surface. In addition, adventitious roots were observed growing into the water from nodes above the soil surface.

**Figure 7.3** Population dynamics of *Polypogon monspeliensis* and *Anguina* sp. in relation to rainfall and flooding events at Stewart's Range in 1993. ■ water depth, ▲ tillers/plant, ● juvenile nematodes/tiller, ■ shoot meristem galls/tiller and ▼ seed galls/tiller.

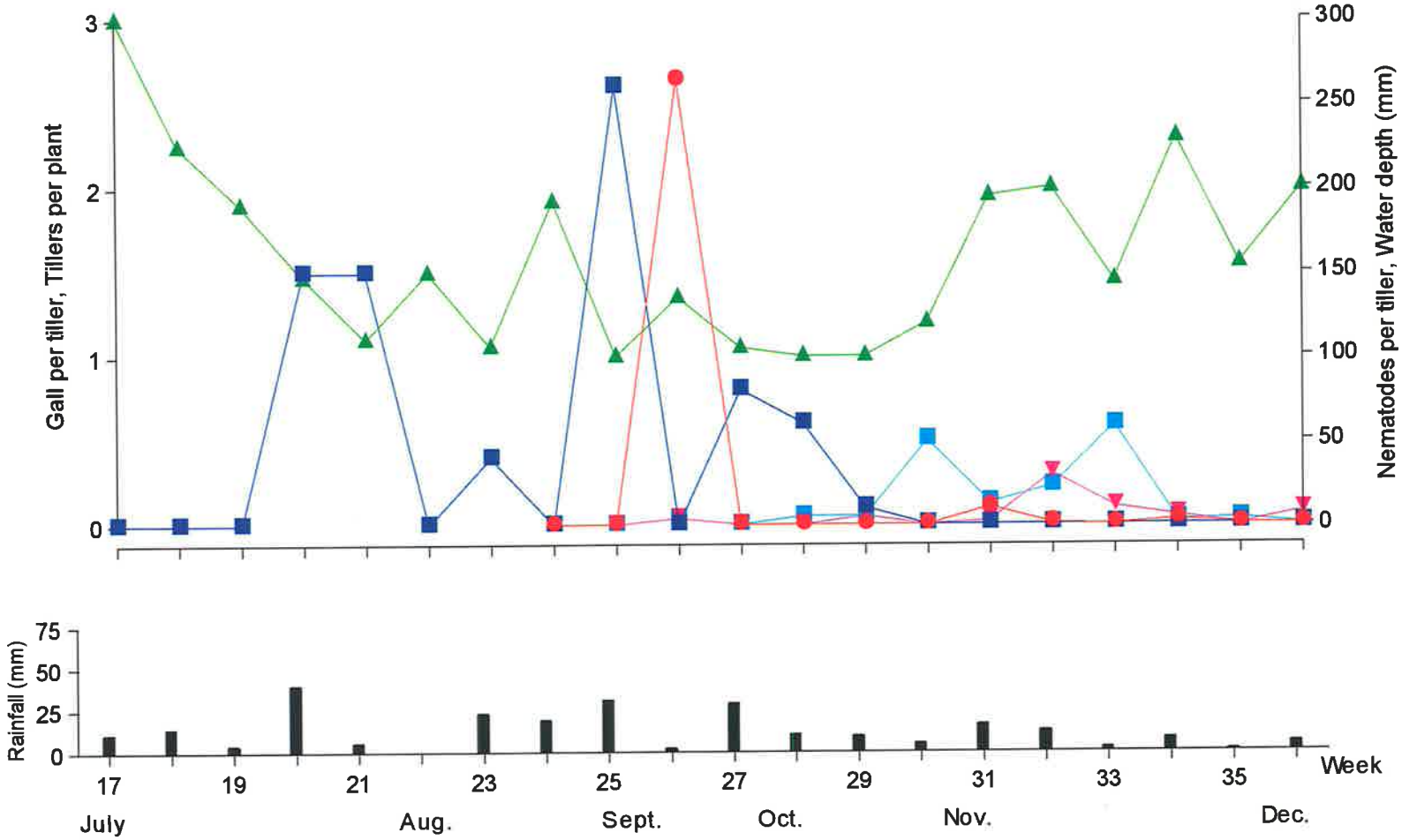
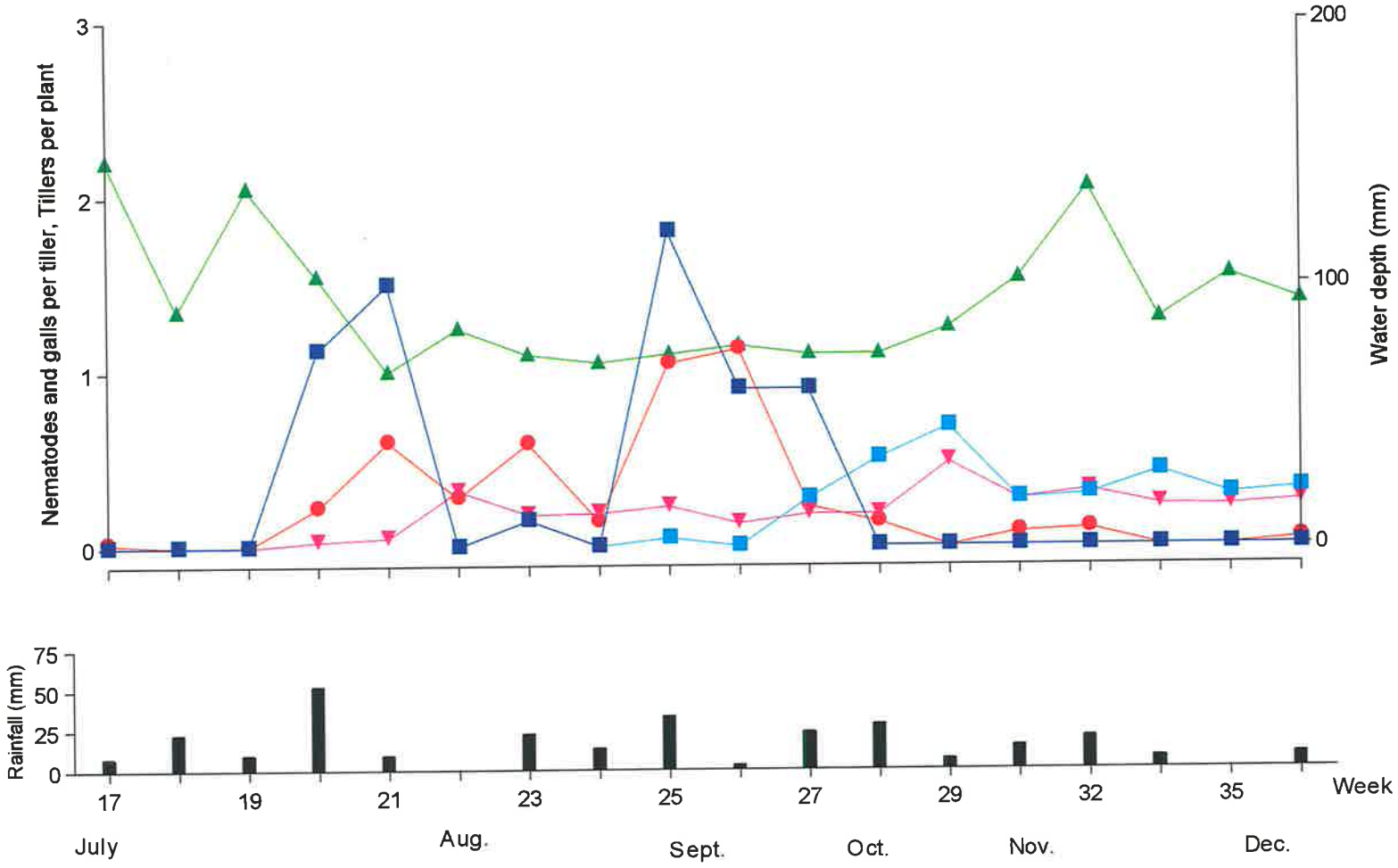


**Figure 7.4** Population dynamics of *Polypogon monspeliensis* and *Anguina* sp. in relation to rainfall and flooding events at Cape Jaffa in 1993. ■ water depth, ▲ tillers/plant, ● juvenile nematodes/tiller, ■ shoot meristem galls/tiller and ▼ seed galls/tiller.



### 7.3.2 Nematode movement into tillers in the field

At Stewart's Range in 1992, tillers containing J2s were found in week 15, 14 weeks after the opening rains (Figure 7.1). The number of J2s per tiller increased to an average of 1.9 per tiller at week 18, declined to almost zero between weeks 24 and 30 and increased once more to 2.7 per tiller by week 36. At Cape Jaffa in 1992, J2s were found in tillers from the first sampling period in week 11. The average number per tiller increased to 0.5 at week 18, declined to 0 between weeks 24 and 31, before increasing again toward the end of the season (Figure 7.2).

At Stewart's Range in 1993, the first J2s were detected in tillers in week 25, 14 weeks after the opening rains (Figure 7.3). Nematode numbers remained low throughout the season except for week 26 where an average of 264 J2s were found per tiller. As in 1992, J2s were found from the first sampling period in week 17, 6 weeks after the opening rains, at Cape Jaffa (Figure 7.4). The maximum number of J2s found per tiller was also at week 26.

### 7.3.3 Gall production

Several types of nematode galls were observed during the study and I will deal with each separately.

#### *Shoot Meristem Galls*

In 1992, galls were found in *P. monspeliensis* samples collected in week 14 from Stewart's Range and week 18 from Cape Jaffa and subsequently were found throughout the year (Figures 7.1 and 7.2). The plants had one to five tillers up to 30 mm high and were actively growing. Green, ovoid galls were observed amongst the tillers on the crown of some of these plants (Plates 7.1 and 7.2). The galls were firmly attached and could not be removed

**Plate 7.1** Nematode gall initiated in the shoot apical meristem of a *Polypogon monspeliensis* tiller. The gall is 3 mm in length

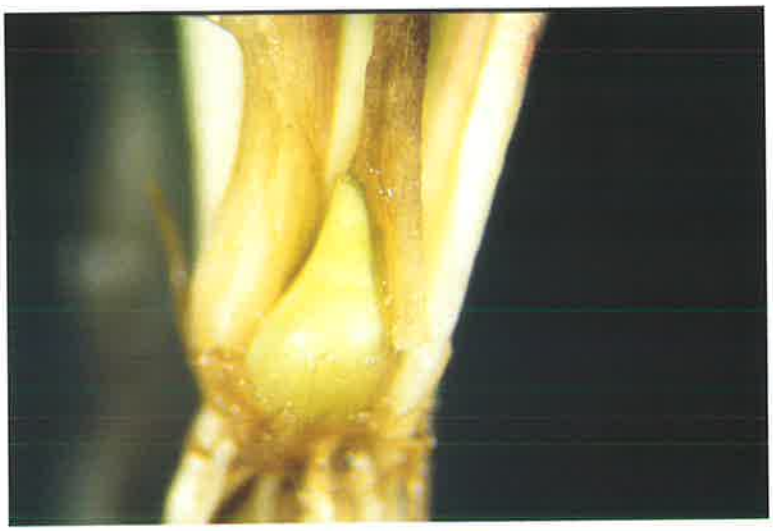


**Plate 7.2** Closeup of the nematode gall depicted in Plate 7.1

without rupturing, unless cut free with a scalpel. Solitary galls with no accompanying tillers were also observed and were anchored to the soil by an apparently healthy root system. Some galls were surrounded by the necrotic remains of leaf sheaths. When galls were dissected, each was found to contain either adult nematodes, eggs and many J2s, or a yellow bacterium, later identified as *R. toxicus*, in a cavity within the gall.

Apparently healthy tillers with varying degrees of swelling in their bases were also present in the samples. Dissection of these tillers revealed the presence of a gall in the innermost leaf sheath. The youngest leaf, still within the outer sheaths and generally surrounding the gall, was often twisted and distorted (Plate 7.3). Galls contained adult nematodes and/or eggs and J2s or *R. toxicus*. Similar galls were also found in tillers with no obvious basal swelling, except that these were much smaller and contained mostly pre-adult nematodes. Very small galls contained J2s with enlarged genital primordia.

In weeks 24 and 22, at Stewart's Range and Cape Jaffa respectively, galls were found in tillers undergoing internode elongation. Single galls were situated in the innermost leaf sheaths at various heights above the crown. The galls resembled those found on the crown and contained nematodes at various stages of development and/or *R. toxicus*. When the galls were large, they were visible as a pronounced swelling in the tiller.

From week 26 at Stewart's Range and week 33 at Cape Jaffa, samples contained galls that had begun to decay. Generally, part of the gall was necrotic and very few nematodes were present in the cavity. However, in week 32 at both sites, newly initiated galls were observed in the base of tillers and subsequently in tillers undergoing internode elongation. Lateral buds forming at the internodes were also replaced by nematode galls. All galls contained nematodes at various developmental stages and/or *R. toxicus*. At the end of the

**Plate 7.3** Distorted *Polypogon monspeliensis* leaf sheath surrounding a nematode gall initiated in the shoot apical meristem (not visible)

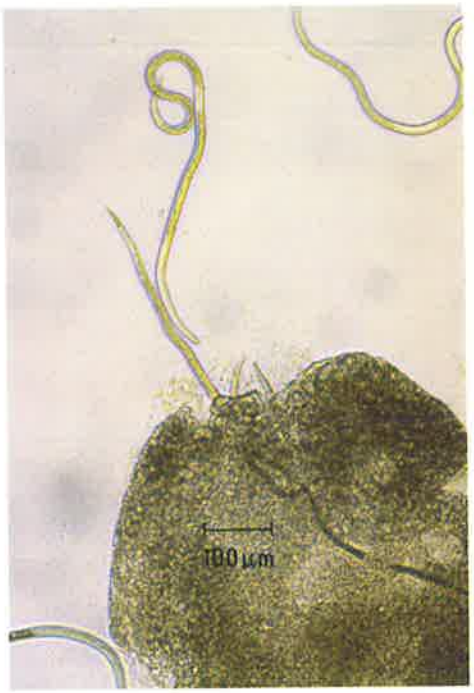



**Plate 7.4** Invasive J2 nematodes protruding from a cavity of a *Polypogon monspeliensis* shoot apical meristem

season brown galls containing anhydrobiotic J2 nematodes were observed on senesced plants. Galls colonised by *R. toxicus* were observed throughout the year and accounted for 38% and 48% of galls examined from Stewart's Range and Cape Jaffa respectively.

In 1993, galls were first observed in week 26 and week 20 at Stewart's Range and Cape Jaffa respectively. Once again galls were sampled throughout the season but the numbers sampled per tiller were greater toward the end of the season than in 1992 (Figures 7.3 and 7.4). Galls were found in tillers undergoing internode elongation and in lateral buds. However, decaying galls were not observed. Seven per cent of the total galls examined from Stewart's range were colonised by *R. toxicus* compared to 37% from Cape Jaffa.

Observations of galls at various developmental stages throughout both seasons indicate that the galls originate in the vicinity of the shoot apical meristem and I will refer to the galls described in this section as "Shoot Meristem Galls" (SMG). High numbers of J2s were observed aggregating around both the youngest leaf surrounding the apical meristem and the meristem itself before any changes in the plant. The first evidence of gall initiation was a shallow depression in the shoot apical meristem, often with the tails of invading J2 nematodes protruding from it (Plate 7.4). Enlargement of the host tissue led to the formation of a gall comprising a cavity with a distinct opening (Plate 7.5). The J2s which occupied the cavity began to increase in size and their genital primordia enlarged. The number of nematodes initiating SMG, for both sites and years, are presented in Figure 7.5. The observed number of male and female nematodes in SMG did not differ significantly from 1:1 ratio for each site/year combination (Table 7.3).

As galls increased in size they became more elongate and the opening at the apex narrowed (Plate 7.6). While the shape of the galls did not change appreciably once they had reached

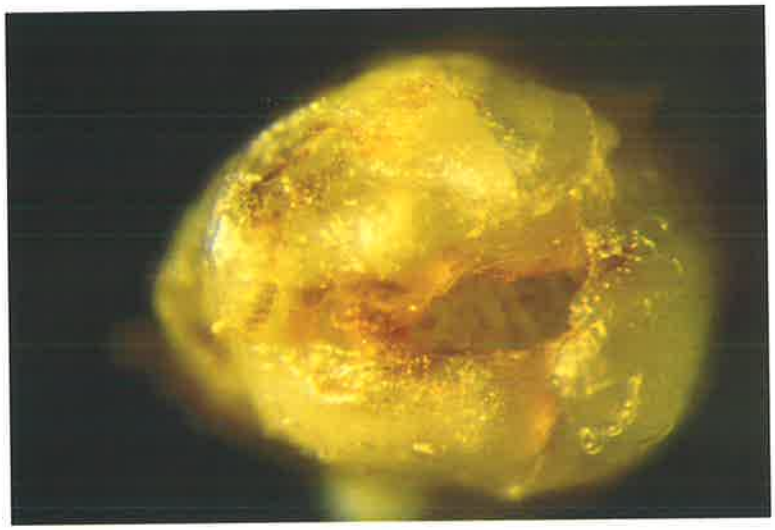


**Plate 7.5** Nematode gall recently initiated in a *Polypogon monspeliensis* shoot apical meristem with a distinct opening and cavity

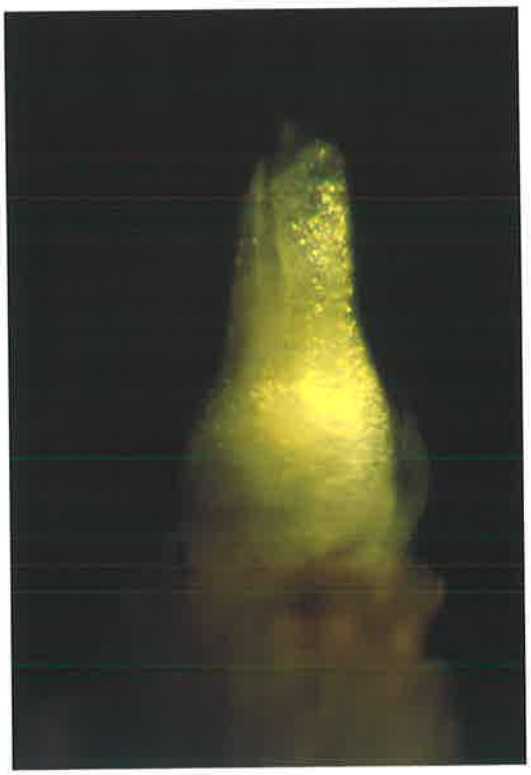
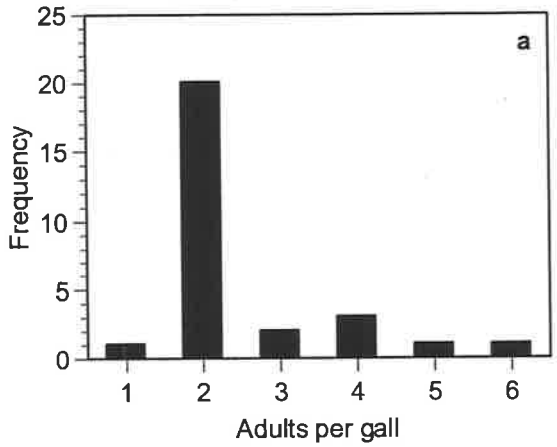
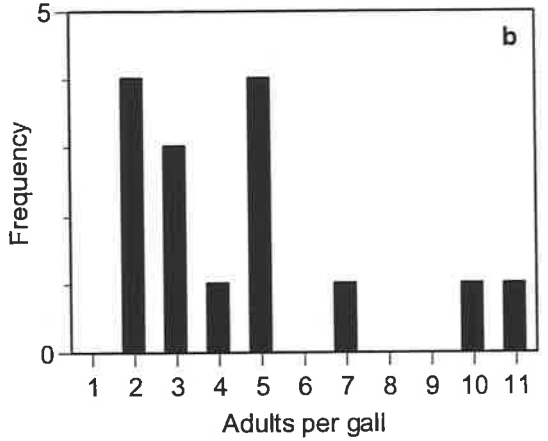


**Plate 7.6** Nematode gall initiated in a *Polypogon monspeliensis* shoot apical meristem. This gall is more elongate and has a narrower opening than the one depicted in Plate 7.5

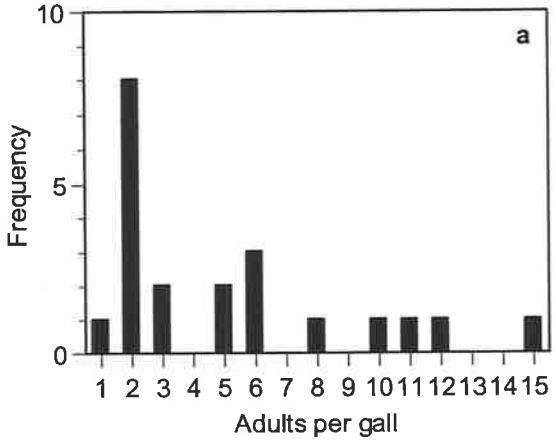
**Figures 7.5** Number of adult nematodes per shoot meristem gall at (a) Stewart's Range and (b) Cape Jaffa in 1992 and 1993 respectively

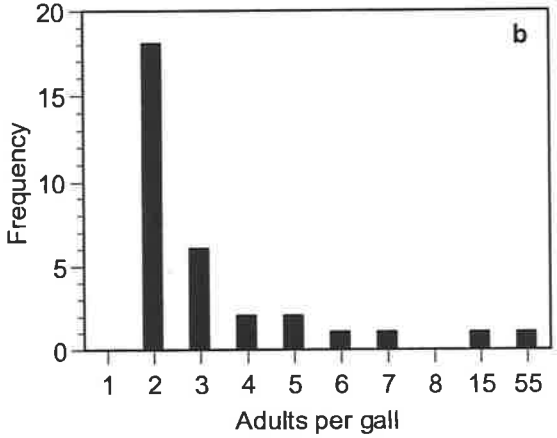
1992




1993



this stage, they continued to increase in size as the nematodes within moulted through three stages to adults and began to lay eggs. Eggs at various stages of embryogenesis were observed within galls. Nematodes hatched as second stage juveniles, undergoing the first moult in the egg. In general, by the time 50% of eggs had hatched, the adults had died and begun to decay. J2s that apparently had been feeding were easily distinguished from recently hatched J2s as their intestines were much darker.

**Table 7.3** Corrected chi-square values from goodness of fit testing of the observed numbers of male and female nematodes in different gall types to an hypothesised 1:1 ratio. The number of observations is in parentheses.

| Site | Shoot Meristem Galls | Seed Galls | Branch Galls |
|---|---|---|---|
| Stewart's Range 1992 | 0.377 (69) | 1[1] (7) | 0.36[1] (5) |
| Cape Jaffa 1992 | 0.377 (69) | 0.252 (258) | 0.4 (25) |
| Stewart's Range 1993 | 1.589 (107) | 0.293 (58) | -[2] (1) |
| Cape Jaffa 1993 | 0.322 (155) | 0.071 (14) | 1[1] (7) |

[1] Values from the binomial test
[2] Not calculated

***Seed galls***

In 1992, the first seed galls were initiated in week 32 at both sites and the number of galls reached a maximum of 2.6 per tiller at week 36 at Stewart's Range and 2.7 per tiller in week 34 at Cape Jaffa. Each gall was initiated by an average of 2.3 and 2.6 nematodes

respectively (Figure 7.6) and the ratio of males to females did not differ significantly from 1:1 (Table 7.3). Eggs were first observed at week 36 at Stewart's Range and some galls already contained hatched J2s. At Cape Jaffa, nematode eggs were first observed at week 34 and hatched J2s at week 35. Twenty percent of the total galls examined from Stewart's Range were colonised by *R. toxicus*, compared to 35% from Cape Jaffa.

In 1993, seed galls were first initiated at week 26 at Stewart's Range and at week 25 at Cape Jaffa. An average of 4.8 and 2.0 nematodes respectively initiated each gall (Figure 7.6). Once again the observed ratio of males to females conformed to the hypothesised 1:1 ratio (Table 7.3). The first galls containing eggs were observed in weeks 32 and 31 at Stewart's Range and Cape Jaffa respectively and some galls contained hatched J2s. While no bacterially colonised seed galls were collected from Stewart's Range, 67% of seed galls from Cape Jaffa were colonised by *R. toxicus*.

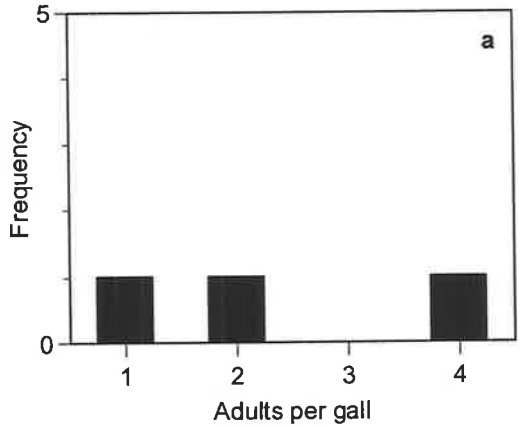
***Branch galls***

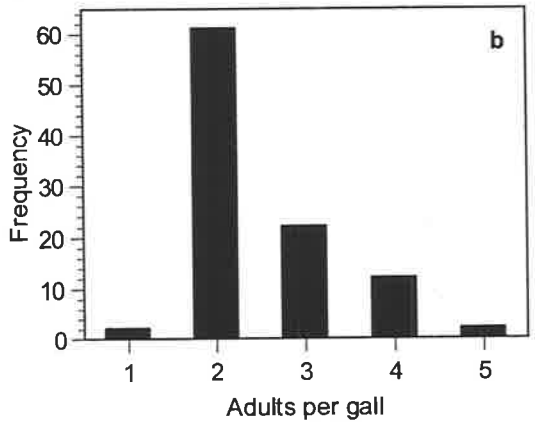
During 1992, branch galls were first observed in weeks 36 and 32 at Stewart's Range and Cape Jaffa respectively. The galls generally resembled SMG and were attached to either the rachis or its first and second order branches. Galls contained nematodes at various stages of development. *R. toxicus* had colonised 41% and 19% of the galls from Stewart's Range and Cape Jaffa respectively.

In 1993, branch galls were first observed in weeks 33 and 32 at Stewart's Range and Cape Jaffa respectively. No galls sampled from Stewart's Range were colonised by *R. toxicus* compared to 57% from Cape Jaffa. The numbers of nematodes initiating branch galls for each site/year combination are presented in Figure 7.7. The male:female ratio for each combination did not differ significantly from the hypothesised 1:1 ratio (Table 7.3).

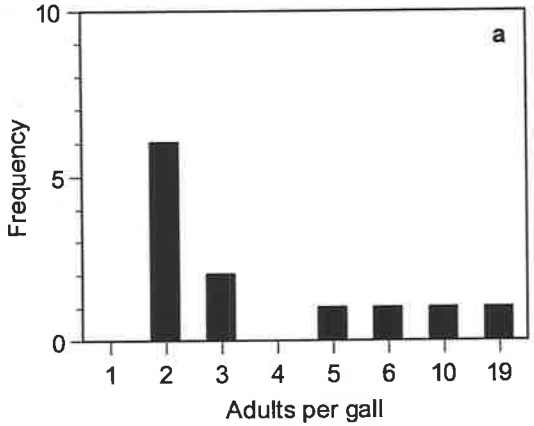
**Figures 7.6** Number of adult nematodes per seed gall at (a) Stewart's Range and (b) Cape Jaffa in 1992 and 1993 respectively

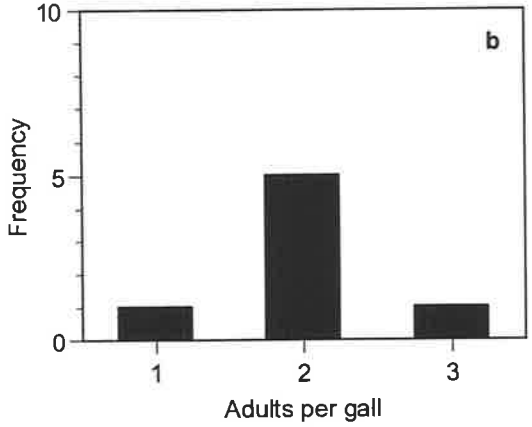
1992



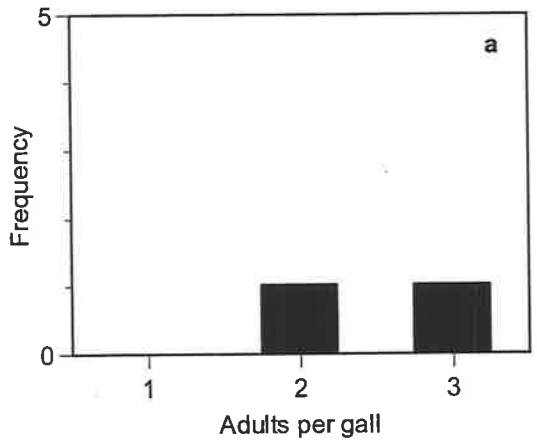

1993

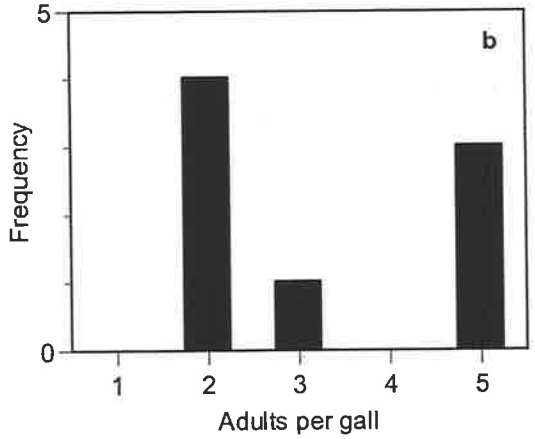


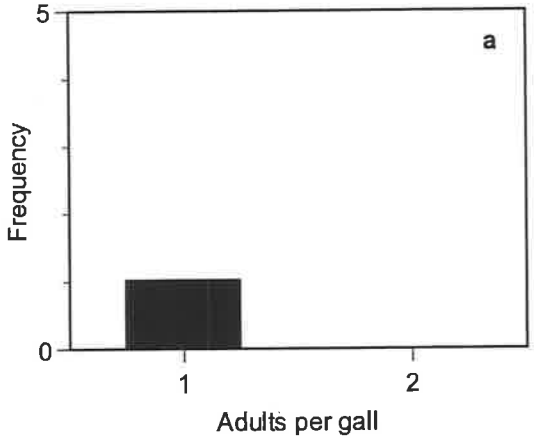
**Figures 7.7** Number of adult nematodes per branch gall at (a) Stewart's Range and (b) Cape Jaffa in 1992 and 1993 respectively

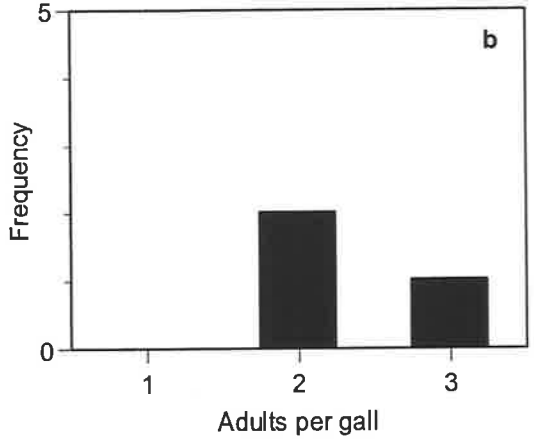
1992




1993



### 7.3.4 Association between nematode and tiller development

Table 7.4 shows the number of SMG, seed galls and branch galls observed containing nematodes at particular developmental stages at various tiller developmental stages. There was a large amount of variation in the number of all types of galls sampled between sites and years and therefore gall counts are composites across both sites and years.

SMG were found only in tillers before the shoot apical meristem became reproductive. Even though galls were observed after internode elongation, none were found after the shoot apical meristem had reached the double ridge stage. The galls sampled contained nematodes at all stages of development before and after elongation of the internodes.

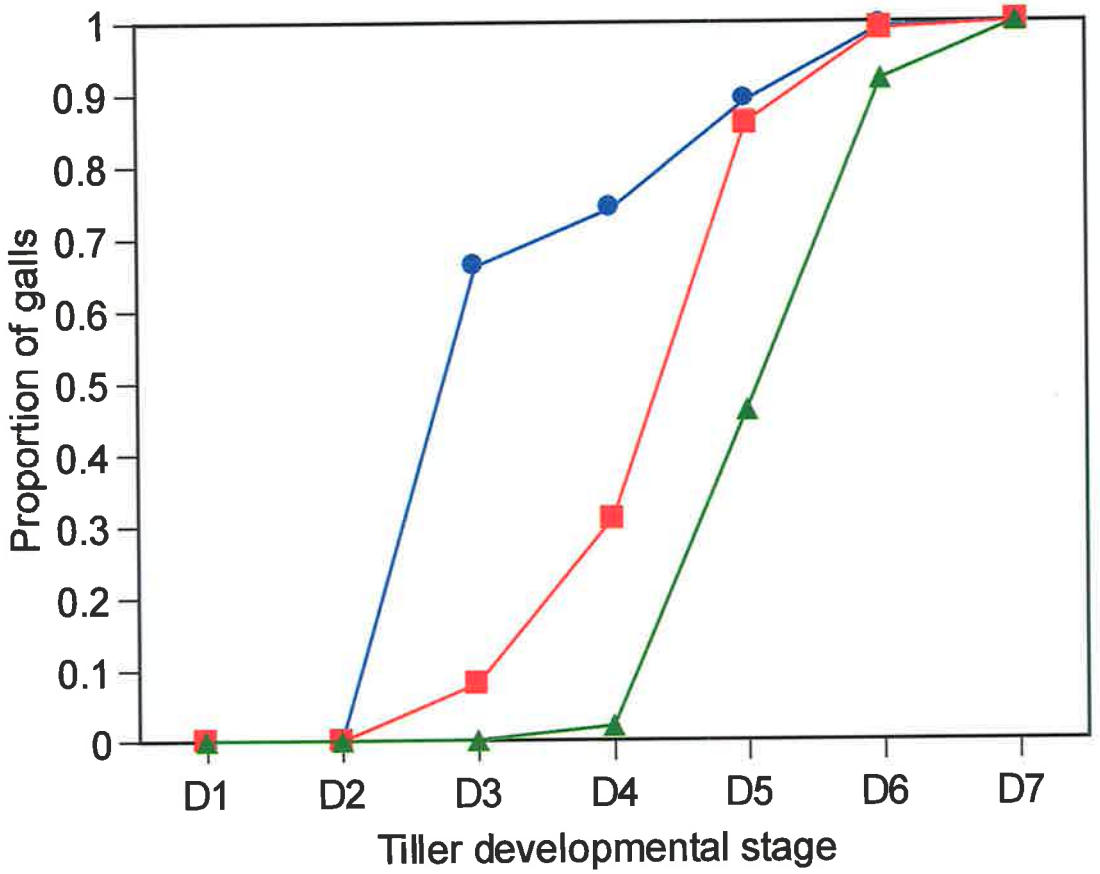
Branch galls were found only after the shoot apical meristem had become reproductive but were formed before the spikelet primordia had developed. While correlations between nematode development within the galls and tiller development are difficult to make, due to the low number of branch galls found during this study, the data indicate that most branch galls contain hatched J2s by the time the inflorescence is fully emerged.

Seed galls were only initiated after the spikelet primordia had developed. Examination of the cumulative relative frequencies of each nematode developmental class at particular tiller developmental stages (Figure 7.8) indicates that the development of the nematode within seed galls can be predicted by tiller development. Just before the inflorescence began to emerge from the boot, the majority of galls contained adults or sub-adult stages with egg laying only in the most advanced galls. As the inflorescence continued to emerge, the probability that galls contained eggs increased markedly, with J2s hatching in only the most advanced galls. By the time the inflorescence was fully emerged, the probability that

**Table 7.4** Number of shoot meristem galls, seed galls and branch galls, and nematodes at particular stages of development from tillers at various developmental stages. Tiller developmental stages are listed in Table 7.1.

| Tiller Developmental Stage | Number of Galls | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Shoot Meristem galls | | | Seed Galls | | | Branch Galls | | |
| | Without eggs | With eggs | With hatched J2s | Without eggs | With eggs | With hatched J2s | Without eggs | With eggs | With hatched J2s |
| D1 | 47 | 18 | 57 | 0 | 0 | 0 | 0 | 0 | 0 |
| D2 | 50 | 20 | 13 | 0 | 0 | 0 | 8 | 2 | 0 |
| D3 | 0 | 0 | 0 | 82 | 6 | 0 | 1 | 1 | 3 |
| D4 | 0 | 0 | 0 | 10 | 16 | 1 | 2 | 3 | 2 |
| D5 | 0 | 0 | 0 | 18 | 39 | 17 | 1 | 0 | 15 |
| D6 | 0 | 0 | 0 | 14 | 9 | 18 | 0 | 0 | 1 |
| D7 | 0 | 0 | 0 | 1 | 1 | 3 | 0 | 0 | 1 |

**Figure 7.8** Cumulative proportion of seed galls containing ● adult nematodes, ■ eggs and ▲ hatched J2s at various tiller developmental stages. Tiller developmental stages are given in Table 7.1.



galls contained eggs had risen to 0.86 and 50% of galls were expected to contain hatched J2s. By anthesis, over 90% of the galls sampled contained hatched J2s.

### 7.3.5 Nematode invasion and movement within the plant

Twenty four hours after inoculation, the 10-day old *P. monspeliensis* plants consisted of a coleoptile surrounding a single tiller of four leaf sheaths. Second stage juvenile nematodes were found in 50% of the plants (four from the potting compost, six from the flood plain soil). Most of the nematodes were concentrated between the coleoptile and first leaf sheath (position A) but a few were found between the first and second leaf sheaths (position B) and between the second and third sheaths (position C) (Table 7.5). Two days later, all but one of the plants sampled contained J2s. The majority of the nematodes were aggregated at position B. The second tiller, comprising two leaf sheaths, had developed and in one case J2s were found between the first and second sheaths of this tiller. By day five, the coleoptiles were almost rotted away and while the majority of the J2s were still located at position B, a few individuals were clustered around the shoot apical meristem of the second tiller (Table 7.5). Six days later, J2s were found at all positions in the second and successive tillers and were progressively moving in towards the more central tissues of the first (Figure 7.9). In only one plant, grown in flood plain soil, had J2s reached the shoot apical meristem of the first tiller by this time. This plant was heavily infested and had significantly more nematodes ($P < 0.01$) in its first tiller than the total number of nematodes in any other plant. By day 18, the plants consisted of between 4-8 tillers and J2s were aggregated around the shoot apical meristems of all the tillers examined.

Twenty five days after inoculation, all but one plant had 1- 4 tillers showing the initial symptoms of shoot meristem gall formation. In one plant, two shoot meristem galls had

**Table 7.5** Number of second stage juvenile nematodes recovered from various positions within *P. monspeliensis* grown in (a) potting compost and (b) flood plain soil at different times after inoculation. Data are means, n = 10.

(a)

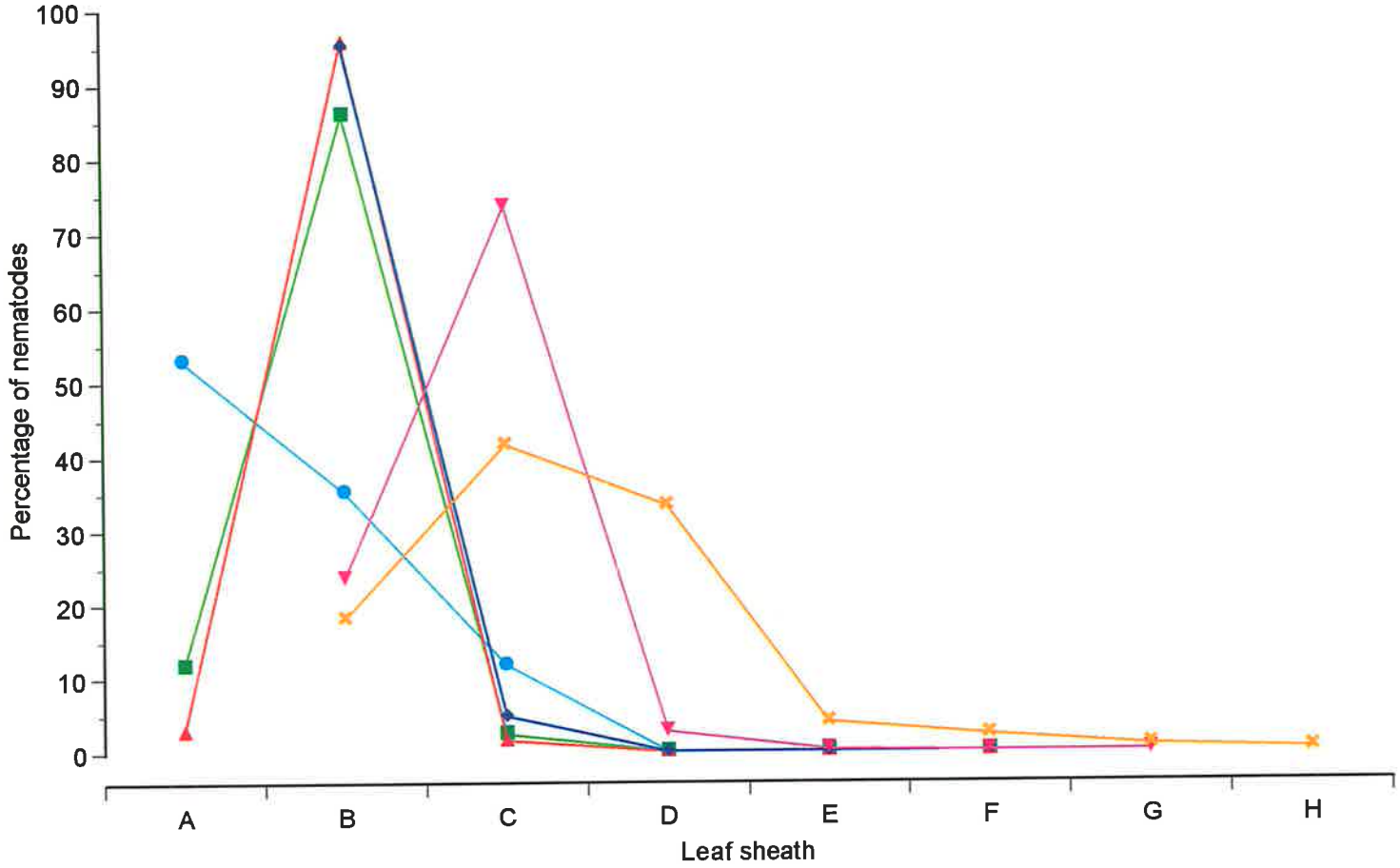
| Days after inoculation | Position within plant | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T1 | | | | | | | | T2 | | | | | T3 | | | | | T4 | | | T5 | | T6 | | | T7 | | T8 |
| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T | U | V | W | X | Y | Z | AA | AB | AC |
| 1 | 0.9 | 0.6 | 0.2 | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 3 | 3.4 | 25.1 | 0.7 | 0 | 0 | 0 | - | - | 0.3 | 0 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | 1.1 | 37.7 | 0.6 | 0 | 0 | 0 | - | - | 2 | 0.5 | - | - | - | 0 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 7 | - | 51.6 | 2.6 | 0 | 0 | 0 | - | - | 5 | 6.1 | 7.6 | 0 | - | 0 | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 11 | - | 70.9 | 222 | 8.2 | 0.6 | 0 | 0 | - | 94.6 | 18.8 | 38.4 | 53.3 | - | 14.3 | 31.4 | 3.4 | - | - | 26 | 33 | - | - | - | - | - | - | - | - | - |
| 18 | - | 132 | 304 | 243 | 28.6 | 16.3 | 5.3 | 1 | 354 | 157 | 129 | 180 | 134 | 34 | 203 | 359 | 323 | 92.5 | 83.4 | 138 | 173 | 109 | 57.5 | 12 | 84.3 | 70 | 46 | 149 | 48 |

(b)

| Days after inoculation | Position within plant | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T1 | | | | | | | | T2 | | | | | T3 | | | | | T4 | | | T5 | | T6 | | | T7 | | T8 |
| | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T | U | V | W | X | Y | Z | AA | AB | AC |
| 1 | 1.7 | 0.5 | 0.1 | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 3 | 3.4 | 25 | 0.7 | 0 | 0 | 0 | - | - | 0.3 | 0 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | - | 23 | 5.6 | 1.9 | .5 | 0 | - | - | 2.7 | 4.1 | 1.5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 7 | - | 21 | 3.7 | 17 | 14 | 21 | - | - | 18 | 14 | 6.3 | - | - | 0 | 0 | 0 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 11 | - | 73 | 143 | 40 | 31 | 26 | 50 | - | 106 | 21 | 21 | 9.6 | - | 32 | 38 | 51 | - | - | 16 | 4.3 | - | 26 | - | - | - | - | - | - | - |
| 18 | - | 74 | 209 | 162 | 34 | 25 | 16 | - | 350 | 146 | 164 | 211 | 49 | 423 | 639 | 472 | 44 | - | 164 | 360 | 318 | 52 | 34 | 13 | 172 | - | 19 | 33 | 145 |

T1 - T8 represent tillers 1 - 8
Position A is between the coleoptile and the outermost leaf sheath (1)
The first position for each tiller is between leaf sheaths 1 and 2, the last is surrounding the shoot apex.
- denotes tissues that either had not formed or had decayed

**Figure 7.9** Percentage of the total number of nematodes invading successive leaf sheaths (A - H) of the first *Polypogon monspeliensis* tiller at ● 1 day, ■ 3 days, ▲ 5 days, ◆ 7 days, ▼ 11 days and ✖ 18 days after inoculation.



been initiated and contained nine and 85 J2s respectively. Young leaves surrounding the galls were distorted and, in heavily infested tillers, galls were also found in the leaf laminae. These galls appeared as swellings on the surface of the leaf lamina and were lighter in colour than the surrounding leaf tissue (Plate 7.7). The galls did not have a distinct cavity or opening and were filled with spongy tissue. Only J2s were found within leaf galls.

There was no significant difference in the number of tillers or leaf sheaths per tiller ($P > 0.09$; approximate for all tests) of plants growing in either soil type at any sampling time. Also, while the mean total number of J2s per plant in both soil types more than doubled at each sampling period (Table 7.6), the only significant difference noted between soil types, in terms of total nematode number, occurred three days after inoculation. Analysis of empirical logits of proportions of tillers penetrated by nematodes showed a statistically significant difference between soils ($P \approx 0.006$), with nematodes penetrating further in any given tiller at any given time on plants growing in flood plain soil. However, the non-significant soil interaction terms indicate similar differences between the two soils for any time or tiller number. This can be easily seen in plots of fitted values derived from the spline models (Figure 7.10).

**Plate 7.7** Nematode galls (indicated by arrows) formed in the leaf laminae of a heavily infested *Polypogon monspeliensis* plant

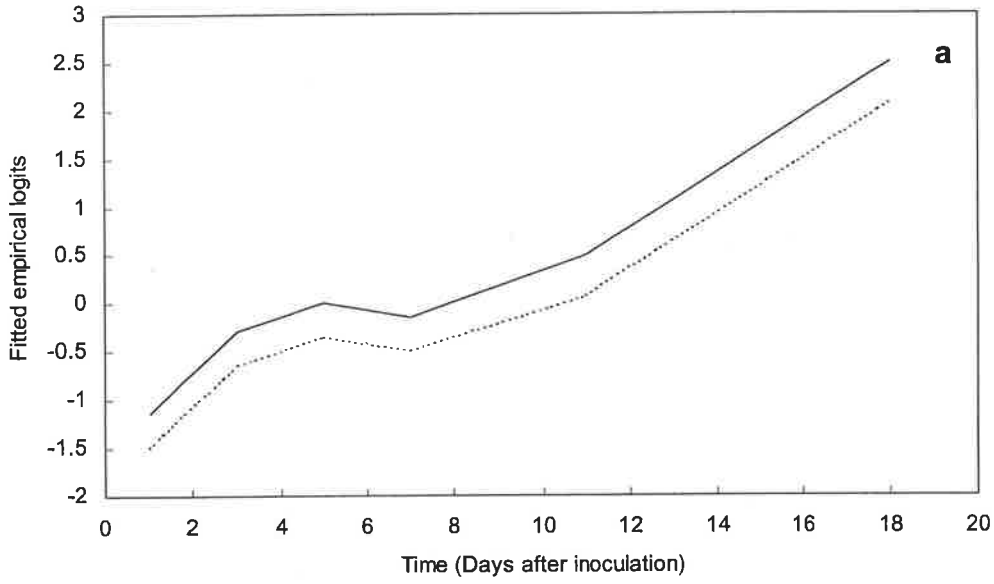
**Table 7.6** Total number of second stage juvenile nematodes in *Polypogon monspeliensis* plants grown in two types of soil at different times after inoculation. Data are means ± SD, n = 10. * Significance is at the 5% level.

| Days after inoculation | Potting Compost | Flood Plain Soil |
|---|---|---|
| 1 | 1.7 ± 2.5 | 2.3 ± 2.6 |
| 3 | 29.5 ± 22.1* | 11.2 ± 9.8* |
| 5 | 40.9 ± 32.0 | 38.1 ± 56.0 |
| 7 | 72.9 ± 111.4 | 106.4 ± 150.5 |
| 11 | 532.7 ± 399.7 | 627.8 ± 488.5 |
| 18 | 3113.4 ± 1524.2 | 3833.2 ± 1940.7 |

### 7.3.6 Optimization of ELISA

Absorbance curves for each bacterial concentration over a range of dilutions are shown in figures 7.11 and 7.12 for the L-standards and P-standards respectively. For both sets of standards, dilutions of 1:1600 or less resulted in saturation of the assays at 16 bacterial galls. A further 1:8 dilution resulted in saturation at 64 bacterial galls. At dilutions of $1:1x10^5$ and greater, the absorbance continued to increase over the range of bacterial concentrations tested and never saturated the assay. By performing a modified logarithmic transformation ($\log_{10}(x + 1)$) on the data, a near linear relationship is established between absorbance and bacterial concentration at a dilution of $1:1x10^5$. Thus, this dilution was chosen to produce the standard curves to quantify the amount of bacteria in the test samples. In addition, a 1:1600 dilution of each of the standards was added to each test plate to aid in the identification of low concentrations of bacteria.

**Figure 7.10** Fitted values (fixed effects soil + linear trend over time, plus best linear predictors for the fitted spline) with fixed soil and random plant effects for (a) tiller 1 and (b) tiller 2. —— flood plain soil, - - - potting compost.



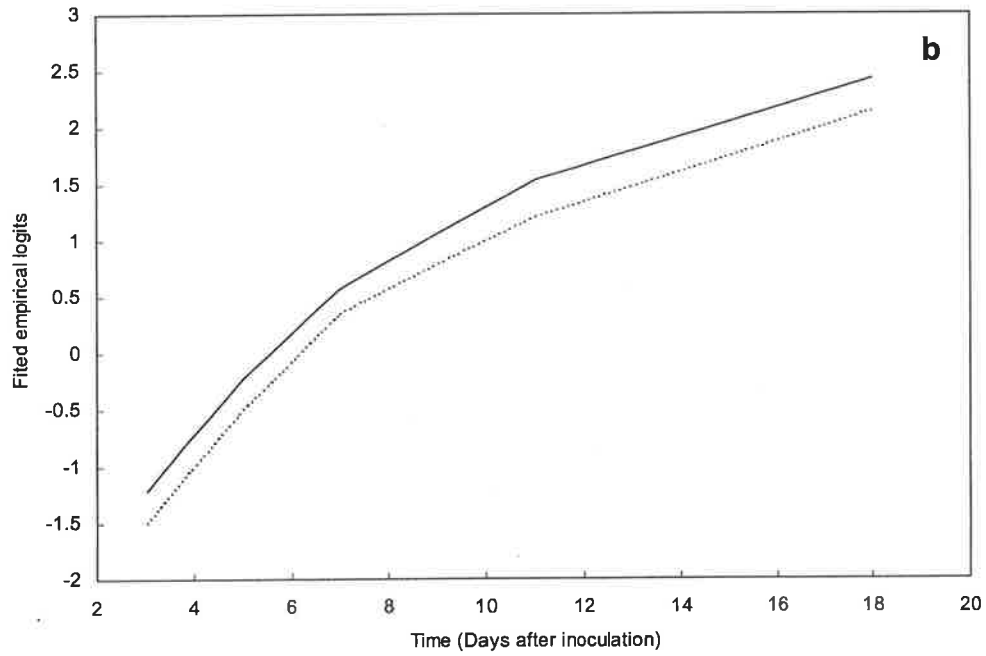



**Figure 7.10** Fitted values (fixed effects soil + linear trend over time, plus best linear predictors for the fitted spline) with fixed soil and random plant effects for (c) tiller 3. —— flood plain soil, - - - potting compost.

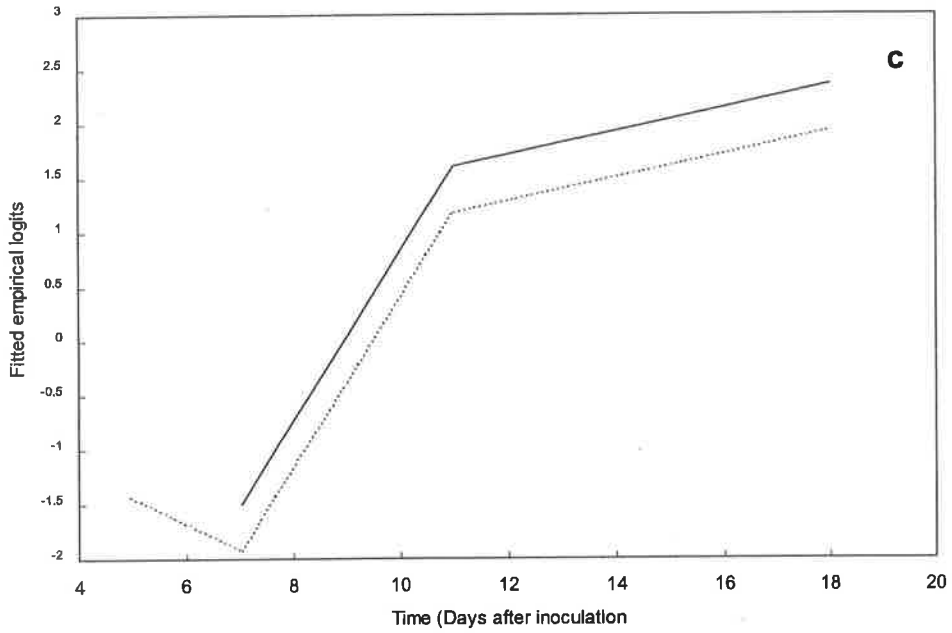


**Figure 7.11** Absorbance curves for bacterially colonised nematode galls originating from *Lolium rigidum* over a range of dilutions ■ 1:25, ● 1:200, ▲ 1:1600, ▼ 1:1.3x10$^4$, □ 1:1x10$^5$, ○ 1:8.2x10$^5$, △ 1:6.6x10$^6$

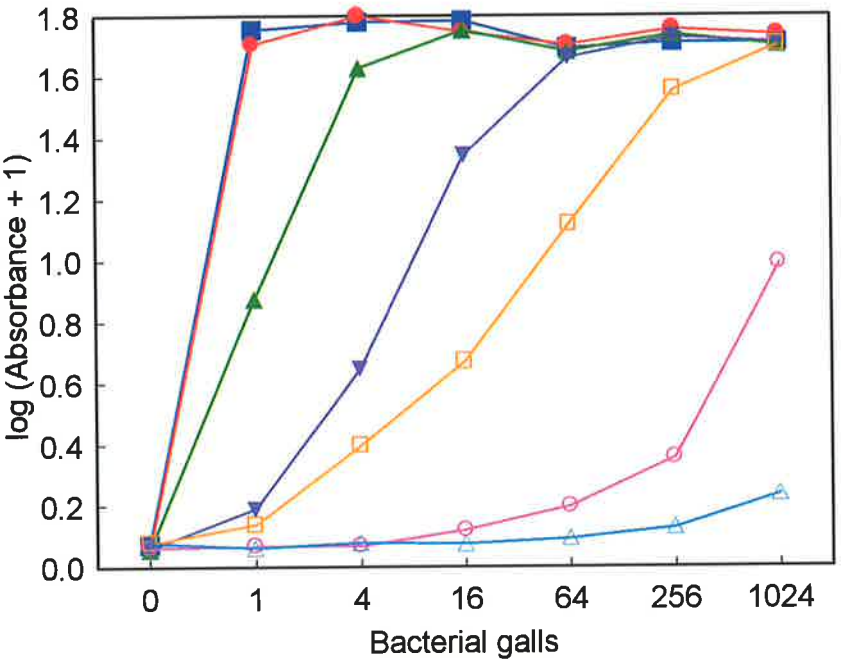
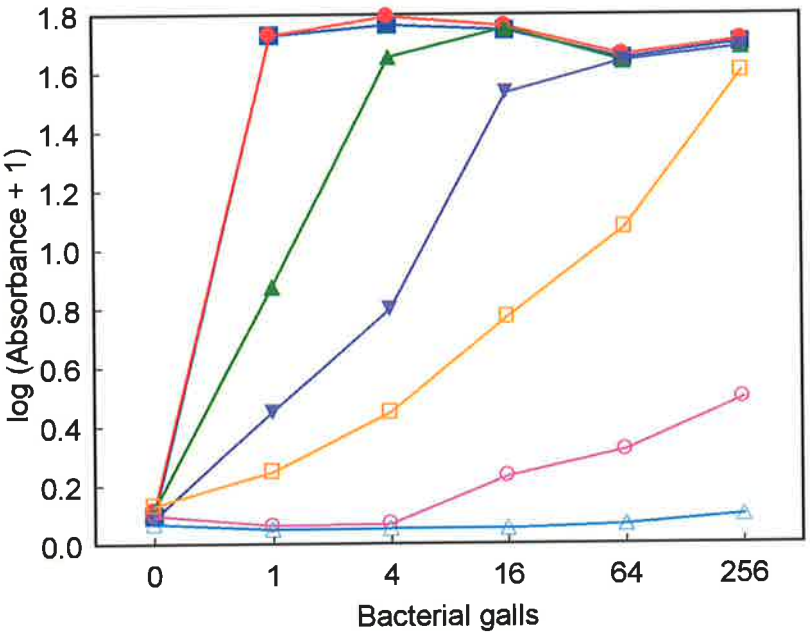



**Figure 7.12** Absorbance curves for bacterially colonised nematode galls originating from *Polypogon monspeliensis* over a range of dilutions ■ 1:25, ● 1:200, ▲ 1:1600, ▼ 1:1.3x10$^4$, □ 1:1x10$^5$, ○ 1:8.2x10$^5$, △ 1:6.6x10$^6$



### 7.3.7 Quantification and distribution of *Rathayibacter toxicus*

The 25 plants sampled had between 1 and 20 tillers (mean= 7). The tillers ranged in length from 1 mm (the size allocated to a solitary SMG) to 500 mm (mean= 83.5 mm) and weighed between 0.009 and 1.7 grams (mean = 0.25 g). *Rathayibacter toxicus* was detected in 39 of the 173 tillers assayed but was only visually evident in 47% of these. However, tillers with visible bacterial colonisation accounted for 70% of the total bacterial population sampled. The tillers with no visible sign of bacterial colonisation that tested positive for *R. toxicus* had symptoms of nematode infestation and SMG formation.

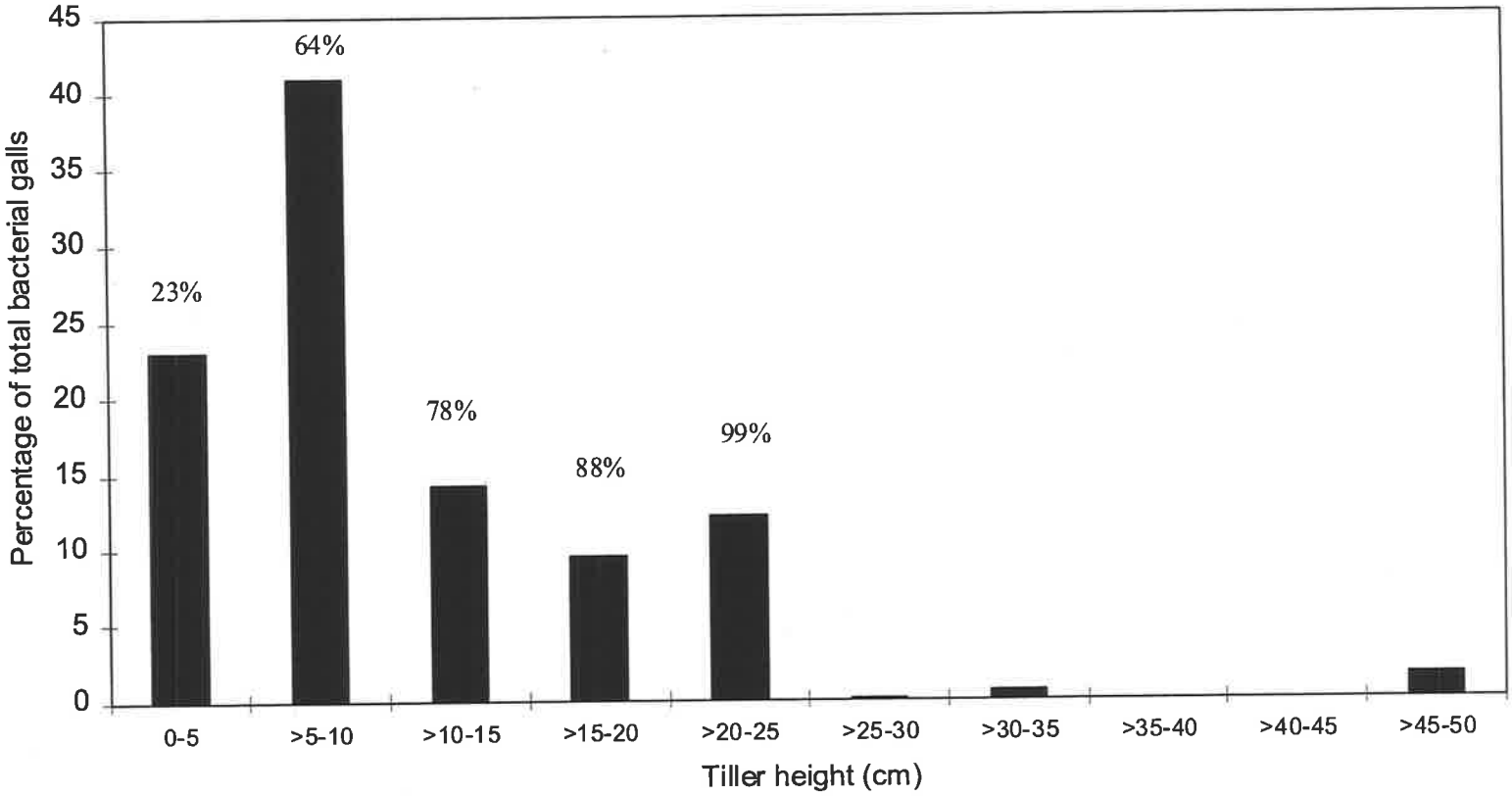
Two-thirds of the total bacteria were present in tillers less than 100 mm long (Figure 7.13) and more than 75% in tillers less than 150 mm. All of these tillers were vegetative (developmental stage D1) and only 10% of the bacterially colonised tillers had inflorescences partially or fully emerged from the boot. The amount of bacteria detected ranged from 0 to 256 bacterial gall equivalents per tiller.

## 7.4 Discussion

### 7.4.1 Climatic conditions and host dynamics

The study period (1992-93) allowed the monitoring of field populations of *Anguina* sp. and *P. monspeliensis* in successive years under different climatic conditions. The unseasonably wet conditions in 1992 contrasted with the below average and inconsistent rainfall in 1993, which persisted in the following years. Consequently, the flooding depth and duration was greater in 1992 than in 1993. Nevertheless, the patterns of tiller production by *P. monspeliensis* were consistent in both years at both sites.

**Figure 7.13** Percentage of total *Rathayibacter toxicus* detected by immunoassay found in *Polypogon monspeliensis* tillers of various heights. The cumulative percentage of total bacteria is indicated above each bar.



Responses to increasing flooding intensity vary widely among flood tolerant plant species but are generally associated with morphological and anatomical changes to improve gas exchange (Jackson and Drew, 1984), altered metabolic features to sustain cell function under oxygen stress (Armstrong *et al.*, 1994) and in some cases biomass allocation patterns (Van Der Sman *et al.*, 1993). The decline in the mean number of tillers per plant observed in *P. monspeliensis* between August and November in each season, may be due to the reallocation of resources to support the rapid extension of the main shoot, thereby shortening the period of submergence and improving gas exchange. This rapid extension, associated with elongation of the internodal cells, has been well studied in deep-water rice (*Oryza sativa* L.) and results from changes in hormone levels, predominantly an increase in gibberellins (GA: Azuma *et al.*, 1990) and a decrease in abscisic acid (ABA: Hoffman-Benning and Kende, 1992), both mediated by accumulation of ethylene (Armstrong *et al.*, 1994). Adventitious root formation is also mediated by ethylene (Drew *et al.*, 1979) and the involvement of GA, ABA and indole-3-acetic acid have been described for a number of species such as *Callitriche platycarpa* Kutz. (Musgrave *et al.*, 1972), *Ranunculus scleratus* L. (Musgrave and Walters, 1973) and *O. sativa* (Hoffman-Benning and Kende, 1992). While other anatomical and morphological changes such as aerenchyma formation, epinastic curvature of leaves and stomatal closure also result from the actions of the same hormones and probably occur in *P. monspeliensis*, no further observations of the effects of flooding on the host plant were made.

As the effects of ethylene are both rapid and reversible (Armstrong *et al.*, 1994), the subsequent increase in the mean number of tillers per plant observed in spring as the flooding decreases, is also likely to be under the influence of hormone levels. Increased gas

diffusion leads to decreased ethylene concentration in plant tissues, restoring the balance of hormones to pre-inundation levels.

### 7.4.2 Nematode dynamics

***Invasion and movement within the plant***

Results from the pot experiment indicate that the nematode is able to rehydrate, emerge from the gall and invade a host within 24 hours. These results were unexpected as experiments conducted by Price *et al.* (1979) indicated that *A. funesta* began to emerge from galls approximately 29 days after the galls were placed in moist soil. Further, Swarup and Gupta (1971) reported that 10-15 days was required for the emergence of *A. tritici* from galls. The differences in the time for emergence may be due to differences in both the structure of the galls and the amount of plant tissue surrounding them. Galls of *A. funesta* are surrounded by a palea and lemma which probably act as a physical barrier, slowing water absorption and requiring microbial degradation before emergence can take place. In contrast, *A. tritici* galls are not covered by a palea and lemma allowing faster absorption of water and presenting less of a barrier. The galls produced by the *Anguina* sp. vary in the amount of plant tissue that is covering them. The seed galls are mostly within enlarged glumes and palea (McKay *et al.*, 1993), although frequently they are only loosely covered. Branch galls have no protective covering and shoot meristem galls are either found within remnant leaf sheaths or fully uncovered if the sheaths have rotted away.

Nematode invasion of field plants varied between sites and years depending on suitable environmental conditions. The early break of the season in 1992, followed by favourable conditions, enabled nematodes to move into the plant earlier than in 1993. In both years, nematodes invaded plants earlier at Cape Jaffa than at Stewart's Range. Whether this was

due to sampling error or environmental factors is unclear. Observation of SMG initiation in field samples six weeks after the opening rains is comparable to the three to four weeks noted in the pot experiment. The rapid emergence, invasion and gall initiation observed for *Anguina* sp. may be an adaptation to survive flooding as it appears that the nematode cannot invade host plants while they are inundated (see Chapter 8). Germination of the host is also rapid, presumably to enable plants to become established before flooding thus enhancing their chance of survival.

The decline in the mean number of J2s per plant during flooding seems to be correlated with a reduction in the number of tillers per plant, suggesting that nematodes cannot easily leave tillers and reinvade new ones. The absence of nematodes from the single tiller which remains during the peak of flooding is interesting. This is likely to be the first tiller produced by the plant and hence the one with the most number of leaves. The results indicate that the central tissues of the first tiller are the last to be infested and thus the nematodes may not have sufficient time to reach the apical meristem before flooding. Once elongation of the internodes commences in response to flooding it is unlikely that the nematodes will be able to penetrate further.

The fate of nematodes which do not successfully initiate a SMG before flooding is unclear. The nematodes can survive in flooded soil for an extended period (see Chapter 8) and it is possible that these are involved in the second nematode invasion that occurs after floods recede. However, this is also the time that J2s emerge from SMGs produced before flooding. These nematodes are likely to be in better condition than any surviving in soil and hence are likely to be responsible for the majority of new invasions in spring.

***Gall production***

The ability of the nematode to initiate galls in the shoot apical meristem was unexpected as only seed and branch galls had been recorded previously by McKay *et al.* (1993). The galls are similar to those produced by *A. agropyri* in couch grass (*Agropyron repens* (L.) Beauv.) and rye (*Secale* sp.) (Figure 6; Krall, 1991), but it is unclear if *A. agropyri* galls originate in the shoot apical meristem. *Mesoanguina amsinckiae* (Steiner and Scott 1935) Chizhov and Subbotin 1985 has also been reported to form galls in terminal apical meristems of *Amsinckia intermedia* Fisch. & Mey. (Nagamine and Maggenti, 1980). The authors did not indicate whether the meristems had become reproductive.

All described species of *Anguina* can be separated into two groups; those that initiate galls and reproduce in reproductive tissue (*A. agrostis*, *A. funesta*, *A. hyparrheniae*, *A. spermophaga*, A. *tritici*) and those that initiate galls and reproduce in vegetative tissue (*A. agropyri*, *A. australis*, *A. danthoniae*, *A. graminis, A. microlaenae*, *A. tumefaciens*). The *Anguina* species under investigation is therefore unique in that it can reproduce in vegetative and reproductive tissue. Southey (1969) reported that an undescribed species of *Anguina* parasitic on Cocksfoot grass (*D. glomerata*), could form galls and reproduce in both tissue types but leaf galls were only observed in pot experiments where a very high inoculum level was used and have not been recorded from the field.

The induction of a SMG has serious implications for the host as it precludes the formation of new leaves, elongation and the development of the inflorescence in the tiller in which it is formed. Pre-existing leaves appear to senesce at a normal rate but are not replaced, leading to the death of the tiller. These events can probably be attributed to the mechanical destruction of the central zone cells at the tip of the shoot apical meristem by feeding

nematodes. These cells are normally recruited to the peripheral or rib zones of the shoot apical meristem to undergo organogenesis and differentiation into new leaves or vasculature. Thus, no new leaves will be produced. In *Arabidopsis thaliana* L., cells at the base of the central zone are also responsible for the expression of the *WUSCHEL* (*WUS*) gene which appears to act as an organising centre for the stem cells of the central zone (Mayer *et al.*, 1998). Expression of this gene is essential for shoot and floral meristem integrity (Laux *et al.*, 1996). Surgical destruction of the central zone is genetically mimicked by mutations in the *WUS* gene and results in adventitious shoot meristem induction across the terminated apex integrity (Laux *et al.*, 1996). The repeated induction of adventitious meristems (Clark, 1996), may provide further potential feeding sites and an avenue of manipulation by nematode feeding.

While the shoot apical meristem is considered vegetative until the onset of flowering, it nevertheless is a site of active cell division and differentiation. During the formation of the inflorescence further sites of meristematic activity develop and it is in these that the nematode is able to induce galls. As branch galls were observed after the apex had become reproductive but before spikelet development had taken place, these probably originate from branch primordia as suggested by McKay *et al.* (1993). Most branch galls were attached to the rachis and hence initiated in first order branch primordia. Galls formed in second order branch primordia were less common and none were observed in third order branch primordia. With most monocots, spikelet differentiation begins at the tip of the main axis (Barnard,1957). Bonnett (1937) noted that spikelet development in the oat panicle then proceeded basally in succession at the tips of the primordia of the branches of the first order. Thus, by the time that most of the third order branches are beginning to form, floral primordia are already present at the top of the inflorescence. If the developing

floral primordia are more attractive to the nematodes as gall initiation sites, this would explain why no galls were observed in third order branch primordia.

While there is a general consensus that the floral primordium is the site of gall induction by the seed-galling *Anguina* species, the specific tissues involved appear to differ. Davaine (1857) (cited in Decker, 1989) reported that initiation of seed-galls by *A. tritici* occurred before differentiation of the palea, stamen and ovary and that initiation did not occur after differentiation had begun. This is in contrast to Marcinowski (1909) who suggested that most galls were formed later in undifferentiated carpellate or staminate tissue. Swarup and Gupta (1971) also found that the floral primordia were involved but did not mention any particular stage of differentiation.

Goodey's (1930) observation that the galls formed by *A. agrostis* in bentgrass (*Agrostis* sp.) were modified ovaries was supported by Jensen *et al.* (1958), who reported that the nematodes attacked the young ovule. Jensen (1961) later reported that the nematodes moved "into the ends of young seeds". Norton (1965) noted that the florets of western wheatgrass (*Pascopyrum smithii* (Rydb.) Bartworth & Dewey) were galled and later reported that gall initiation occurred before stamen and pistil differentiation (Norton and Sass, 1966). More recent studies of *A. funesta* in annual ryegrass by Price *et al.* (1979) indicated that floret primordia were attacked after differentiation of the palea but before lodicule, stamen or ovary development. Stynes and Bird (1982) concluded that the gall developed in place of the ovules and less frequently in place of the stamens.

The seed galls produced in *P. monspeliensis* and *A. avenacea* are surrounded by glumes and a lemma (McKay *et al.*, 1993), which indicates that they are initiated before any differentiation of the floral parts. In the rounded stage before differentiation of any floral

parts, the young flower primordium has the same characteristic arrangement of cells as the vegetative shoot apical meristem (Barnard, 1955). Thus, it is likely that the nematodes seek out undifferentiated cells at the tip of the floral primordium to begin feeding.

The mechanism by which the J2 nematodes stimulate meristematic tissue to develop into a gall is unknown but it is likely that enzymatic secretions or hormonal mimics liberated from the nematode pharyngeal glands are involved. Common enzymes detected in secretions from nematodes include: esterases (*Ditylenchus* spp., *Meloidogyne hapla* Chitwood 1949, *Meloidogyne javanica* (Treub 1885) Chitwood 1949), alkaline phosphatase (*Meloidogyne* spp.), acid phosphatase (*Ditylenchus triformis* Hirschmann and Sasser 1955, *Meloidogyne* spp., *Tylenchulus semipenetrans* Cobb 1913); amylase (*T. semipenetrans*, *Ditylenchus dipsaci* (Kühn 1857) Filipjev 1936, *Heterodera schachtii* Schmidt 1871, *Globodera rostochiensis* (Wollenweber 1923) Behrens 1975, *M. javanica*), callulase (*D. dipsaci*, *Heterodera trifolii* Goffart 1932, *H. schachtii*, *Meloidogyne arenaria* (Neal 1889) Chitwood 1949); B-galactosidae and B-glucosidase (*G. rostochiensis*), proteinase (*G. rostochiensis*, *H. schachtii*, *D. dipsaci*) and chitinase (*D. dipsaci*, *Ditylenchus destructor* Thorne 1945) (Giebel, 1974). While these secretions aid in the digestion of cell walls and ingestion of food, they may also induce metabolic changes in the host (Hussey, 1989). In contrast to the distinctive changes produced in relatively few cells by nematodes in the Heteroderidae (Dropkin, 1969), Stynes and Bird (1982) suggested that species of *Anguina* induced more subtle changes in a large number of cells. Further studies, which include sectioning and histological staining of developing galls, would elucidate both which cell lines are involved in gall production and the changes that occur within them.

*Nematode development*

Nematode development within all three gall types appears to be similar to that described for other species of *Anguina*. As mentioned by Price *et al.* (1979) and re-iterated by Bird and Stynes (1981b), it is difficult to determine the duration of the various stages of nematode development from field populations due to environmental fluctuations and an inability to precisely determine when invasion occurs. Nevertheless, estimation of development times within seed galls will be more accurate than from SMG, as flowering is mostly synchronous and takes place over a shorter time frame than the vegetative cycle.

Once feeding commences, the gonad primordium begins to enlarge and the nematodes grow and moult three times inside the developing seed gall to become adult in 7-14 days. This is comparable to the 5-14 days reported for *A. funesta* (Price *et al.*, 1979) and 3-14 days reported for *A. tritici* (Swarup and Gupta, 1971). The 1:1 ratio of females to males in all three gall types differs from other species of *Anguina*. Price *et al.* (1979) noted that in *A. funesta* galls there were frequently more females than males with ratios ranging from 1:1 to 4:1. Similar results were found for *A. australis*, where the average ratio of females to males is significantly different from 1:1, with galls containing more females on average (Shedley, 1995).

Egg laying began between the seventh and fourteenth day but the sampling interval was too long to determine the exact timing. Nevertheless, the timing is comparable to both *A. funesta* (Price *et al.*, 1979) and *A. tritici* (Swarup and Gupta, 1971). The nematodes undergo the first moult in the egg and newly hatched second stage juveniles are present within seven days. There is a marked difference between reports of embryogenesis in *A. funesta,* ranging from 4-7 days in field studies (Price *et al.*, 1979) to 9-10 days in

laboratory studies (Bird and Stynes, 1981a). Subsequent field sampling by McKay *et al.* (1985) confirmed the estimate of Price *et al.* (1979). The whole life-cycle takes between 4-6 weeks in the field and there is only one generation per gall.

### 7.4.3 Association between nematode and tiller development

*Polypogon monspeliensis* tillers remain vegetative for a long period of time under field conditions and could be continuously subjected to nematode invasion when not inundated. The timing of SMG initiation will depend on a number of factors, predominantly suitable environmental conditions and the number of leaf sheaths the nematode must negotiate to reach the shoot apical meristem. The asynchronous initiation of SMG makes it impossible to match the stage of nematode development with development of the host. Some measure of when the SMG were initiated can gauged by the number of leaves present on the tiller and the tiller number in which it is formed but this also becomes unreliable once more than a few tillers are present.

In contrast to the SMG, the data indicate that there is some correlation between nematode and tiller development within both branch and seed galls. Floral initiation in *P. monspeliensis* is under the control of photoperiod and it has been described as a facultative long day plant for flowering (Gutterman, 1992). Once flowering begins it will only cease or be delayed if day length is reduced (Pearson and Ison, 1997). Thus, the branch and floral primordia should form at the same relative time in relation to the development of the inflorescence. While the number of branch galls sampled was too low to permit correlations to be made with tiller development, it seems reasonable to accept that nematode development in branch galls will be more advanced than in seed galls in the same inflorescence.

The first sign of the inflorescence emerging from the boot is well correlated with the start of egg laying by the nematodes, with 8% of galls expected to contain eggs at this stage. Similarly, the period in which the inflorescence is emerging from the boot can be used to determine when the first J2s are hatching. This is in contrast to the findings of McKay *et al.* (1985) that "head fully emerged" was the most conservative indicator of *A. funesta* galls containing hatched J2s but that anthesis was also acceptable. In *P. monspeliensis*, once the inflorescence has fully emerged and the culm is elongating, 50% of galls are expected to contain hatched J2s and by the time the tillers reach anthesis most of the galls contain hatched J2s.

### 7.4.4 Quantification and distribution of *Rathayibacter toxicus*

As *R. toxicus* is carried into the host by the nematode and remains tightly attached (Bird, 1985), it is likely that bacterial proliferation will be seen at sites of nematode activity. Thus, in association with *A. funesta* the bacterium colonises the seed galls induced by the nematode and occasionally produces a gummosis of the inflorescence (Price *et al.*, 1979a) often referred to as "bacterial slime". The gummosis can cause distortion of the inflorescence, preventing its emergence from the boot (McKay and Ophel, 1993) and subsequent visual detection. In the survey of *P. monspeliensis* in southeastern SA, reported in Chapter 4, bacterial gummosis was observed at all sites where the bacterium was recorded, indicating high levels of bacteria in the field. However, only half of the total number of tillers detected by ELISA as contaminated with *R. toxicus* had any evidence of bacterial contamination and only a further 20% had evidence of nematode infestation.

The estimation of bacterial contamination in the field is likely to be underestimated even further, due to the large proportion of very short tillers colonised by *R. toxicus*. This results

from bacterial colonisation of the SMG which, as previously discussed, are generally found at the base of the tillers. As SMG initiation stops further development of the tiller, it is not surprising that only 10% of bacterially colonised tillers had inflorescences at least partially emerged from the boot.

Estimation of risk for ARGT is based primarily on an estimate of bacterial levels, expressed as bacterial gall equivalents, found in a sample of annual ryegrass (McKay and Riley, 1993). It has been suggested that between 16,000 and 25,600 bacterial galls constitute a lethal dose for a 40kg sheep (Jago and Culvenor, 1987). This study determined that in *P. monspeliensis*, bacterial slime can be equivalent to 256 bacterial galls. Thus, a 40 kg sheep would only need to ingest 62 – 100 such tillers to consume a lethal dose of corynetoxins

# Chapter 8

# The Effect of Flooding on Nematode Invasion and Survival

## 8.1 Introduction

In southeastern SA, pastures are flooded annually for between one and five months (McKay and Ophel, 1993). Flooding of this duration often limits resources and biota that flourish in such conditions often have adaptations to overcome these limitations. Ecological field studies on *Anguina* sp., discussed in Chapter 7, indicated that the shoot meristem galls (SMG) may be adaptive for surviving floods. Infective J2 nematodes were almost completely absent from plants during flooding, except in SMGs and breakdown of these galls in spring was correlated with nematode invasion.

This chapter describes experiments to determine the importance of the SMG in the nematode lifecycle by a) investigating the effect of flooding on invasion of *P. monspeliensis* by J2s and b) determining how nematode survival is affected by prolonged flooding.

## 8.2 Materials and Methods

### 8.2.1 Effect of flooding on invasion

Forty seeds of *P. monspeliensis* were surface sterilised and germinated in 30 ml polycarbonate tubes as described in Chapter 3.2. As soon as the seed had germinated, 20 tubes were inoculated with 10 µl of sterile distilled water containing approximately 25 J2 nematodes, by placing the inoculum directly onto the germinated seed. Ten microlitres of sterile distilled water was added to the remaining 20 tubes as uninoculated controls. Immediately after inoculation, 2 ml of sterile distilled water was added to 10 inoculated tubes and 10 uninoculated tubes, to simulate flooding. The tubes were arranged in a

randomised complete block design and placed in growth cabinet at 20°C with 16 hour photoperiod supplied by 30 W fluorescent cool white tubes.

Plants were assessed after seven days by removing each from its tube, carefully removing successive leaf sheaths with fine forceps and counting the number of nematodes that had invaded the plant. Care was taken to exclude nematodes that were entangled in the root hairs and empty seed coat. The number of plants invaded in each treatment were compared using a two-tailed Fisher's exact test (Fisher, 1934) under the hypothesis that nematode invasion of *P. monspeliensis in vitro* is independent of the presence of water.

### 8.2.2 Nematode movement in water

Considering that flooding is usually an annual event in many areas where *Anguina* sp. is found, the movement of J2 nematodes in water was investigated to determine if they could swim. Nematodes were extracted from *P. monspeliensis* seed galls as described in Chapter 3.4 and transferred to a small glass petri dish containing water (about 20 mm deep). The frequency of the waves of muscle contractions passing along the length of 20 randomly selected nematodes was determined as they moved in the water.

### 8.2.3 Nematode survival in flooded soil

Nematode survival in flooded soil was compared with survival in soil at field capacity (-10 kPa suction). The device used to control water potential was a variant of the methods described by Topp *et al.* (1993), as shown in Plate 8.1, and consisted of a 1 bar ceramic plate (Plate 8.2) connected to a water reservoir (Plate 8.3) via an airlock apparatus (Plate 8.4). Two ceramic plates were soaked for 24 hours in distilled water to remove air. The airlock apparatus, reservoir and reservoir tube were filled with water and attached to one of the ceramic plates underwater to ensure air did not enter the system. Each ceramic plate

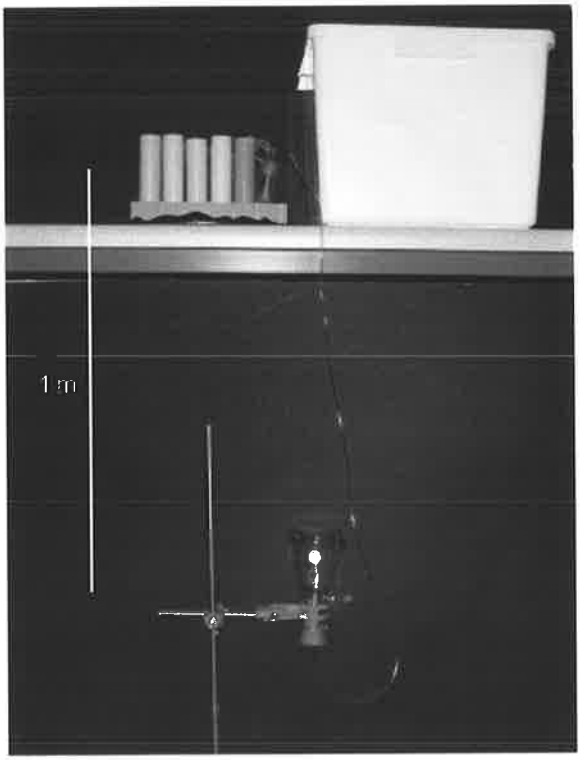



**Plate 8.1** The device used to control water potential in the nematode survival experiments

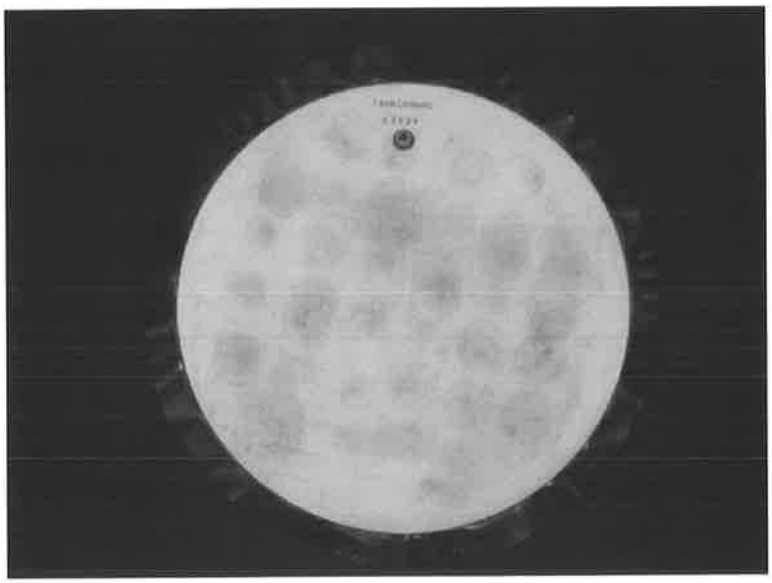


**Plate 8.2** 1 bar ceramic plate

**Plate 8.3** Büchner flask used as a water reservoir. As water moves through the tube at the base of the flask to the ceramic plate, it is replaced by air which enters through the side arm

**Plate 8.4** Airlock apparatus to trap any air entering the ceramic plate from the water reservoir and breaking the water potential. Air can be flushed from the device by raising the reservoir briefly and then releasing the clamp

was placed into a tub, supported by three glass petri dishes. Thirty PVC tubes (30 mm diameter x 100 mm in length) were arranged on the plates in a PVC grid (Plate 8.5), which was affixed to the plates by rubber bands to ensure the tubes would not lose contact with the surface of the plates. Each tube had a piece of 250 μm nylon mesh attached to its base with a rubber band. The tubes were 75% filled with cooled, steam pasteurised soil collected from field site 5 (see Chapter 3.6), once the tubes were securely in place on the ceramic plate. This ensured that soil particles did not lodge under the bottom edges of the tubes, which could result in the breaking of the water potential. The water level in each tub was raised to half the height of the tubes to wet the soil thoroughly. The opening of the water reservoir was placed 1 m below the water level, to create a suction pressure of –10 kPa about 250 mm below the soil surface (Topp *et al.*, 1993) and the water allowed to drain through the system for three days. The water level in the other tub was raised so that the water level was 20 mm above the soil surface but not above the top of the tubes. Approximately 5000 nematodes in 50 μl of water were added to the soil surface of each tube.

The experiment was conducted in a 15°C constant environment room with a 12 hour photoperiod supplied by three 400 W metal halide lights. Suction pressure was maintained by refilling the reservoir under water before it completely emptied, forcing any air bubbles from the reservoir tube into the airlock by raising the reservoir and then removing the air from the airlock by opening the clamp briefly while the reservoir was still raised. The water level in the second tub was maintained at 20 mm above the soil surface by adding more water.

**Plate 8.5** Arrangement of the PVC tubes on the ceramic plate using a PVC grid

Ten tubes were randomly removed from each tub at monthly intervals. Nematodes were extracted from the soil in each tube using a modified Baermann funnel and counted (see Chapter 3.5). The first 50 nematodes counted were categorised into three groups (0-33%, 34-66%, 67-100%) according to the proportion of intestinal contents that was present as lipid droplets. Once counted, the nematode suspension was diluted with sterile distilled water so that 10 µl contained approximately 25 J2s. The viability of the nematodes was investigated by inoculating *P. monspeliensis* plants under *in vitro* conditions as described in Chapter 5.2.2. Nematodes freshly extracted from dry galls at each sampling time were used as controls. The plants were assessed for gall production after six weeks and any galls opened to determine the stage of nematode development. Data were examined by analysis of variance where applicable and the least significant difference method with a Bonferroni correction (Miller, 1981) was used to make pairwise comparisons between the treatment means.

## 8.3 Results

### 8.3.1 Effect of flooding on invasion

Plants were invaded by nematodes in both non-flooded and flooded treatments. Eight of the ten plants were invaded in the non-flooded treatment by between one and 14 nematodes (mean 4.3). In contrast, two of the ten plants in the flooded treatment were invaded by a single nematode. No nematodes were found in plants that were not inoculated. Analysis of the 2 x 2 contingency table does not support the null hypothesis ($P < 0.05$) that nematode invasion is independent of the presence of flooding.

### 8.3.2 Nematode movement in water

The J2s do not appear to be able to support themselves in standing water by their own activity. The mean frequency of waves of muscular contractions per second passing along their lengths while moving through the water was 1.07 and ranged from 0.77 to 1.47.

### 8.3.3 Nematode survival in flooded soil

Nematodes were recovered from both flooded and moist soil treatments at each sampling time during the experiment (Table 8.1). There was a highly significant interaction between treatment and sampling time ($P < 0.001$), limiting comparisons to cell rather than treatment means. In flooded soil, the number of nematodes recovered decreased significantly ($P < 0.001$) at each sampling time, in contrast to moist soil where the number of nematodes was not significantly different at any sampling time. The number of nematodes recovered from moist soil was significantly less than that recovered from flooded soil at 4 weeks ($P < 0.001$) and significantly more at 12 weeks ($P < 0.01$).

The proportion of the 50 nematodes which fell into the three categories according to the amount of remaining lipid in the intestine is shown in Table 8.2. The majority of nematodes were placed in the 34-66% and 67-100% categories, although nematode numbers in each category differed between treatments. To facilitate analysis of the data, the proportions at each sampling time were converted to a "mean lipid content" variate by multiplying the number of nematodes within each category by the mean of the category and dividing by the total number of nematodes examined (Table 8.3). Analysis of variance revealed a highly significant interaction between treatment and sampling time ($P < 0.001$). Comparisons of cell means showed that nematodes from the flooded treatment had significantly higher body contents ovcrall ( $P < 0.01$) at every sampling time. Within treatments, the mean overall body contents decreased significantly between weeks 4 and 8

**Table 8.1** Mean number of nematodes recovered from moist and flooded soil at monthly intervals. The Bonerroni corrected LSD for comparison of cell means within strata = 479.64.

| Sampling Time | Treatment | |
|---|---|---|
| | Moist | Flooded |
| 4 Weeks | 1049.0 | 1920.7 |
| 8 Weeks | 788.2 | 1186.3 |
| 12 Weeks | 1101.0 | 448.8 |

**Table 8.2** Proportion of nematodes recovered from moist and flooded soil at monthly intervals categorised according to percentage of intestinal lipid content present. Data are means ± standard deviations.

| Sampling Time | Treatment | | | | | |
|---|---|---|---|---|---|---|
| | Moist | | | Flooded | | |
| | 0–33% | 34–66% | 67–100% | 0–33% | 34–66% | 67–100% |
| 4 Weeks | 0.2 ± 0.6 | 34.1 ± 5.6 | 15.6 ± 5.3 | 0.2 ± 0.4 | 17.2 ± 5.7 | 32.6 ± 5.5 |
| 8 Weeks | 1.0 ± 1.6 | 41.1 ± 4.4 | 7.9 ± 3.6 | 0.8 ± 1.0 | 16.6 ± 3.6 | 32.5 ± 3.5 |
| 12 Weeks | 0 | 48.8 ± 0.8 | 1.2 ± 0.8 | 0 | 9.8 ± 6.9 | 40.2 ± 6.9 |

**Table 8.3** Mean intestinal lipid content of nematodes recovered from moist and flooded soil at monthly intervals. The Bonerroni corrected LSD for comparison of cell means within strata = 4.1.

| Sampling Time | Treatment | |
|---|---|---|
| | Moist | Flooded |
| 4 Weeks | 60.3 | 71.7 |
| 8 Weeks | 54.6 | 71.3 |
| 12 Weeks | 50.8 | 76.9 |

for the moist treatment ($P < 0.01$) and between weeks 8 and 12 for the flooded treatment ($P < 0.01$).

Nematodes recovered at each sampling time induced galls in *P. monspeliensis in vitro*, regardless of the treatment from which they were extracted. However, at 12 weeks, only half of the plants inoculated with nematodes from the moist soil had galls compared to all plants inoculated with nematodes from the flooded soil (Table 8.4). The majority of galls contained adults, eggs and/or hatched juvenile nematodes regardless of sampling time or the treatment from which the inoculum was recovered.

**Table 8.4** Percentage of *in vitro Polypogon monspeliensis* plants with shoot meristem galls after inoculation with nematodes recovered from two soil treatments at monthly intervals.

| Sampling Time | Treatment | | |
|---|---|---|---|
| | Moist Soil | Flooded Soil | Control |
| 4 Weeks | 90 | 90 | 100 |
| 8 Weeks | 100 | 100 | 100 |
| 12 Weeks | 50 | 100 | 100 |

## 8.4 Discussion

### 8.4.1 Invasion and movement during flooding

Second stage juvenile nematodes of *Anguina* sp. were unable to effectively invade *P. monspeliensis in vitro* under simulated flooding. This is in agreement with the field observations discussed in Chapter 7.3.2, where invasion was noted before and after flooding but no invasion of plants occurred during inundation. The exact mechanism by which species of *Anguina* invade their hosts is unclear. Observations by Price (1973) indicate that *A. funesta* J2s move up the leaf sheath in water films and enter at the junction of the leaf blade. Experiments with *A. tritici* (Limber, 1973) and observations by McKay *et al.* (1981), that invasion of *L. rigidum* by *A. funesta* followed rainfall events, support the entry model suggested by Price (1973). However, there is a critical water film thickness at which the forward movement of nematodes is inhibited (Wallace, 1959). Assuming that invasion occurs in this manner in *Anguina* sp., flooding would interfere with the nematodes' ability to move up the leaf sheath by negating the thin film of water needed for successful forward progression and hence invasion.

Furthermore, it appears that *Anguina* sp. is unable to swim. While some forward progression is made by the nematodes on the horizontal axis, the frequency of waves of muscular contraction exhibited by the nematodes is not sufficient to support their bodies in a water environment. Nematodes such as *Haemonchus contortus* Rudolphi 1803, *Panagrellus redivivus* (Linne 1767) Goodey 1945 and *Turbatrix aceti* (Muller 1783) Peters 1927 exhibit considerably faster frequencies of 1.7, 3.0 and 5.2 waves per second respectively, yet *T. aceti* is the only one of these that can truly swim (Gray and Lissman, 1964). The form of the waves also appears to be important in swimming. In *T. aceti*, the amplitude of transverse movements of the tail is about four times greater than the head

(Gray and Lissman, 1964), which allows forward progression without yawing from side to side (Gray, 1958). *Anguina* sp. moves in a similar manner to *H. contortus*, where the anterior and posterior move around two nodes, causing the whole body to yaw and a decrease in the amplitude of the wave as it moves posteriorly (Gray and Lissman, 1964).

### 8.4.2 Nematode survival in flooded soil

Field observations (Chapter 7.3.2) indicate that two nematode invasion events occur; one before flooding and one after flooding. As it seems unlikely that invasion takes place during inundation, this study set out to determine if nematodes failing to invade before flooding could be responsible for invasion after flooding . The results presented above show that *Anguina* sp. is able to survive in flooded conditions for at least three months and is still able to produce galls in the host after this time. Nematodes from the flooded treatments had more intestinal lipid content than those from moist soil, where conditions are optimal for quick progressive movement (Wallace, 1958). It is possible that the flooded conditions may induce some kind of quiescence in the nematodes as suggested by Wallace (1968) for *M. javanica* and by Cooper and Van Gundy (1971) for *Aphelenchus avenae* Bastian 1865. Thus, the infectivity of the nematode would be maintained ready to invade a host plant when movement became optimised, as floods receded and soil pores drained. The decrease in body contents associated with the loss of infectivity is in agreement with the results of Van Gundy *et al.* (1967). They found that in *M. javanica* and *T. semipenetrans*, a decrease in infectivity was correlated with a decrease in mobility and body contents when maintained at high temperatures in dry soils or oxygenated solutions. Conversely, body contents were conserved and infectivity increased at low temperatures in wet soils and in solutions with low oxygen.

Nematodes inducing galls were able to complete their life cycle and there was no indication that those that did initiate galls were less fit. Thus, it appears that *Anguina* sp. has several strategies for overcoming the constraints imposed by flooding. Firstly, nematodes unable to invade *P. monspeliensis* before flooding are able to survive until conditions improve and secondly, induction of SMG in autumn guarantees a significant population of infective juveniles in spring.

# Chapter 9

# Agronomic Management of Flood Plain Staggers

## 9.1 Introduction

Agronomic methods have been effective in controlling *A. agrostis* in *Agrostis* spp. (Jensen *et al.*, 1958; Apt *et al.*, 1960; Courtney *et al.*, 1962), *A. tritici* in wheat (Paruthi *et al.*, 1992) and *A. funesta* in *L. rigidum* (McKay *et al.*, 1982), provided that host grasses are controlled during crop rotational phases. In permanent pastures, rotations are not a viable option for nematode management. Nevertheless, methods based on disrupting the nematode life cycle during pasture phases have been successful in preventing stock losses from annual ryegrass toxicity (ARGT). Price (1973) and McKay *et al.* (1981) demonstrated that *A. funesta* was susceptible to desiccation before the ryegrass reached anthesis. Removal of ryegrass inflorescences by mowing, high stocking rates, use of selective herbicides or desiccant herbicides followed by grazing produced effective control (McKay *et al.*, 1982).

This chapter reports on field experiments conducted in southeastern SA to evaluate some agronomic methods for the management of FPS. The methods evaluated were similar to those which have proven successful for ARGT, since at the commencement of this work the biology of the nematode was not completely known but thought to be similar to that of *A. funesta*.

## 9.2 Materials and Methods

### 9.2.1 Strategic stocking of pastures

Management of nematode populations by strategic stocking of pastures was investigated at Lucindale (Site 2), Reedy Creek (Site 7) and Biscuit Flat (Sites 4 and 5: see Chapter 3.6 for site details). In December 1992, pairs of sampling sites were identified within each paddock with about equal numbers of *P. monspeliensis* tillers, nematode seed galls and bacterially infected inflorescences per square metre. These estimations were based on the mean of three random counts taken within a 250 x 250 mm quadrat and selected areas marked with a steel peg.

Livestock were excluded from one of each pair of sampling sites with a circular weldmesh enclosure, about 5.5 m in diameter, centred on the peg (Plate 9.1). Sheep were moved into the paddocks and allowed to graze the pasture. Fences were erected around the other of the pair of steel pegs in February 1993 giving replicate enclosures, one grazed and one ungrazed. The ungrazed sites were always down-slope of the grazed sites to prevent the exchange of material by water flow during flooding. Counts of the number of *P. monspeliensis* tillers, nematode seed galls and bacterially infected inflorescences per square metre were taken with the same 250 x 250mm quadrat used to select the sites. Both grazed and ungrazed plots were then reassessed in November 1993 and 1994 in the same manner as described above, except that counts of SMGs and tillers infested by *D. alopecuri* (see Chapter 10) were also included, following their recognition in mid 1993.

### 9.2.2 Herbicides

Herbicide experiments were established at Lucindale (Site 2), Reedy Creek (Site 7) and Biscuit Flat (Site 5: see Chapter 3.6 for site details). Sites were selected on the basis of

**Plate 9.1** Circular weldmesh enclosure used to exclude livestock from one of the sampling sites

good numbers of *P. monspeliensis* plants in the preceding year. Fluazifop-p (1.06 g a.i./ha), sethoxydim (60 g a.i./ha) plus the wetting agent DC-tron oil at 14 ml/L and a simazine (500 g a.i./ha) / paraquat (37.5 g a.i./ha) mix were applied in autumn before the pastures became inundated. Before treatment, the plots were grazed at the standard stocking rate for the farm. Stock were excluded from the sites after application.

Randomised complete blocks of 7 treatments (including one untreated), replicated three times with individual plots 10 x 3 m, were used. Treatments were applied with a bicycle wheel plot sprayer with 2 m boom. Grasses ranged between 40 -100 mm tall at application.

Plots were assessed 12 weeks after herbicide application. The efficacy of each treatment was estimated by counting the number of grass plants of each species in three random 500 x 250 mm quadrats per plot at each site.

### 9.2.3 Manipulation of clover composition

The stocking experiments outlined in section 9.2.1, indicated that the proportion of clover present in the pasture may have an effect on the number of nematode galls and bacterially infected inflorescences observed in *P. monspeliensis*. Four experiments were established at 3 sites, *viz*. Lucindale (Site 1), Cape Jaffa (Site 6) and Biscuit Flat (Site 5), in areas where nematode infested *P. monspeliensis* was growing among clover. Each trial consisted of 3 to 6 replicates of both an untreated control plot (1.5 x 3 m) and a treated plot, where clover was removed using the selective herbicide dicamba (140 g a.i./ha). The herbicide was applied with a bicycle wheel plot sprayer with 2 m boom in spring, immediately after flooding had receded.

The treatments were evaluated by taking three random quadrats 500 x 250 mm from each plot at each site. The contents of each quadrat were removed by cutting the plants just below the soil surface and transported back to the laboratory. For each quadrat taken, individual plants were sorted according to species, placed in paper bags and dried at 60°C for 14 days. *Polypogon monspeliensis* and *A. avenacea* were examined for shoot meristem and seed galls before drying. The dry weights were used to determine the percentage of the pasture attributable to each plant species.

### 9.2.4 Statistical analysis

Treatment means were compared using analysis of variance. Where significant differences were detected between the means, the Tukey multiple comparison test (Tukey, 1953) was used to examine the differences between all possible pairs of means.

## 9.3 Results

### 9.3.1 Strategic stocking of pastures

The numbers of tillers produced by *P. monspeliensis*, nematode seed galls and inflorescences colonised by *R. toxicus* observed during the experiment are shown in Table 9.1. There was an overall decline in tiller and gall numbers per $m^2$ from December 1992 to November 1994, which can be attributed to a lower than average rainfall in 1993 and 1994.

By the end of the period of grazing (February 1993), livestock had significantly reduced the total number of seed galls ($P < 0.05$) compared to the ungrazed plots. However, by the end of November 1993, there was no significant difference in the number of seed galls between the grazed and ungrazed plots. The number of tillers and bacterially infected inflorescences in the grazed and ungrazed plots over the same period were not significantly

**Table 9.1** Mean numbers of *Polypogon monspeliensis* tillers, nematode seed and shoot meristem galls and inflorescences colonised by *Rathayibacter toxicus* in grazed and ungrazed plots over a two year period. Data are expressed as numbers per square metre.

| | | Sampling date | | | |
|---|---|---|---|---|---|
| | | December 1992 | February 1993 | November 1993 | November 1994 |
| Tillers | Grazed | 1573 | 1120 | 1052 | 379 |
| | Ungrazed | 1445 | 1461 | 1061 | 541 |
| | | n.s. | n.s. | n.s. | n.s. |
| Seed galls | Grazed | 757 | 448 | 460 | 45 |
| | Ungrazed | 800 | 842 | 579 | 70 |
| | | n.s. | * | n.s. | n.s. |
| Bacterial inflorescences | Grazed | 26 | 11 | 85 | 1 |
| | Ungrazed | 38 | 13 | 142 | 0.7 |
| | | n.s. | n.s. | n.s. | n.s. |
| Shoot meristem galls[1] | Grazed | | | | 54 |
| | Ungrazed | | | | 43 |
| | | | | | n.s. |

* = $P < 0.05$; n.s. = not significant

[1] Shoot meristem galls were first recognised in 1993 and thus were not able to be scored until 1994.

different. Likewise, in November 1994, there was no significant difference in number of tillers, seed galls and bacterially colonised inflorescences between grazed and ungrazed plots. SMGs were only assessed at the end of the experiment as it was necessary to remove the *P.monspeliensis* plants to score them. Although the number of SMGs present at the start of the experiment were unknown, there was no significant difference in their numbers between the grazed and ungrazed plots at the end of the experiment.

The exclusion of livestock from the ungrazed plots for two years encouraged the growth of clover and it was noted that the number of SMGs produced per plant was lower where the clover component was high enough to form a canopy. Regression analysis of the data (Figure 9.1) indicates that an increase in the proportion of clover (as determined by dry matter production) results in a decrease in the number of SMGs per $m^2$ ($P < 0.05$) but has no effect on the number of seed galls per $m^2$.

### 9.3.2 Herbicide

*Polypogon monspeliensis*, *Hordeum* sp., *Lolium* sp. and *Poa annua* L. were the major components of the pastures in which the herbicide experiments were conducted. Despite good growth in the preceding year, *P. monspeliensis* was not present in the Reedy Creek site when the treatments were evaluated. At the two remaining sites, each of the herbicides tested gave excellent control of *P. monspeliensis* (Table 9.2). The efficacy of the treatments on the other grasses varied between herbicides, species and sites. For control of grasses other than *P. annua*, fluazifop-p was the most effective treatment, followed by sethoxydim and the simazine\paraquat mixture. Plots in which *P. monspeliensis* was controlled before flooding remained bare throughout the season.

**Figure 9.1** The relationship between the percentage of clover in experimental plots and the number of shoot meristem galls recovered from those plots. The regression line describes an exponential function such that the number of shoot meristem galls = -1.03 + exp (5.26 + (-0.11) * clover%).

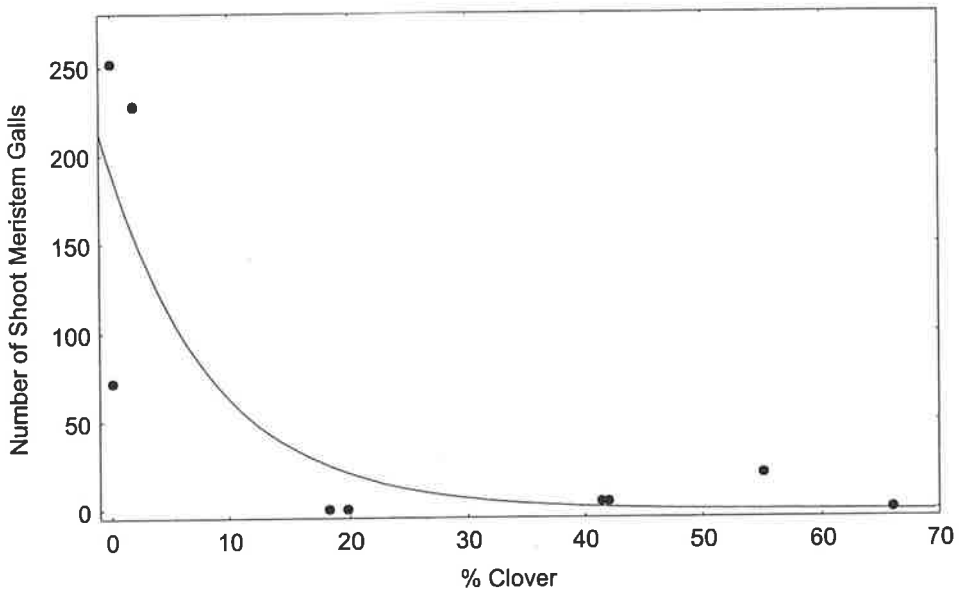


**Table 9.2** Efficacy of herbicides applied during autumn to control *Polypogon monspeliensis* and other grasses at three sites. Data are mean numbers of plants per square metre ± standard deviation

| Site | Treatment | *Polypogon monspeliensis* | *Hordeum* sp. | *Lolium* sp. | *Poa annua* |
|---|---|---|---|---|---|
| Biscuit Flat | Untreated | 100 ± 141 | 12878 ± 4517 | 2276 ± 2083 | 4807 ± 3650 |
| | Fluazifop-p | 0 | 7 ± 10 | 108 ± 81 | 418 ± 232 |
| | Sethoxydim | 0 | 7004 ± 2569 | 526 ± 613 | 448 ± 477 |
| | Simazine / Paraquat | 0 | 10375 ± 914 | 1707 ± 933 | 0 |
| Reedy Creek | Untreated | 0 | 6240 ± 1825 | 1223 ± 145 | 0 |
| | Fluazifop-p | 0 | 0 | 24 ± 9 | 0 |
| | Sethoxydim | 0 | 3243 ± 544 | 107 ± 136 | 0 |
| | Simazine / Paraquat | 0 | 5532 ± 745 | 772 ± 43 | 0 |
| Lucindale | Untreated | 1522 ± 939 | 36 ± 50 | 128 ± 76 | 5788 ± 412 |
| | Fluazifop-p | 0 | 0 | 0 | 544 ± 130 |
| | Sethoxydim | 64 ± 91 | 0 | 0 | 1438 ± 1083 |
| | Simazine / Paraquat | 71 ± 61 | 0 | 123 ± 72 | 100 ± 141 |

### 9.3.3 Manipulation of clover composition

Three of the experiments (Sites 5 and 6) were abandoned because the *P. monspeliensis* population was overrun by other species, due to lack of flooding. The changes in pasture composition in experimental plots at the remaining site after removing clover with a selective herbicide are shown in Table 9.3. The presence of clover significantly reduced the dry matter production of *P. monspeliensis* ($P < 0.001$), *A. avenacea* ($P < 0.001$) and *Hordeum* sp. ($P < 0.05$). However, the biggest impact was on *P. monspeliensis* where its proportion of the total grass dry matter was reduced by 10.4% when clover was present. *Polypogon monspeliensis* also produced signicantly fewer tillers per plant ($P < 0.01$) when clover was present in the plots (Plate 9.2). The effect of the treatments on nematode gall numbers could not be determined as galls were only found in a single plot.

## 9.4 Discussion

The results presented in this chapter must be considered preliminary as many important features of the nematode life cycle, such as the occurrence of SMG, only became apparent after experiments had commenced. Drier than average conditions during the experiments also resulted in *P. monspeliensis* being out-competed by less flood tolerant species and subsequently there was a reduction in nematode populations. Some field experiments failed to establish and these have not been discussed. While recommendations can not be made on the data presented herein, the experiments have raised interesting possibilities that should be explored further.

### 9.4.1 Strategic stocking of pastures

This study set out to determine if better management of livestock could be used to reduce nematode populations, thereby reducing the risk of flood plain staggers. McKay (1985)

**Table 9.3** Changes in pasture composition as a result of removing clover with the selective herbicide dicamba. Data are percentage compositions based on dry weight.

| Pasture Species | Untreated | Treated | |
|---|---|---|---|
| *Polypogon monspeliensis* | 2.55 | 17.85 | *** |
| *Agrostis avenacea* | 13.80 | 39.05 | *** |
| *Lolium* sp. | 2.60 | 15.10 | n.s. |
| Clover | 62.71 | 0 | not testable |
| *Hordeum* sp. | 7.14 | 16.38 | * |
| Other (Broadleaf) | 11.20 | 11.62 | n.s. |

* = $P < 0.05$; **=$P < 0.01$; ***=$P < 0.001$; n.s. = not significant

**Plate 9.2** *Polypogon monspeliensis* plants before and after the removal of clover with the selective herbicide dicamba, respectively

suggested that if annual ryegrass inflorescences are consumed before shattering occurred, that there would be a high reduction in the *A. funesta* populations, given that the survival rate of nematodes passing through animals is low (Price, 1993). The seed galls produced on *P. monspeliensis* remain attached to the rachis even after the seed has been shed (McKay et al., 1993). Thus, the reduction in seed gall numbers observed is likely to be a reasonable estimate of the effectiveness of grazing. Even though livestock effectively removed 40% of seed galls, the reduction in inoculum did not appear to influence the number of seed galls produced at the end of the following year. This is probably due to the occurrence of SMGs, which are produced in tillers close to the ground (Chapter 7.3.3) and are unlikely to be consumed by livestock.

The proportion of SMGs was lower where a reasonably high clover component was present. Shading from the clover canopy probably inhibited development of new tillers in spring, resulting in less opportunity for nematodes to produce SMGs. The opposite was true in grass dominant plots where a more open canopy allowed new tillers to be initiated. Tillers produced in the spring tend to remain short and can be heavily infected by *R. toxicus* (Chapter 7.3.7), which inhibits the emergence of the inflorescence. These short tillers are likely to be consumed when livestock graze dry annual clover residues on the ground or short summer growth on perennial clovers. Thus, these short tillers are likely to be the main source of corynetoxins consumed by livestock.

The grazing pressure that is required to totally remove the seed galls would be hard to sustain in both SA and NSW, but for different reasons. *Polypogon monspeliensis* inflorescences are not very palatable to livestock and are not significantly grazed (Black, 1980). Thus, using grazing as a strategy to disrupt the nematode lifecycle before nematodes in the gall reach their survival stage is unlikely to be successful. The method works for

ARGT because inflorescences of *L. rigidum* are palatable and stock often preferentially graze them (McKay *et al.*, 1985). The pastures in northern NSW are extensive, with low stocking rates, making this method untenable. In NSW, methods of control that require low inputs are needed.

The discovery of *D. alopecuri* in *P. monspeliensis* is worth pursuing. This is the first record of the pathogen on this host and associated with this nematode. While strictly a weak plant pathogen, *D. alopecuri* appears to be providing useful control of *A. funesta* populations in Western Australia (Riley, 1994) by destroying the galls in which the nematode reproduces. The potential of this new isolate as a biological control agent is investigated in Chapter 10.

### 9.4.2 Herbicide

All the herbicides tested reduced the number *P. monspeliensis* plants. Although the grass selective herbicides were more effective, their use requires careful planning as repeated use can lead to herbicide resistance. Increased herbicide resistance has been reported to be associated with higher populations of *A. funesta* (Riley and Gill, 1994). There are currently no reports of resistant *P. monspeliensis* in the literature.

While *P. monspeliensis* is able to out-compete other plant species in flood prone areas, it only becomes a significant pasture component when flooding is prolonged. In low lying areas that remain inundated for extended periods, *P. monspeliensis* is often the only plant species present of any significance. When *P. monspeliensis* is removed from these areas, the ground remains bare except for plants such as *Crassula* sp. and the effects can still be seen in the following season. This is undesirable as there will be a total loss of productivity.

Other barriers to herbicide use as a result of flooding include inappropriate timing of application due to free water or boggy conditions. Application of herbicide directly into free standing water is unlikely to be environmentally sound. In northern NSW, the logistics of treating large areas of pasture makes this management option non-viable, particularly with selective herbicides which are more expensive.

### 9.4.3 Clover

The results indicate that allowing the clover to produce a canopy will not only reduce the overall amount of *P. monspeliensis* but also reduce the total number of tillers produced. A similar reduction in tiller number has been observed in *L. rigidum* when growing with subterranean clover (Mckay et al., 1981; 1982). Thus, fewer tillers will be available for colonisation by the nematode and bacterium. In addition, a closed canopy will reduce the incident light at the base of the plants, which should discourage the production of short tillers.

In grass dominant plots, many short tillers were produced in spring. These short tillers were found to be the most heavily infected with *R. toxicus* (Chapter 7.3.7) and are likely to be consumed while livestock graze dry clover residues or short summer growth. They are likely to be the main source of toxin consumed by stock.

## 9.5 Conclusions

The results obtained so far indicate that grazing will reduce the production of the seed galls but will only have a limited effect on the nematode population, possibly because of the

contribution of the SMG galls. Thus, in a tightly grazed pasture it is expected that grazing will only reduce total inoculum by 50%. The actual reduction is likely to be less as the mature inflorescence of *P. monspeliensis* is unpalatable, and a short canopy could result in higher numbers of tillers per plant in the spring, increasing the potential number of SMG produced. Herbicides can succesfully remove *P. monspeliensis* from pastures but care must be taken ensure it is replaced by other pasture species. New cultivars of Persian clover (e.g. Kyambro) which are tolerant of some flooding have been developed but were not able to be tested during this study.

A better option may be to graze the pasture lightly in spring to allow the clover to form a canopy. This, along with *D. alopecuri*, are the only treatments so far that have potential to reduce the number of SMG. It is clear that treatments that do not control these galls will have a limited effect in reducing the problem. Inundation adds a further constraint to possible management strategies as pastures can not be grazed or easily managed during this period.

# Chapter 10

# Evaluation of strains of *Dilophospora alopecuri* as biological control agents

## 10.1 Introduction

The potential for using *D. alopecuri* for biological control of ARGT has been recognised for some time. Naturally occurring populations of the fungus are providing some control (Riley, 1994) and are spreading (Riley, 1994b), albeit slowly. Experimental field inoculations have demonstrated that it is possible to successfully introduce the fungus into an area and impact on nematode numbers in the year of application (Riley, 1994), paving the way for introductions of *D. alopecuri* into areas where outbreaks of ARGT are still common and the fungus has not reached.

Investigations into the biocontrol potential of *D. alopecuri* have primarily been centred on isolates from *L. rigidum* (McKay *et al.*, 1981; Riley, 1994b). However, the fungus is also known to colonise *H. lanatus* (Riley and McKay, 1990) and was discovered infecting *P. monspeliensis* in the strategic stocking experiments described in Chapter 9.2.1. It is unclear whether these strains of *D. alopecuri* are identical to that found on *L. rigidum* and may represent a potential source for the selection of improved biocontrol strains

In this chapter, isolates of *D. alopecuri* from different host grasses were evaluated for possible differences in pathogenicity and suppression of nematode reproduction. The genetic diversity between the isolates was then examined by allozyme electrophoresis to determine if there is a genetic basis for the differences observed. The allozyme analysis of isolates was part of a larger study, in association with researchers from Agriculture

Western Australia and the Evolutionary Biology Unit of the South Australian Museum, investigating genetic variation in *D. alopecuri* within Australia. The allozyme results have been published in Riley *et al.* (1998).

## 10.2 Materials and Methods

### 10.2.1 Fungal isolates and cultures

The fungal isolates referred to throughout this chapter are listed in Table 10.1. These were isolated on potato dextrose agar (Sigma) amended with 0.01% aureomycin and then maintained on PDA at 25°C under cool white and ultraviolet fluorescent tubes with a 12 hour photoperiod to promote sporulation.

Cultures for allozyme analysis were grown in 50 ml of broth containing sucrose (10 g/L), hydrolysed casein (Sigma, 2 g/L), yeast extract (Difco, 2 g/L), $K_2HPO_4$ (2 g/L) and $MgSO_4 \cdot 7H_2O$ (0.3 g/L) in 250 ml Erlenmeyer flasks on an orbital shaker at 250 rpm at 25°C for 14 days. *D. alopecuri* cultures grew as a single suspended mass with a band of growth on the glass at the upper liquid level. The growth was harvested and washed three times in sterile phosphate buffered saline (PBS, 0.01M $NaPO_4$, 0.15M NaCl, pH 7.2) at 5°C.

### 10.2.2 Preparation of fungal inocula

Fungal inocula to be used for testing isolate pathogenicity and effect on nematode reproduction were prepared by Dr I. Riley using a combination of isolates DA34 and DA35 for *D. alopecuri* ex *Polypogon*, DA29 - DA33 for *D. alopecuri* ex *Holcus* and DA28 for *D. alopecuri* ex *Lolium* (Refer to Table 10.1). Cultures of each isolate were used to inoculate

**Table 10.1** Laboratory designations, collection details (host plant, location, date, collector and other designations) and electrophoretic type (ET) of *Dilophospora alopecuri* strains used. The derivation of the ETs follows in the results (10.3.3).

| Lab. No. | Collection details | ET |
|---|---|---|
| DA20 | *Lolium rigidum*, Narrogin, WA, 1986, M. J. Barbetti. WAC 5513 | E1 |
| DA23 | *Avena sativa,* Katanning, WA, 1984, M. J. Barbetti. WAC 8135. | E2 |
| DA25 | *Hordeum vulgare*. Katanning, WA, 1984, M. J. Barbetti. WAC 8137 | E1 |
| DA28 | *Lolium rigidum*, Corrigin (Lomos), WA, 1992, I. T. Riley. WAC 8367 | E1 |
| DA29 | *Holcus lanatus*, Glencoe, SA, 1994, A. C. McKay. WAC 8368, IMI 366177 | E4 |
| DA30 | *Holcus lanatus,* Glencoe, SA, 1994, A. C. McKay. WAC 8369 | E4 |
| DA31 | *Holcus lanatus,* Glencoe, SA, 1994, A. C. McKay. WAC 8370 | E4 |
| DA32 | *Holcus lanatus*, Glencoe, SA, 1994, A. C. McKay. WAC 8371 | E4 |
| DA33 | *Holcus lanatus*, Glencoe, SA, 1994, A. C. McKay. WAC 8372 | E1 |
| DA34 | *Polypogon monspeliensis*, Lucindale, SA, 1993, T. Bertozzi. WAC 8373, IMI 366178 | E9 |
| DA35 | *Polypogon monspeliensis*, Lucindale, SA, 1993, T. Bertozzi. WAC 8374 | E9 |
| DA52 | *Lolium rigidum*, Broomehill, WA, 1992, I. T. Riley | E1 |
| DA68 | *Lolium rigidum*, Boundain, WA, | E1 |
| DA77 | *Lolium rigidum*, Jerramungup, WA, 1992, I. T. Riley. | E1 |
| DA78 | *Agrostis tenuis*. UK, 1961, N. Jackson. IMI 93039 | E5 |
| DA79 | *Holcus lanatus*, Oregon, USA, 1938, R. Sprague. CBS 165.38 | E7 |
| DA80 | *Phalaris arundinacea*, Germany, 1968, U. G. Schlosser. CBS 315.68 | E6 |
| DA81 | *Lolium rigidum*, Black Springs, SA, 1994, A. C. McKay | E8 |
| DA82 | *Lolium rigidum,* Horsham, Vic, 1994, A. C. McKay. | E1 |
| DA83 | *Holcus lanatus,* Mount Gambier, SA, 1995, T. Bertozzi | E3 |
| DA85 | *Lolium rigidum,* Neales Flat via Eudunda, SA, 1995, A. C. McKay | E10 |

wheat, which was prepared by autoclaving (121°C for 20 min) Schott bottles containing 500 g of grain three times, leaving one day between the first and second treatments and two days between the second and third. Cultures were grown at 25°C under the same conditions as for isolate maintenance and after an initial period of four days were shaken regularly every 2 days. Once the grains had blackened (due to the presence of pycnidia), the wheat inoculum was air-dried for one week by spreading on paper towel and maintaining at room temperature. Where a mixture of isolates was used, these were combined after drying.

### 10.2.3 Isolate pathogenicity and effect on nematode reproduction

Eighty seedlings each of *P. monspeliensis*, *A. avenacea* (NSW ecotype) and *L. rigidum* were prepared and transplanted into tubes containing potting compost as described in Chapter 3.7. Seed of *P. monspeliensis* and *A. avenacea* came from the same sources listed in Table 5.1. Seed of *L. rigidum* was provided by Dr A. C. McKay. The tubes were arranged in mesh crates (see Chapter 3.7), with each crate containing 10 replicate tubes of a single grass species (a total of eight crates per species), and the crates randomly arranged in solarised sand beds. After 21 days of growth, the tubes in four crates of each grass species were inoculated with two seed galls of their associated nematode parasite, *viz. P. monspeliensis* with *Anguina* sp. ex *Polypogon*, *A. avenacea* with *Anguina* sp. ex *Agrostis* and *L. rigidum* with *A. funesta*. The seed galls were placed just below the soil surface to ensure they would not be dislodged by wind or rain. One and a half grams of each of the fungal inocula described in section 10.2.2 and a wheat inoculum control was applied to the tubes in two crates (one inoculated with nematodes and one without nematodes) for each grass species and 500 mm high partitions were erected between them in an effort to control splash dispersal of nematode or fungal inocula.

The pathogenicity of each isolate on each host was assessed by scoring the percentage leaf area affected by the fungus on four leaves for each plant, seven weeks after inoculation. The two uppermost fully emerged leaves on the tiller judged closest to due East and the one immediately to the left of it were visually scored from 0 - 100% using leaf disease assessment keys (James, 1971). As no assessment keys were available for *D. alopecuri*, the rating schemes for *Septoria* leaf blotch of cereals and bacterial black chaff of wheat (*Xanthomonas translucens* (Jones, Johnson & Reddy 1917) Vauterin *et al.* 1995), which most closely matched the symptoms observed, were used. The smallest lesion was designated as 0.1%.

The efficacy of each fungal isolate in controlling nematode reproduction in each host was assessed by recording the number of nematode seed galls produced by *A. funesta* in *L. rigidum* and both the number of SMG and seed galls produced by the other nematodes in *P. monspeliensis* and *A. avenacea*. For *A. funesta*, all inflorescences from a single replicate were combined, threshed and the seed examined using a light box to detect seed galls.

Treatment means were compared using analysis of variance. However, as the possibility of cross-contamination of fungal and nematode inocula necessitated an experimental design in which the error of each observation may not be independent, the significance level was set at 1%. In addition, the percentage leaf area and gall count data sets were arcsine($\sqrt{(x/100)}$) and $\ln(x + 1)$ transformed respectively before analysis to satisfy the assumptions of normality and homoscedasticity. Where significant differences were detected between the means, the Tukey multiple comparison test (Tukey, 1953) was used to examine the differences between all possible pairs of means.

### 10.2.4 Allozyme analysis of isolates

***Sample preparation***

The harvested fungal cultures were washed, squeezed between blotting paper to remove excess PBS and then diced into 2 mm square pieces. About 100 mg of diced fungus was placed in a 2 ml Eppendorf tube to which two volumes of buffered lysing solution (0.02M Tris-HCl, pH 8.0, containing 0.2% β-mercaptoethanol and 0.02% NADP ) per weight of fungus was added. The fungus was disrupted by brief sonication bursts with a Branson sonifier (model B12 ), with a micro-tip attachment. The samples were then centrifuged for 5 min at 10000 *g* and 20 µl aliquots of the supernatant were drawn into glass capillary tubes which were stored at -20°C until used for electrophoresis.

***Electrophoresis***

Allozyme electrophoresis was conducted on cellulose acetate gels (Cellogel®), following Richardson *et al.* (1986). Twenty three enzymes produced zymograms of sufficient intensity and resolution for genetic interpretation. The enzymes tested, their Enzyme Commission numbers, the loci scored and run buffers are given in Appendix 1. Allozymes were designated alphabetically and multiple loci, where present, were designated numerically, both in order of increasing electrophoretic mobility. Those strains which had identical alleles for all loci were grouped together as electrophoretic types (ETs). A matrix of pairwise comparisons between ETs based on the pecentage of loci that had different alleles between pairs, was constructed. From this matrix, phenograms showing relationships between ETs were generated using the unweighted pair group method of analysis (UPGMA) and average linkage cluster method (Sneath & Sokal, 1973).

## 10.3 Results

### 10.3.1 Pathogenicity of isolates

Lesions characteristic of *D. alopecuri* infection were observed in all three grass species in treatments containing nematodes and *D. alopecuri* inoculum. In the absence of nematodes or *D. alopecuri* inoculum, no lesioning was observed on any plant part for any treatment / grass combination. *D. alopecuri* ex *Polypogon* infection was characterised mainly by leaf spots while the other two strains also produced shiny black striate lesions on both leaves and stems. The mean percentage area of the leaves assayed that were covered by lesions is presented in Table 10.2. The interaction between *D. alopecuri* strain and host grass was highly significant ($P < 0.001$), indicating that the differences observed in the area lesioned for each strain was not constant in all the host grasses tested. This was principally due to the *D. alopecuri* ex *Polypogon* strain. While this strain produced a significantly larger area of lesions ($P < 0.001$) than the other strains in all three host grasses, the variability in lesioning between hosts was also highly significant ($P < 0.001$). Although the *D. alopecuri* ex *Holcus* strain also showed a variable response, the variability was limited to *P. monspeliensis*.

### 10.3.2 Effect on gall production

The mean total number of galls produced by the nematodes on each of the three grasses in response to inoculation with different strains of *D. alopecuri* are shown in Table 10.3. For *P. monspeliensis* and *A. avenacea*, this is a combination of both seed galls and SMG. The interaction between *D. alopecuri* strain and host grass was again highly significant ($P < 0.001$), limiting comparisons to cell rather than treatment means. In *L. rigidum*, *A. funesta* produced significantly fewer seed galls ($P < 0.001$) when inoculated with the *D. alopecuri*

**Table 10.2** Mean percentage leaf area covered by lesions in three host grasses by three strains of *Dilophospora alopecuri*. Data are back-transformed means. Superscripts indicate significance of cell means at the 0.1% level both within columns (a, b) and rows (x-z). The control treatment has been excluded as no lesions were observed.

| Fungal inoculum source | Host Grass | | | Mean |
|---|---|---|---|---|
| | *Lolium rigidum* | *Polypogon monspeliensis* | *Agrostis avenacea* | |
| ex *Lolium* | $0.008^{ax}$ | $0.024^{ax}$ | $0.018^{ax}$ | 0.016 |
| ex *Holcus* | $0.026^{ax}$ | $0.933^{ay}$ | $0.068^{ax}$ | 0.215 |
| ex *Polypogon* | $6.935^{bx}$ | $16.249^{by}$ | $0.625^{bz}$ | 6.291 |
| Mean | 0.942 | 3.055 | 0.156 | |

**Table 10.3** Mean total number of galls recovered from three host grasses after inoculation with three strains of *Dilophospora alopecuri*. Data are back-transformed means. Superscripts indicate significance of cell means at the 0.1% level both within columns (a-c) and rows (x, y).

| Fungal inoculum source | Host Grass | | | Mean |
|---|---|---|---|---|
| | *Lolium rigidum* | *Polypogon monspeliensis* | *Agrostis avenacea* | |
| Control | $23.567^{bx}$ | $14.123^{ax}$ | $1.446^{ay}$ | 9.464 |
| ex *Lolium* | $20.338^{bx}$ | $19.641^{ax}$ | $20.033^{cx}$ | 21.162 |
| ex *Holcus* | $18.279^{bx}$ | $8.199^{ax}$ | $6.936^{bx}$ | 10.207 |
| ex *Polypogon* | $0.149^{ax}$ | $4.999^{ay}$ | $0.374^{ax}$ | 1.116 |
| Mean | 10.451 | 10.457 | 3.867 | |

ex *Polypogon* strain. The other strains were not significantly different from the control or each other. In *P. monspeliensis*, there was no significant difference in the number of nematode galls recovered between any of the strains. In *A. avenacea*, the number of nematode galls produced by *Anguina* sp. was significantly different for each strain of *D. alopecuri* ($P < 0.001$). Surprisingly, significantly more galls were recovered after inoculation with the ex *Lolium* and ex *Holcus* strains than from the control treatment.

If the number of seed and shoot meristem galls produced by the nematodes in *P. monspeliensis* and *A. avenacea* are considered separately, reanalysis indicates that the interaction between gall type and fungal strain is highly significant for both hosts ($P < 0.001$). In *P. monspeliensis*, this is almost solely due to the variability in the control treatment (Table 10.4). If the control treatment data is omitted from the analysis, there is no significant interaction or gall type effect, only a significant fungal strain effect which is reflected in the significance of the cell means for SMG shown in Table 10.4. In *A. avenacea*, very few SMG galls were produced in any of the treatments (Table 10.5). This was the main contributor to the highly significant interaction effect observed, but had little influence on the ranking of the fungal treatments. Inoculation with *D. alopecuri* ex *Polypogon* significantly reduced the number of seed galls that were produced in comparison with the other strains. The other strains were not significantly different from each other but were significantly different from the control treatment.

### 10.3.3 Allozyme analysis

A total of 27 presumptive loci were scorable and the 21 isolates of *D. alopecuri* and the *Phoma* grouped into eleven electrotypes according to their allelic profiles (Table 10.6). Twenty five of the loci showed polymorphisms and the allelic profiles were consistent with

**Table 10.4** Mean number of seed and shoot meristem galls (SMG) produced on *Polypogon monspeliensis* after inoculation with three strains of *Dilophospora alopecuri*. Data are back-transformed means. Superscripts indicate significance of cell means at the 1% level both within columns (a-c) and rows (x, y).

| Fungal inoculum source | Gall type | | Mean |
|---|---|---|---|
| | Seed gall | Shoot meristem gall | |
| Control | $0.750^{a\,x}$ | $12.503^{c\,y}$ | 3.862 |
| ex *Lolium* | $10.858^{b\,x}$ | $8.136^{bc\,x}$ | 9.408 |
| ex *Holcus* | $4.521^{ab\,x}$ | $3.074^{ab\,x}$ | 3.743 |
| ex *Polypogon* | $3.595^{ab\,x}$ | $1.120^{a\,y}$ | 2.121 |
| Mean | 3.790 | 4.713 | |

**Table 10.5** Mean number of seed and shoot meristem galls (SMG) produced on *Agrostis avenacea* after inoculation with three strains of *Dilophospora alopecuri*. Data are back-transformed means. Superscripts indicate significance of cell means at the 1% level both within columns (a, b) and rows (x, y).

| Fungal strain | Gall type | | Mean |
|---|---|---|---|
| | Seed gall | Shoot meristem gall | |
| Control | $1.218^{a\,x}$ | $0.149^{a\,y}$ | 0.596 |
| ex *Lolium* | $19.697^{b\,x}$ | $0.231^{a\,y}$ | 4.048 |
| ex *Holcus* | $6.761^{b\,x}$ | $0.149^{a\,y}$ | 1.986 |
| ex *Polypogon* | $0.196^{a\,x}$ | $0.149^{a\,x}$ | 0.172 |
| Mean | 3.544 | 0.169 | |

**Table 10.6** Allelic profiles of ten *Dilophospora alopecuri* strains (E1-E10) and one *Phoma* strain (E11).

| Locus | Electrophoretic type | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | E1 | E2 | E3 | E4 | E5 | E6 | E7 | E8 | E9 | E10 | E11 |
| *Acon-1* | a | a | a | a | a | b | e | c | c | c | d |
| *Enol* | c | c | c | c | c | - | a | b | b | b | b |
| *Est-5* | b | b | b | b | b | b | b | a | a | b | c |
| *Fdp* | b | b | b | b | b | - | b | a | a | a | b |
| *Fum* | b | b | b | b | b | b | - | c | c | c | a |
| *Ga3pd* | a | a | a | a | a | a | b | a | a | a | a |
| *Glo* | c | a | c | a | a | a | - | c | c | c | b |
| *Got-1* | c | c | c | c | c | c | - | b | a | a | c |
| *Got-2* | b | b | c | c | b | b | d | a | a | a | b |
| *G6pd* | a | a | a | a | a | a | a | b | b | b | a |
| *Gpi* | b | b | a | a | b | c | ad | c | c | c | e |
| *Hk* | a | a | a | a | a | a | a | a | a | a | b |
| *Idh* | a | a | a | a | a | a | b | b | b | b | cd |
| *Lap-1* | b | b | b | b | a | b | c | c | c | c | d |
| *Lap-2* | b | b | a | a | b | c | c | c | c | c | d |
| *Mdh-1* | b | b | b | b | b | b | d | a | a | b | c |
| *Mdh-2* | b | b | b | b | b | b | b | b | b | b | a |
| *Mpi* | b | b | b | b | b | b | c | b | b | a | b |
| *Ndpk* | a | a | a | a | a | a | c | a | a | a | b |
| *Pep-A* | a | a | a | a | a | a | c | b | b | b | b |
| *Pep-C* | b | b | b | b | a | b | d | c | c | c | d |
| *Pep-D1* | c | c | c | c | b | c | - | ab | ab | ab | d |
| *Pep-D2* | b | b | b | b | b | a | c | e | e | e | d |
| *6pgd* | b | b | b | b | b | c | a | b | b | b | b |
| *Pgk* | b | b | b | b | b | b | c | a | a | a | d |
| *Pgm-2* | a | a | a | a | a | a | b | c | c | c | d |
| *Tpi* | b | b | b | b | b | b | bc | a | a | a | b |

*D. alopecuri* being a haploid asexually reproducing organism. An extra allele was present in several cases; for GPI and TPI in electrophoretic type E7, for PepD1 in electrophoretic types E8-E10 and for IDH in electrophoretic type E11. However, in each instance, the allelic patterns were not typical of heterozygous patterns shown by diploid organisms.

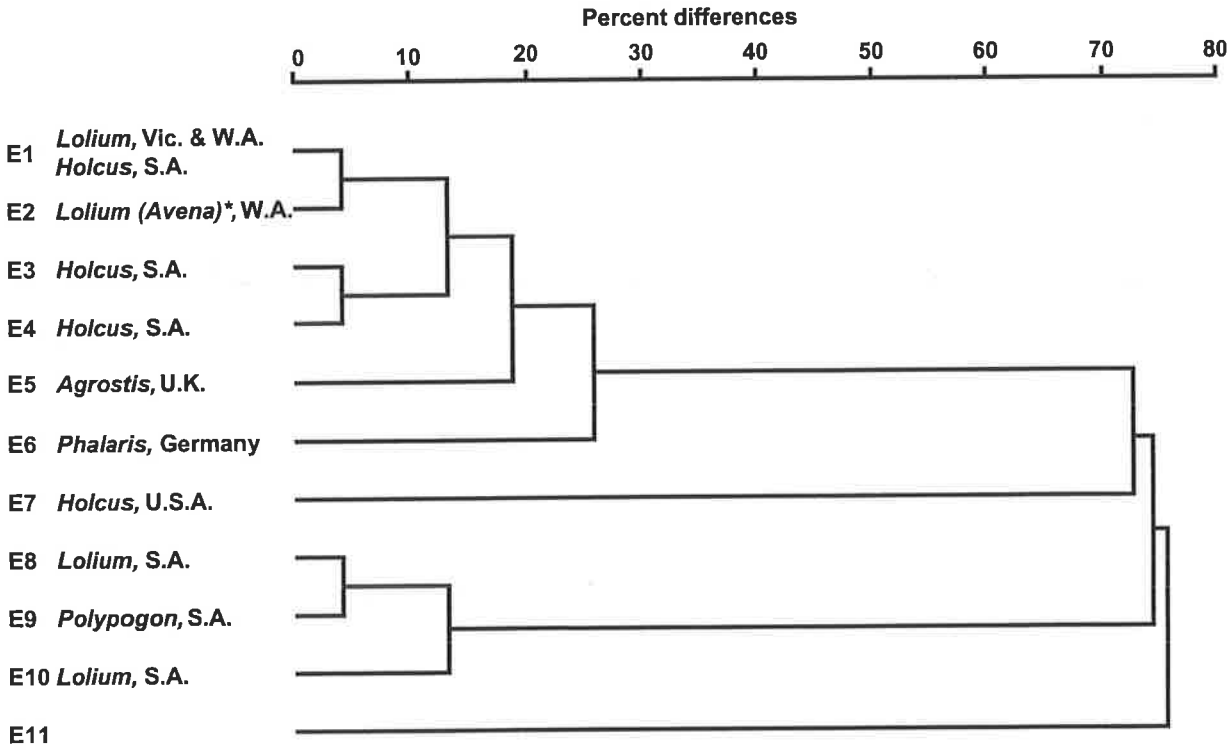
The matrix of pairwise comparisons between electrophoretic types based on percent of loci differing between electrophoretic types is given in Table 10.7. The relationship between ETs given by the UPGMA dendrogram (Figure 10.1) differed only slightly from that of the average linkage cluster method dendrogram, thus only the former is presented.

Electrophoretic types E1 and E2 differed by a single locus and contained all seven strains from *L. rigidum* from WA, the only strain from *L. rigidum* from Victoria and one strain (DA33) from *H. lanatus* from SA. Electrophoretic types E3 and E4 also differed by a single locus and contained strains isolated from *H. lanatus* from SA. These four electrophoretic types formed a group that differed by less than an average of 15%. Each of the strains obtained from international culture collections were distinct. E5 and E6 were most closely related to the group E1 to E4, differing by an average of 23 to 27%. However, electrophoretic type E7 (strain DA79 from *H. lanatus*), was quite distinct from all other strains, including those from *H. lanatus* from Australia., differing by at least an average of 70%. Electrophoretic types E8 to E10 formed another distinct group differing from other *D. alopecuri* strains by an average of over 74%. This group consisted of strains from *L. rigidum* and *P. monspeliensis* from SA. The *Phoma* strain (electrophoretic type E11) differed by an average of 76% from the *D. alopecuri* strains.

**Table 10.7** Matrix of percent differences of loci between pairs of electrophoretic types of *Dilophospora alopecuri* strains (E1-E10) and one *Phoma* strain (E11)

| | E1 | E2 | E3 | E4 | E5 | E6 | E7 | E8 | E9 | E10 | E11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **E1** | 0 | | | | | | | | | | |
| **E2** | 4 | 0 | | | | | | | | | |
| **E3** | 15 | 11 | 0 | | | | | | | | |
| **E4** | 11 | 15 | 4 | 0 | | | | | | | |
| **E5** | 26 | 22 | 11 | 15 | 0 | | | | | | |
| **E6** | 28 | 24 | 20 | 24 | 32 | 0 | | | | | |
| **E7** | 70 | 70 | 74 | 74 | 74 | 71 | 0 | | | | |
| **E8** | 74 | 78 | 78 | 74 | 74 | 72 | 78 | 0 | | | |
| **E9** | 74 | 78 | 78 | 74 | 74 | 72 | 78 | 4 | 0 | | |
| **E10** | 70 | 74 | 74 | 70 | 70 | 68 | 74 | 15 | 11 | 0 | |
| **E11** | 74 | 74 | 70 | 70 | 70 | 76 | 83 | 81 | 81 | 85 | 0 |

**Figure 10.1** Phenogram showing relationships among *Dilophospora* electrophoretic types (E1-E10, host and region indicated) and one *Phoma* electrophoretic type (E11) from allozyme data based on unweighted pair group method analysis. *E2 was isolated from *Avena* from a site where *Lolium* was the predominent host.



## 10.4 Discussion

### 10.4.1 Pathogenicity of isolates

The strains of *D. alopecuri* investigated in this study appear to be neither host nor vector specific. However, this may not hold for all combinations of *D. alopecuri* strains and *Anguina* nematodes, as differences in adhesion characteristics of the spores to different nematodes have already been demonstrated (Riley and McKay, 1990). The fact that no lesions were found on hosts in the absence of nematodes, indicates that *Anguina* nematodes are required for the successful colonisation of host grasses by *D. alopecuri* under local conditions. These findings are in agreement with recent studies on *D. alopecuri* within Australia (Riley, 1994; Riley,1996). Recent international studies have reported infection in the absence of nematodes but the methods used to inoculate plants were not typical of normal field infection. Macek and Koncan (1988) studied resistance to *D. alopecuri* in eight wheat cultivars but could only achieve infection following wounding. Winder (1999) investigated the use of *D. alopecuri* as a biological control agent for marsh reed grass (*Calamagrostis canadensis* [Michx.] Beauv.) by inoculating seedlings with an artists paint brush. It is possible that this inoculation method may have damaged the plant cuticle, assisting pathogen entry into the plant. Interestingly, both grass genera are known hosts of species of *Anguina*.

*D. alopecuri* ex *Polypogon* was the most pathogenic strain tested and the ex *Lolium* strain the least for each host. However, the mean leaf area covered by lesions produced by all strains was almost always higher in *P. monspeliensis* and *A. avenacea*. As each host has a unique nematode vector associated with it, the individual contributions of the nematode and the host cannot be separated. Nevertheless, there are some important differences

between the nematode species in these hosts compared to *A. funesta* in *Lolium* that may partly explain the differences.

Both *P. monspeliensis* and *A. avenacea* have nematode vectors that feed and initiate galls in the shoot apical meristem and this may influence disease expression in a number of ways. Firstly, the mechanical damage to plant tissues by feeding nematodes may increase potential infection sites by creating points for opportunistic entry of the fungal hyphae. Wounding has been demonstrated to assist *D. alopecuri* infection (Macek and Koncan, 1988) and has been documented for a number of nematode/fungus interactions such as *Meloidogyne incognita* (Kofoid and White 1919) Chitwood 1949 and *Thielaviopsis basicola* (Berk. And Broome) Ferraris (Walker *et al.*, 1999) and *Radopholus similis* Cobb 1893 and *Cylindrocarpon musae* Booth and Stover (Booth and Stover, 1974). While this may be a factor in colonisation of developing galls by the fungus, it does not adequately explain the presence of lesions on leaves, which cease to be formed after gall initiation begins (see Chapter 7).

It is more likely that the interaction of the nematode with the host grass makes the host more susceptible to fungal attack. It has been demonstrated that the syncytia/giant cells produced by species of *Meloidogyne* and other sedentary nematodes provide a nutritionally enriched medium resulting in aggressive growth of invading fungi such as *Fusarium oxysporum* (Schltdl.) Fr.(Upadhyay and Dwivedi, 1987), *Rhizoctonia solani* Kühn (Khan and Müller, 1982) and *Phytophthora megasperma* Drechs. (Griffin *et al.*, 1988). Furthermore, the effects can be translocated, with healthy parts of the nematode-infested plant also more susceptible to fungal attack (Storey and Evans, 1987). Developing *Anguina* galls have also been shown to be sites of greatly increased metabolite production (Stynes

and Bird, 1982) and thus SMG initiation in *A. avenacea* and *P. monspeliensis* may provide an earlier opportunity for fungal attack, resulting in a greater number of lesions.

Further, exudates from the feeding nematodes or damaged plant tissue may alter gene expression within the fungus, resulting in increased pathogenicity. This mechanism has been well documented for the crown gall bacterium *Agrobacterium tumefaciens* (Smith and Townsend 1907) Conn 1942, where wounding of the host promotes the release of lignin precursors and acetosyringone which stimulate an increase in expression of the virulence (*vir*) genes resulting in increased infection (Shimoda *et al.*, 1990).

### 10.4.2 Effect on gall production

Reproduction in the genus *Anguina* takes place exclusively inside the nematode gall, which provides nutrition and protection for the sedentary adults and subsequent generation of second stage juveniles. McKay *et al.* (1981) observed that *D. alopecuri* dramatically reduced the number of seed galls produced by *A. funesta*, by colonising and subsequently destroying the gall tissues, preventing feeding and reproduction. In addition, nematode galls cannot be produced in plant tissue that has been attacked by *D. alopecuri* (Khan and Pathak, 1993). Thus, we would expect that strain pathogenicity will have a marked effect on gall production.

The results presented herein show that the efficacy of the *D. alopecuri* strains is directly related to their pathogenicity. The more pathogenic the strain, the smaller the total number of galls recovered from the host following inoculation. Although there was no statistical difference between the treatments on *P. monspeliensis* when total gall numbers were examined, separate analysis of seed galls and SMG did revcal differences between fungal strains. The ex *P. monspeliensis* strain was the most effective strain in reducing both seed

galls and SMG. The effect on SMG is significant in that few agronomic control measures are able to effectively minimise the production of these galls (see Chapter 9). It is interesting that the ratio of seed galls to SMG increases with increasing fungal pathogenicity. This is because the production of seed galls and SMG is not mutually exclusive. Tillers that have SMG produced in them do not produce seed heads and thus an increase in SMG production will have a negative effect on the number of seed galls formed and vice versa.

While it appears that this effect is reversed in *A. avenacea*, with the seed gall to SMG ratio decreasing with increasing fungal pathogenicity, this is probably due to the low number of SMG that occurred in this host. It appears that the conditions under which this experiment was performed, and in fact conditions throughout SA, are not conducive to SMG formation even though seed gall production remains largely unaffected (see also Chapter 5). SMG galls have been observed in field populations of the nematode in NSW but it is uncertain whether the low numbers observed during this study is typical of field dynamics.

While pathogenicity may be the primary cause of the reduction in gall numbers, it is also likely that the adhesive characteristics of the fungus and nematodes may also be involved. The movement of nematodes is restricted when tightly bound by adhering conidia (Riley and McKay, 1990) and is likely to impede invasion and movement within the plant. Experiments by Atanasoff (1925) showed that inoculation with moderate to heavy spore suspensions resulted in totally disease free plants indicating the nematodes were unable to invade the host plants. However, populations of *A. funesta* exist to which *D. alopecuri* does not adhere (Riley and McKay, 1990). In mixed populations of adhesive and non-adhesive nematodes, introduction of *D. alopecuri* may actually increase gall production as this will

effectively reduce the initial number of invading individuals, which has been shown to increase the number of galls produced (McKay *et al.*, 1986).

In contrast to the decline in the incidence of ARGT in the original infested areas of WA (Roberts, 1992), no such decline has been reported in original FPS areas even though *D. alopecuri* has been found in these areas. It is possible that it was introduced only recently and is still establishing but this can only be determined by further monitoring of field populations. If the fungus has been present for some time, then the organisms involved may have reached a state of equilibrium but it is more likely that changes in host and nematode populations due to environmental conditions make fungal dynamics inherently unstable.

### 10.4.3 Allozyme analysis

The strains of *D. alopecuri* exotic to Australia were not strongly related to each other or any of the Australian strains. The Australian strains can be divided into two main groups, electrophoretic types E1 to E4 and electrophoretic types E8 to E10. These two groups differed from each other by an average of over 70% and crossed both regional and host boundaries. Fixed differences of this magnitude suggest that *D. alopecuri* is either highly variable or consists of several species.

Allozyme analysis has been used to make recommendations for both the separation and combination of fungal species. Bonde *et al.* (1991) found less than an average of 20% intraspecific variation at 14 loci for *Colletotrichum fragariae* Brooks and *C. gloeosporioides* (Penz.) Penz. & Sacc. and considered 60% variation between the species sufficient to suggest they are distinct. Leung and Williams' (1986) study of 18 loci from *Pyricularia oryzae* Cavara found only about 10% variation within 335 strains isolated from

rice and about 50% for 34 strains from other poaceous hosts. Hodges *et al.* (1986) and Micales *et al.* (1987) considered 15 strains of *Endothia eugeniae* (Nutman and Roberts) Reid and Booth and *Cryphonectria cubensis* (Bruner) Hodges to be conspecific with less than an average of 20% differences at 16 loci or less than 40% differences when 4 strains that were null at several loci were included. The high level of polymorphisms (92.5%) found in *D. alopecuri* is considerably more than reported in other haplophase fungi and is comparable to the high levels of polymorphism found in dikaryotic edible fungi such as *Pleurotus ostreatus* (Pers. ex Fr.) Sing. (Kulkarni *et al.*, 1986) and *Agaricus campestris* Fr. (May and Royse, 1982). Clearly, further work will be required to determine the taxonomic significance of these polymorphisms.

### 10.4.4 Conclusions

The strains of *D. alopecuri* investigated in this study are neither host nor vector specific. All three strains were able to suppress nematode reproduction by reducing to varying degrees the number of both seed galls and SMG produced. The ex *P. monspeliensis* strain is clearly the most aggressive of the three strains examined and while care needs to be taken that it is not carried into important non-host species, the literature indicates that this is unlikely in the absence of a suitable vector. In selecting the most effective strain for dissemination as a biocontrol agent, the ex *Lolium* strains from SA warrant further investigation as they appear to be closely related to the ex *Polypogon* strains. Undescribed species of *Anguina*, of which we know little, exist in Australia and further characterisation of their ecology and associated micro-organisms may reveal new strains of *D. alopecuri* for evaluation as biological control agents.

# General Discussion

The main objectives of this study were to define the distribution of the organisms associated with FPS, to examine the biology and ecology of the nematode involved and to indicate potential pasture and livestock management practices that could be employed to reduce the impact of the toxicosis.

## Distribution of the organisms associated with flood plain staggers

The surveys of areas prone to prolonged flooding in southeastern SA and northern NSW described in Chapter 4 indicated that the nematode *Anguina* sp. and bacterium *R. toxicus* are more widely distributed than indicated by reports of livestock deaths in either State. In southeastern SA, the incidence of both organisms mirrored that of *P. monspeliensis* indicating that the introduction of the organisms to this area was not a recent event. In northern NSW, both organisms were also widely distributed but populations were more fragmented rather than spread across the entire region.

The incidence of *Anguina* sp. and *R. toxicus* is influenced primarily by distribution of *P. monspeliensis* in southeastern SA and *A. avenacea* in northern NSW. Although *P. monspeliensis* was also found infested with nematodes in NSW, *A. avenacea* appears to be the major host in that State. The nematode is an obligate parasite and so can only spread into areas occupied by its host. Likewise, while *R. toxicus* is able to colonise several different grasses, it is reliant on *Anguina* nematodes to gain entry into its hosts and thus requires the nematode to be present. Observations of field experiments in southeastern SA over several years, detailed in Chapter 7, indicate that populations of *P. monspeliensis* can fluctuate dramatically according to environmental conditions. Areas that receive reliable

rainfall and are prone to flooding during winter support the highest populations of *P. monspeliensis* as they are able to dominate other pasture species. Northern NSW, has not received reliable winter rainfall since the reported outbreaks of FPS in 1990/91. Thus populations of *A. avenacea* have become restricted, causing the fragmented distribution of nematodes and bacteria observed.

It is clear from the host range experiments described in Chapter 5 and observations made during survey work, that ecological factors also govern the incidence of *Anguina* sp. and *R. toxicus*. Even though *A. avenacea* from southeastern SA and northern NSW are equally good hosts for *Anguina* sp., no infested *A. avenacea* has been found in SA, even when growing around infested *P. monspeliensis*. It is not clear what factors are responsible but they need to be determined to identify other areas where the nematode and bacterium may be present.

*P. monspeliensis* is a common weed in other Australian States and in other areas of SA and NSW but it is not known if *Anguina* sp. occurs in any of these areas. Pastures in southeastern SA often have an abundance of feed in spring which prompts many producers to cut them for hay. The hay is then transported to other areas of southeastern SA and also interstate, making it an important mechanism for the movement of the nematode and bacterium to other areas.

**Biology and ecology of *Anguina* sp.**

Evidence presented in Chapters 5 and 6 indicates that the nematodes from *P. monspeliensis* and *A. avenacea* in NSW and SA are not only the same species but a new species of *Anguina*. Although the taxonomy of this group has been hampered by similarity of

morphological measurements, there are morphological characters that distinguish the species under study from other described *Anguina*. A combination of direction of curvature, the number of flexures of the testis, the size of the spicules and the shape of the copulatory bursa and tail tip allows the diagnosis of adult males of this species from other species of *Anguina*. However, adult nematodes are present in galls for only a short time and the most commonly encountered stage is the J2, as it is both the invasive and survival stage. While the morphological similarity of J2s among species of *Anguina* precludes precise identification, the molecular methods of identification such as allozyme electrophoresis or DNA sequencing examined in Chapter 6, clearly resolve the species boundaries. Furthermore, now that DNA sequences reside on public databases, such as GENBANK, accurate identification of species can easily be made. This will prove especially useful for regulatory authorities who need to identify commercially important *Anguina* species that impinge on import/export markets.

While accurate identification is pivotal in decision making processes, it needs to be combined with biological data before it is of use to primary producers. Host range tests discussed in Chapter 5 indicated that *P. monspeliensis* and *A. avenacea* are likely to be the only two suitable hosts for the nematode under study. *In vitro* studies indicated that they are equally good hosts, although in pot trials *P. monspeliensis* appears to be superior. The limiting factor is likely to be the ability of the nematodes to invade each host plant, which is not only influenced by environmental conditions but also by nematode population density, phenology and relative growth rates of the hosts under different varying conditions.

Before this study, the biology of *Anguina* sp was thought to follow most closely that of *A. funesta, A. tritici* and *A. agrostis sensu stricto* in that the J2s emerge from their anhydrobiotic over-summering state in autumn to colonise hosts, where they induce galls in developing floral primordia producing a single generation in each gall. However, the biology of *Anguina* sp differs from other species of *Anguina* currently described in the literature in a number of different ways. Unlike other members of the genus, *Anguina* sp. can induce galls in a variety of meristematic tissues. Thus, instead of being limited to either floral or vegetative tissue, gall induction can take place in the shoot apical meristem and in various meristematic tissues in the developing inflorescence. This strategy allows the nematode to complete at least two generations per year and is possibly an adaptation to survive prolonged flooding. Also, data presented in Chapter 7 indicates that the J2 stage of this species can leave its anhydrobiotic state, exit the gall and invade hosts much faster than other species of *Anguina*. This also appears to be an environmental adaptation, ensuring that invasion and gall induction take place before any flooding occurs.

Flooding favours proliferation of both *P. monspeliensis* and *A. avenacea* by removing competition from less flood tolerant pasture species such as *Hordeum* sp. and thus is necessary for increases in nematode populations or expansion of range. However, flooding is not necessary for nematode development and experiments performed in Chapter 8 indicate that the nematode is not able to invade inundated host plants. Nematodes that are unable to invade host plants before flooding are able to survive in flooded soil for at least three months and still induce galls in host plants when conditions become more favourable. Also, the mechanisms which allow the host plants to survive flooding may actually reduce nematode populations by causing plants to shed tillers, and hence potential gall induction sites.

**Management of flood plain staggers**

Logically, the biology of the nematode should have been completely studied before management trials were commenced but this was not possible within the time frame of the project. Thus, the management options explored were based on methods that have been used to successfully manage ARGT. However, the studies outlined in Chapters 5, 7 and 9 clearly indicate management methods used for the control of ARGT will not have the same impact on the organisms responsible for FPS. Methods to reduce nematode numbers must target both shoot meristem and seed galls. In addition, because different gall types are induced throughout the growing season, management methods such as grazing and hay cutting, which rely on synchronous nematode development, will only affect a portion of the nematode population. In fact, grazing in spring may actually increase nematode populations as it promotes an open pasture and increases the number of tillers produced per plant. The increased number of tillers provides many new opportunities for the induction of shoot meristem galls and thus colonisation by *R. toxicus*. Experiments in Chapter 7 indicated that tillers in which galls were initiated in spring were usually less than 100 mm in length but contained two thirds of the bacterial population.

A better option may be to promote the growth of clovers in spring. The clovers exclude light from the base of the host plant reducing the total number of tillers produced per plant and preventing the production of short tillers. As the inflorescences emerge, they can be removed by using desiccant herbicides While these methods may prove effective for pastures in southeastern SA, the large size of paddocks and low stocking rates in NSW makes these treatments non viable. The use of the plant parasitic fungus *D. alopecuri* as a biocontrol agent may prove to be one of the few options available.

*D. alopecuri* was discovered in southeastern SA infecting *P. monspeliensis*. A comparison of strains isolated from *P. monspeliensis* with others from around Australia reveal a great diversity with this monotypic genus that should be examined further. Strains of the fungus are already providing useful control of *A. funesta* in the cropping areas of SA and WA but as yet, no information is available regarding its persistence in the areas where FPS is known to occur. It was not found in northern NSW but has been found further south in the State on *Holcus lanatus*. Experiments in Chapter 10 showed that the isolates collected from *P. monspeliensis* are more aggressive and reduce nematode populations to a greater degree than other isolates studied thus far. These isolates should be investigated further as potential biocontrol agents as should those from *L. rigidum* in SA, which appear to be closely related.

**Appendix 1** List of enzymes tested in Chapter 6 and Chapter 10, their Enzyme Commission numbers, the number of presumed loci for each enzyme and the run buffer/s used for each.

| Enzyme | EC number | Chapter 6 | | | Chapter 10 | | |
|---|---|---|---|---|---|---|---|
| | | No. of loci | Locus abbreviation[1] | Buffer/s[2] | No. of loci | Locus abbreviation[1] | Buffer/s[2] |
| Acid phosphatase | 3.1.3.2 | 1 | Ap | Phos | 1 | Ap | Phos;TM |
| Aconitase | 4.2.1.3 | 1 | Acon | CP | 2 | **Acon-1** | Phos;CP |
| | | | | | | Acon-2 | Phos |
| Aminoacylase | 3.5.1.14 | ? | Acyc | TM | | | |
| Alcohol dehydrogenase (ethanol) | 1.1.1.1 | | | | 1? | Adh | Phos |
| Aldolase | 4.1.2.13 | 2 | Ald-1 | Phos | ? | Ald | Phos |
| | | | Ald-2 | Phos | | | |
| Enolase | 4.2.1.11 | 1 | Enol | CP | 1 | **Enol** | Phos |
| Esterase (4 methylumbelliferyl acetate) | 3.1.1... | 2 | Est-1 | TM | 5 | Est-1 | Phos |
| | | | Est-2 | | | Est-2 | |
| | | | | | | Est-3 | |
| | | | | | | Est-4 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | **Est-5** | |
| Fructose biphosphatase | 3.1.3.11 | | | | 1 | **Fdp** | Phos |
| Fumarate hydratase | 4.2.1.2 | 1 | Fum | Phos | 1 | **Fum** | Phos |
| βGlucosidase | 3.2.1.21 | | | | ? | βGlu | Phos |
| Glycerol-3-phosphate dehydrogenase (NAD) | 1.1.1.8 | 1 | αGpd | TM | | | |
| Glyceraldehyde-3-phosphate dehydrogenase (NAD) | 1.2.1.12 | 1 | Ga3pd | Phos | 1 | **Ga3pd** | Phos |
| Glyoxalase | 4.4.1.5 | ? | Glo | TM | 1 | **Glo** | Phos |
| Glutamic-oxaloacetatic transaminase | 2.6.1.1 | 1 | Got | Phos | 2 | **Got-1** | Phos, TM, |
| | | | | | | **Got-2** | Tec, CP |
| Glucose-6-phosphate dehydrogenase | 1.1.1.49 | 1 | **G6pd** | CP | 1 | **G6pd** | Phos |
| Glucose-6-isomerase | 5.3.1.9 | 1 | **Gpi** | TM | 1 | **Gpi** | Phos |
| Guanine deaminase | 3.5.4.3 | | | | 1 | Gda | Phos |
| Hexokinase | 2.7.1.1 | | | | 1 | **Hk** | Phos |
| Isocitrate dehydrogenase (NAPD) | 1.1.1.42 | 1 | **Idh** | CP | | **Idh** | Phos;CP |
| Lactate dehydrogenase | 1.1.1.27 | | | | | Ldh | Phos |
| Leucine aminopeptidase | 3.4.11.1 | | | | 2 | **Lap-1** | Phos;TM |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | **Lap-2** | Phos |
| Malate dehydrogenase (NAD) | 1.1.1.37 | 2 | **Mdh-1** | TM | 2 | **Mdh-1** | CP |
| | | | **Mdh-2** | TM | | **Mdh-2** | CP |
| Mannose-6-phosphate isomerase | 5.3.1.8 | 1 | **Mpi** | Phos | 1 | **Mpi** | Phos |
| Nucleoside-diphosphate kinase | 2.7.4.6 | | | | 1 | **Ndpk** | Phos |
| Valine-leucine dipeptidase | 3.4.13.11 | 1 | **PepA** | CP | 1 | **PepA** | Phos;CP |
| Tripeptide aminopeptidase | 3.4.11.4 | 1 | **PepB** | CP | | | |
| Lysine-leucine dipeptidase | 3.4.13.11? | | | | 1 | **PepC** | Phos;CP |
| Phenylalanine-proline dipeptidase | 3.4.1.3.9 | ? | **PepD** | CP | 2 | **PepD-1** | Phos;CP |
| | | | | | | **PepD-2** | |
| 6-phosphogluconate dehydrogenase | 1.1.1.44 | 1 | **6Pgd** | Phos | 1 | **6Pgd** | Phos;TM |
| Phosphoglycerate kinase | 2.7.2.3 | 1 | **Pgk** | Phos | 1 | **Pgk** | Phos |
| | | ? | Pgam | Phos | | | |
| Phosphoglucomutase | 5.4.2.2 | 1 | Pgm | Phos | 2 | Pgm-1 | Phos |
| | | | | | | **Pgm-2** | |
| Pyruvate kinase | 2.7.1.40 | 1 | **Pk** | CP | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Triose-phosphate isomerase | 5.3.1.1 | 1 | **Tpi** | Phos | 1 | **Tpi** | Phos |
| Uridine monophosphate kinase | 2.7.4.4 | | | | 1 | Umpk | Phos |

[1] Loci in **bold** used in the analysis

[2] Buffers: TM, 0.05MTris-maleate pH 7.8; Phos, 0.02MNaPhosphate pH7.0; CP, 0.01MCitrate-phosphate pH6.4; Tec, 0.02MTris-EDTA-citrate pH7.5 (details in Richardson *et al.,* 1986).

**Appendix 2** Microsoft Excel macros used to convert ELISA plate optical densities into bacterial gall equivalents. The macros assume that the mass of the samples processed are stored in a spreadsheet named "weights.xls".

```
Option Explicit

'All variables must be defined

Option Base 1

Dim fname, weights As String
Dim maxstd As Integer
Dim stdgalls() As Integer
Dim stdlog() As Single, stdodadj() As Single

Const Dilns = 2

---------- Subroutine OpenFile
Sub OpenFile()

' Uses Excel's File Open dialog to select and open a "plate.***" file output
' from the plate reader

   Const iTitle = "Select Plate File"
   Const Filterlist = "Plate Reader Files ( plate.* ),plate.*"

   With Application
      fname = .GetOpenFilename(Title:=iTitle, filefilter:=Filterlist)
   End With

   If fname = "False" Then
      MsgBox prompt:="Operation Cancelled", Title:=iTitle
      Exit Sub
   End If
   Application.ScreenUpdating = False
End Sub

---------- Subroutine StartNotePad
Sub StartNotePad()

'Starts Notepad.exe, loads the plate file, copies text to clipboard and quits

   On Error GoTo BadStart

   Shell "notepad.exe " & fname, 1
   SendKeys "%ea", True
   SendKeys "^c", True
```

```
    SendKeys "%{F4}"

    Exit Sub

BadStart:
    MsgBox "Could not start Notepad!", vbOKOnly

End Sub

------------------------------------------------------------------------ Subroutine FormatSheet
Sub FormatSheet()

' Prepares the worksheet for calculations

Dim newname, sheetname As String

    newname = Right(fname, 9)
    sheetname = Application.activesheet.Name
    With Sheets(sheetname)
       .Select
       .Name = newname
    End With

   activesheet.Paste destination:=Range("A1")
   Columns("A:A").Select
   Selection.TextToColumns DataType:=xlFixedWidth
   Columns("A:I").Select
   Selection.ColumnWidth = 7
   Range("A1").Select
   ActiveCell.FormulaR1C1 = "Optical Densities"
End Sub

------------------------------------------------------------------------ Subroutine PlotChart
Sub PlotChart()

'Plots the standard curves for the current plate for assessment

     Dim prange As Object

    activesheet.ChartObjects.Add(40.5, 210, 325.5, 150.75).Select
    Application.CutCopyMode = False

    If Range("I7") < 0.3 Then
       Set prange = Range("c7:h8")  'sets datarange for ABG standards
       maxstd = 6
    Else
      Set prange = Range("c7:i8")   'sets datarange for ryegrass standards
      maxstd = 7
```

```
    End If

      ActiveChart.ChartWizard Source:=prange, Gallery:=xlLine, _
       Format:=1, PlotBy:=xlRows, CategoryLabels:=0, SeriesLabels _
       :=0, CategoryTitle:="Standards", ValueTitle:= _
       "Optical Density"
      Application.ScreenUpdating = True
End Sub

------------------------------------------------------------------------------ Subroutine SampleList
Sub SampleList()

'Opens "weights.xls" to load mass of samples processed

    Dim index As Integer
    Dim sample As String
    Dim dialogbook As Workbook
    Set dialogbook = Workbooks("weights.xls")

    With dialogbook.DialogSheets("dialog1").ListBoxes(1)
       .RemoveAllItems
       .AddItem ("A1 - C9")
       .AddItem ("C10 - H2")
       .AddItem ("H3 - K3")
       .AddItem ("K4 - P4")
       .AddItem ("P5 - S8")
       .AddItem ("S9 - Y1")

       index = .Value
       If index > 0 Then
          sample = .List(index)
       End If
    End With

    If dialogbook.DialogSheets("dialog1").Show Then
       With dialogbook.DialogSheets("dialog1")
          index = .ListBoxes(1).Value
          sample = .ListBoxes(1).List(index)
       End With
    End If

    Select Case sample
       Case "A1 - C9"
          weights = "samples 1"
       Case "C10 - H2"
          weights = "samples 2"
       Case "H3 - K3"
          weights = "samples 3"
```

```
    Case "K4 - P4"
      weights = "samples 4"
    Case "P5 - S8"
      weights = "samples 5"
    Case "S9 - Y1"
      weights = "samples 6"
  End Select

End Sub

---------------------------------------------------------------------------- Function Interpolate
Function Iinterpolate(sampod As Single, dilution As Integer) As Single

'Returns the number of bacterial gall equivalents by interpolation of the standard curves

  Dim i, j As Integer
  Dim sampodlog, dummyvar As Single

  sampodlog = Log(sampod + 1)
  If sampodlog > stdodadj(maxstd, dilution) Then
    interpolate = stdgalls(maxstd)
  Else
    i = 1
    Do While sampodlog > stdodadj(i, dilution)
      i = i + 1
    Loop
    If i = 1 Then
      interpolate = stdgalls(1)
    Else
      j = i - 1
      dummyvar = (stdlog(i) - stdlog(j)) * (sampodlog - stdodadj(j, dilution)) / _
        (stdodadj(i, dilution) - stdodadj(j, dilution)) + stdlog(j)
      interpolate = Exp(dummyvar) - 1
    End If
  End If
End Function

---------------------------------------------------------------------------- Subroutine Calculate
Sub Calculate()

'Calculates gall numbers from ELISA Optical Densities using the interpolate function

  ReDim stdgalls(maxstd)
  ReDim stdlog(maxstd), stdodadj(1 To maxstd, 1 To Dilns)
  Dim i, j As Integer
  Dim sampod As Single

  stdgalls(1) = 0
```

```
  For i = 2 To maxstd
    stdgalls(i) = 4 ^ (i - 2)
  Next i

  For i = 1 To maxstd
    stdlog(i) = Log(stdgalls(i) + 1)
    For j = 1 To Dilns
      stdodadj(i, j) = Log(Cells(6 + j, 2 + i) + 1)
    Next j
  Next i

  For i = 3 To 6
    For j = 2 To 9
      With Cells
        .Value(i + 13, j) = CLng((interpolate(Cells(i, j), 1) * 100))
        .Value(i + 19, j) = CLng((interpolate(Cells(i + 6, j), 2) * 100))
      End With
    Next j
  Next i
End Sub

---------------------------------------------------------------------------- Subroutine Bacteria
Sub Bacteria()

'Calculates bacterial gall equivalents according to the weight of the sample processed

  Dim i, j As Integer

  For i = 16 To 19
    For j = 2 To 9
      If Cells(i, j) < 50 Then
        Cells(i + 12, j).Value = 0
      Else
        If Cells(i + 6, j) < 50 Then
          Cells(i + 12, j).Value = CLng((100 /
Workbooks("weights.xls").Worksheets(weights).Cells(i - 15, j - 1)) / 100)
        Else
          Cells(i + 12, j).Value = CLng((Cells(i + 6, j) /
Workbooks("weights.xls").Worksheets(weights).Cells(i - 15, j - 1)) / 100)
        End If

        If maxstd = 7 Then
          If Cells(i + 12, j) > 1024 Then
            Cells(i + 12, j).Value = 1024
          End If
        Else
          If Cells(i + 12, j) > 256 Then
            Cells(i + 12, j).Value = 256
```

```
            End If
          End If
          If Cells(i + 12, j) < 1 Then
              Cells(i + 12, j).Value = 1
          End If
        End If
      Next j
  Next i
End Sub

---------- Subroutine Main
Sub Main()

'Uses four subroutines to open a platereader file, copy the data to "Excel" via "Notepad",
format the data and plot the standards for examination.

  OpenFile
  StartNotePad
  FormatSheet
  Plotchart
End Sub

---------- Subroutine Continue
 Sub Continue()

'Assuming the standards are OK, allows selection of the sample weights, determines the
bacterial gall equivalents by interpolation of the standards and corrects the estimation
according to the mass of the sample processed.

   SampleList
   Calculate
   Bacteria
End Sub
```

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