CHAPTER 4

Geographical differentiation and genetic variation of *Lolium* spp in the Western Cape: identification of the hybrid *Lolium multiflorum x perenne* and isolation of the pathogen *Fusarium pseudograminearum*

Ferreira, MI[1], CF Reinhardt[2*], Lamprecht, SC[3], Rees, DJG[4#], Sinclair M[5] and Landman, L[6]

[1]*Institute for Plant Production, Private Bag X1, Elsenburg 7607, South Africa*
[2*]*Department of Plant Production and Soil Science, University of Pretoria, Pretoria 0002, South Africa*
[3]*ARC-Plant Protection Research Institute, Private Bag X5017, Stellenbosch 7599, South Africa*
[4]*Department of Biotechnology, University of the Western Cape, Private Bag X17, Bellville 7530, South Africa*
[5]*Veterinary Services, Private Bag X1, Elsenburg 7607, South Africa*
[6]*Institute for Resource Utilisation, Private Bag X1, Elsenburg 7607, South Africa*

Corresponding authors: Institute for Plant Production, Private Bag X1, Elsenburg 7607, South Africa
E-mail address: mikefe@elsenburg.com

*Present address: South African Sugarcane Research Institute, Private Bag X02, Mount Edgecombe 4300, South Africa
#Present address: ARC Biotechnology Platform, Private Bag X5, Onderstepoort 0110, South Africa

INTRODUCTION

While conducting research on rye grass v. Energa described in Chapter 2 Exp 1 and all experiments conducted in Chapter 3, it became increasingly evident that identification of the different *Lolium* spp is problematic. Since representative rye grass weed seeds, as opposed to rye grass pasture type v. Energa used in Chapter 2 & 3, were required for further representative research, it was decided to do relatively quick, but extensive tests in order to gather background information and establish a reliable seed source for research conducted in Chapter 2 Exp 2 and Chapter 5. A random site adjacent to a wheat field was identified at Hermon (18°97'E, 33°43'S, Western Cape) and rye grass samples were collected, prepared and sent for morphological identification. When results from the Compton Herbarium at Kirstenbosch indicated a rye grass hybrid type, the study described in this chapter, followed. As it was a hybrid never before described in South Africa, it was decided that various aspects should be covered by research

collaborators to gain as much information as possible in a relatively short period of time.

Economically, there is no doubt that herbicides and herbicide-resistant crops have drastically improved agricultural efficiency and yields. However, the broad application and/or sometimes the misuse of herbicides has also created problems. The major problem is the evolution of weeds with resistance to herbicides which refers to the capacity of a plant to grow and reproduce under the dose of herbicide that is normally lethal to the species (Yuan *et al.*, 2007). Weed resistance to herbicides presents one of the greatest current economic challenges to agriculture (Baucom, 2009) with more than 346 biotypes of weed known to be resistant to herbicides (Heap, 2010). A species' ability to adapt to changing environmental conditions is found in the genetic diversity of its populations. Success in weed populations facing changing agricultural ecosystems often correlates with an abundance of genetic polymorphisms within those populations (Jasieniuk & Maxwell, 2001). Through the process of mutation and selection, however, weeds evolve resistance to herbicides when they are used repeatedly (Tranel & Trucco, 2009). *L. rigidum* (rigid ryegrass) (Monaghan, 1980) was regarded by Tranel and Trucco (2009) to be the most important weed in terms of it having evolved resistance to multiple herbicides.

According to O'Hanlon *et al.* (2000), there is a widespread concern that weed species with higher levels of genetic diversity will exhibit considerable potential for adaptation and, therefore, may be able to reduce the effectiveness of weed control. Weeds have genetic traits that give them remarkable plasticity, allowing them to adapt, regenerate, survive, and thrive in a multitude of ecosystems (Chao *et al.*, 2005). Many agronomic weeds are close relatives of crop plants and studies on the sequencing of a weed genome are likely to provide clues concerning weed phenotypes and their underlying gene networks (Broz & Vivanco, 2009).

Molecular marker studies have revealed differences in population structure and diversity between the native and introduced types for many invasive weed species (Lee, 2002; Bossdorf *et al.,* 2005). The development of polymerase

chain reaction (PCR)-based techniques to assess genetic diversity has since proven to be a quick, easy, and cost-effective way of performing genetic analysis. In cases where genomic sequence data is available, it is possible to work with microsatellite markers (SSRs - simple sequence repeats) as these tend to give a single unique PCR product and in many cases have numerous alleles, making the evaluation of genotypes much more informative. For rye grass a large number of SSRs have been characterised and published (Gill *et al.,* 2006, Hirata *et al.*, 2006, Jones *et al.,* 2002, Mian *et al.*, 2005, Saha *et al.*, 2004, Saha *et al.*, 2005, Senda *et al.*, 2005, Studer *et al.,* 2006), and these SSRs provided the basis for the analysis required in the present study.

L. perenne L. (perennial ryegrass) (Charmet & Balfourier, 1994) is native to most of Europe and parts of the Mediterranean and Middle East areas, whereas rigid rye grass is distributed all around the Mediterranean. The genus *Lolium* consists of two groups of species, which are outbreeding and inbreeding, respectively (Senda *et al.*, 2005). *L. temulentum* L. (darnel) is an inbreeding species and regarded as a mimic weed and has convergently evolved with cereal crops as a result of unconscious selection by farmers (Senda *et al.,* 2005). The genetic diversity of outbreeding rye grass has been studied in relation to the characterisation of genetic resources of *L. multiflorum* Lam. (Italian ryegrass) (Charmet & Balfourier, 1994) and perennial ryegrass. Analysis of the frequency and distribution of genetic variation in natural populations of perennial ryegrass has supported the view that its centre of origin is the Fertile Crescent (Middle East) and that its distribution expanded following a clinical geographical pattern (Senda *et al.*, 2005). Both perennial and rigid rye grass are wind-pollinated, self-incompatible outbreeding species (Balfourier *et al.*, 2000). Balfourier *et al.* (2000) reported on the weak genetic differentiation, but significant patterns of geographical variation with respect to diversity indices and allele frequencies have been observed in perennial rye grass. In contrast, no spatial organisation of diversity has been detected in rigid rye grass (Balfourier *et al.*, 2000).

Herbicide resistant rye grass is a serious problem in Western Cape grain producing areas as it is threatening more than 100 000 ha of productive grain

fields. Wheat fields have become so heavily infested that economic grain production, will be impossible in certain areas in the foreseeable future, leading to huge production losses and less sustainable grain production.

Knowledge about the genetic constitution of rye grass and its populations is increasingly becoming crucial, particularly given the extent of herbicide-resistance within the Western Cape. These data will in turn further enhance our understanding of the genetics and evolution of herbicide-resistant weeds. Descriptive studies of patterns of genetic diversity in weedy populations can be an extremely important tool for helping to minimise the evolvement of resistance to herbicides (Madhou *et al.*, 2005).

Crown rot, caused by *Fusarium pseudograminearum*, is one of the most important soilborne diseases of wheat in South Africa and also poses a major threat to barley and wheat production in the Western Cape Province. The disease can be significantly reduced by crop rotation with non-susceptible crops such as *Brassica napus* (canola), (*Lupinus angustifolius*) (lupine), annual *Medicago* spp (medic) and *Trifolium* spp (clover) (Lamprecht *et al.*, 2006). However, it is known that grass weed infestation in the non-crop phase of the rotation can favour the disease, and grass weed control is therefore recommended as part of an integrated strategy to manage crown rot (Burgess *et al.*, 2001). Currently there is no information available on grass weed hosts of crown rot in South Africa.

The aims of this study were to: 1) assess the distribution of genetic variability of rye grass; 2) determine its botanical classification by morphological analyses; 3) determine the presence of the crown rot pathogen of barley and wheat on rye grass; and 4) analyse soil samples from each collection point where rye grass were sampled to determine its preference for soil chemical properties.

MATERIALS AND METHODS

Four agricultural production areas of the Western Cape Province, as indicated in Figures 1 & 2, were included in this study, namely Malmesbury-

Moorreesburg (Swartland) - area A, Worcester-Robertson (Breede River Valley) - area B, Stellenbosch-Paarl (Winelands) - area C, and Caledon-Swellendam (Overberg) - area D. These areas were used for *Lolium* spp sampling in 2008 from August until October at 10 localities in each area. Two additional localities with known resistant and susceptible populations of rye grass were also sampled and designated F (Fairview Farm, multiple resistant) and G (Glencairn, susceptible).

Collection points

A simple random sampling strategy, using geographic coordinate points, was followed to ensure representative sample collection. To achieve this, the Random Geographic Coordinate Sampling function of the software program Survey Toolbox© was used to determine 40 randomly selected geographic coordinate points in the main agricultural production areas for grain, fruit, vineyards and mixed agricultural production in the Western Cape. ArcView 8.3 software was used for GIS manipulation of these collection points for easy reference during collection. A Magellan® SporTrak GPS system (with 3 meter accuracy) was utilised in the location of these randomly selected collection points.

Genetic analyses

The first specimen taken at each collection point was used for genetic analyses. Total DNA was extracted from leaves according to the modified CTAB protocol (Senda *et al*., 2004). DNA was prepared twice for experimental replication in each analysis. The SSR technique is a high-resolution genetic marker analysis used to assess genetic relationships in many species. The polymerase chain reaction (PCR) enables the development of powerful genetic markers for the measurement of genotype variation. By measuring genotype, rather than phenotype, genetic markers avoid complicating environmental effects and provide ideal tools for assessing genetic variation, identifying species and other locally adapted forms, as well as the definition of genetic relationships.

SSRs were analysed using an appropriate selection of the published primer pairs for *Lolium*, distributed across the genetic map to ensure a random selection of genetic markers. The SSRs were chosen from those, which were known to work across species, and to have the largest number of alleles. Primers were synthesised with fluorescent labels for subsequent analysis (Madhou *et al.*, 2005). Primer optimisation was undertaken to obtain conditions of selective PCR giving unique products for each primer set. Where appropriate, multiple reactions containing several sets of primers were used (Madhou *et al.*, 2005). When this was completed the analysis of a range of *Lolium* isolates was undertaken. Alleles were scored by analysis using the ABI Genetic Analyser, and scored using the GenoTyper™ software.

SSR similarities between isolates were calculated by the simple matching coefficient, *m/n*, where m is the number of alleles matched and n is the total number of alleles. Cluster analysis was performed using the un-weighted pair-group method with arithmetic averages (UPGMA) (Senda *et al*., 2005). For each dendrogram, the correlation coefficient between the matrix of genetic similarities and the matrix of co-phenetic values was computed, and data produced by AFLP were compared using the Mantel test (Senda *et al*., 2005).

Morphological analyses

A second specimen of each sample was collected and morphologically analysed at the Compton Herbarium, Kirstenbosch Botanical Gardens, Cape Town, in order to identify the different species or hybrids.

Pathogenic analyses

A third specimen of each sample was collected and analysed for the soilborne pathogen crown rot at the Agricultural Research Council - Plant Protection Research Institute's laboratory at Stellenbosch. The number of plants collected from each area for isolation of the fungus varied from three and five for areas F and G, respectively, to 50 each for areas A, B, C and D. The protocol described by Lamprecht *et al*. (2006) was used for the isolation and identification of crown rot.

Soil analyses

A soil sample was taken at each rye grass collection point and analysed at the Soil Science Laboratory at Elsenburg.

**RESULTS AND DISCUSSION**

Genetic analyses

SSRs use an appropriate selection of the published primer pairs for *Lolium*, but as these were only published for Italian -, perennial - and rigid rye grass, all specimens were categorised as one of these species. Therefore, no SSRs have been characterised and published for hybrids among rye grass species, creating contrasts in results between genetic and morphological analyses. However, evaluation of these two methods for identification of Italian rye grass revealed that 90% of specimens occurring as weeds were morphologically classified as a hybrid.

Huge genetic variation was detected between Italian rye grass weed populations with no identifiable alleles associated with herbicide resistance. This finding was complicated by the number of alleles per locus for grass species which is $8n$ as opposed to $2n$ for humans, and the occurrence of quantitative trait loci (http://wikipedia.org) which occurs in organisms displaying chemical resistance. Rigid rye grass showed similarity in genetic make-up in the eastern part of area D and perennial rye grass to a lesser extent in area B from samples collected at Robertson and Montagu, but there was no consistent correlation between geographical and genetic distance of specimen pairs.

Overall, SSRs indicated 47.6% of specimens as rigid rye grass, 42.9% as Italian rye grass and 9.5% as perennial rye grass (Figure 1 & Appendix A, Tables A2-A5). Genetic variation analyses indicated 38% of specimens as rigid rye grass from the areas A and D (Swartland-Overberg), while 9.5% classified as rigid rye grass was sampled in areas B and C (Breede River Valley-Winelands). Only four specimens (9.5%) were classified as perennial

rye grass, of which three occurred in areas B and C and a single specimen in area D.
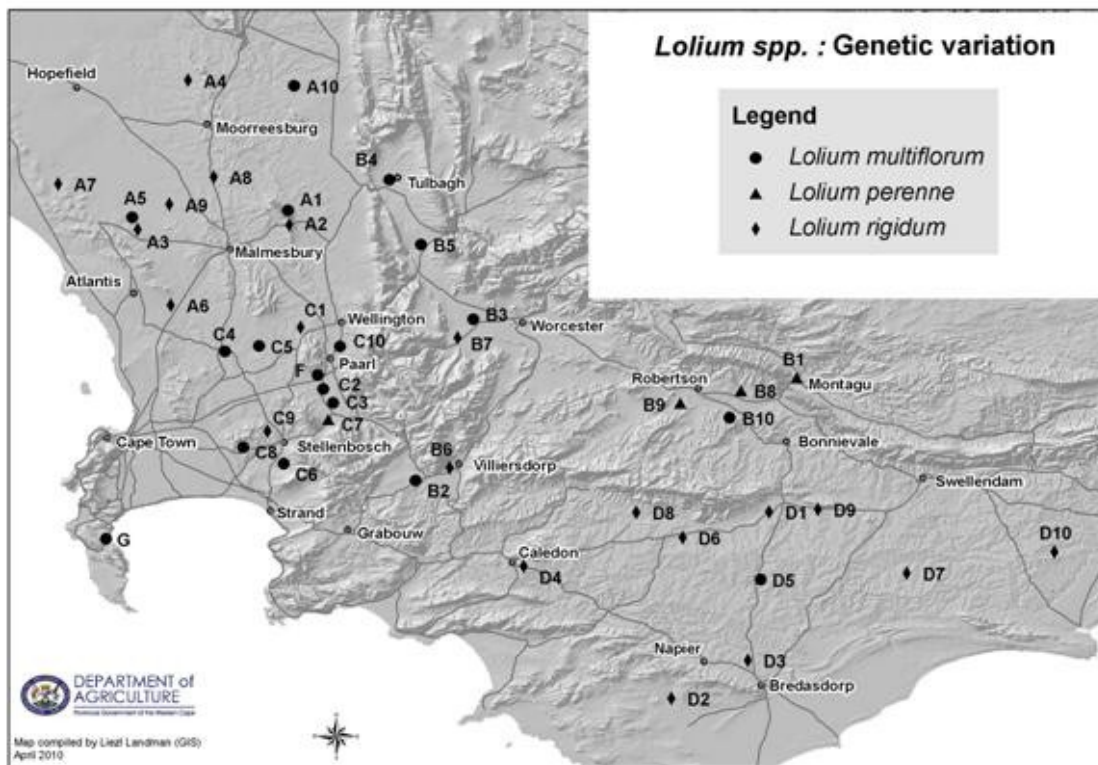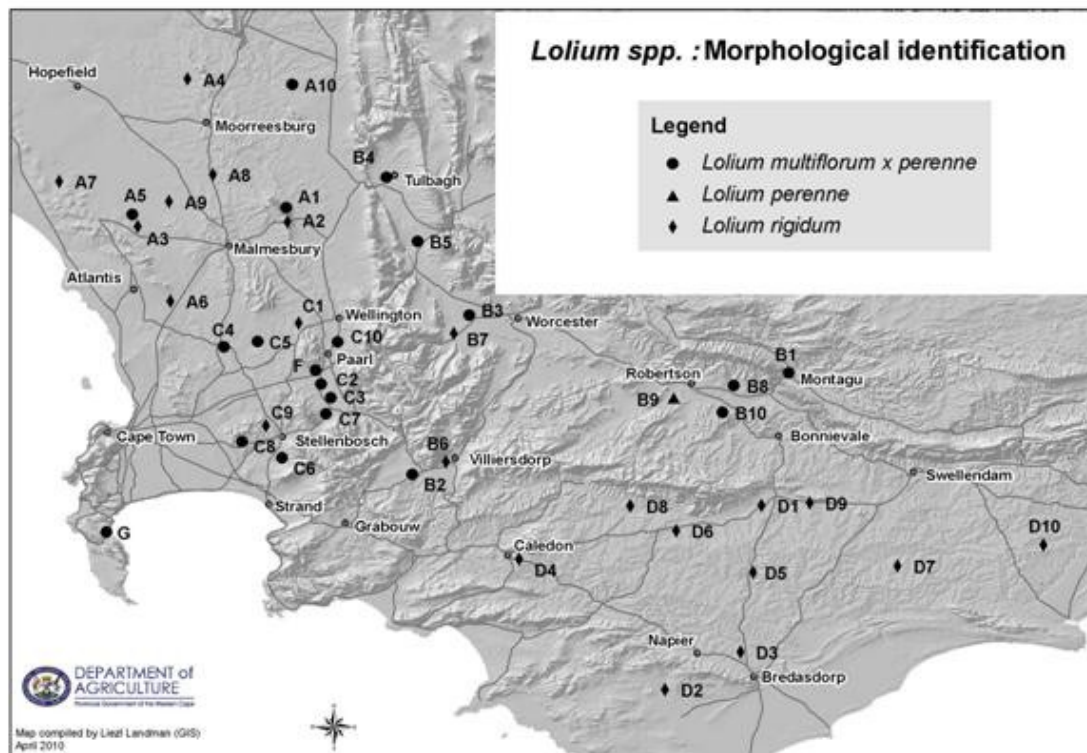

Figure 1 Distribution of rye grass based on genetic classification in the Western Cape

Morphological analyses

Morphologically, 50% of the total number of specimens was classified as rigid rye grass, 48% as the hybrid, namely *L. multiflorum x perenne* and 2% as perennial rye grass. Both the proven herbicide resistant (F) and susceptible specimens (G) were identified as the hybrid *L. multiflorum x perenne* (Figure 2 & Appendix A, Tables A2-A5). Although genetic analyses could only identify Italian rye grass, it would be safe to assume that if published primer pairs were available, many samples would be classified as *L. multiflorum x perenne*.

Of the specimens collected from the wheat, barley and sheep production areas of the Swartland and Overberg (areas A and D), 40.5% was morphologically identified as rigid rye grass, 7% as *L. multiflorum x perenne,* and 52.5% as Italian rye grass. Morphological analyses identified 40.5% of specimens sampled in areas B and C as *L. multiflorum x perenne,* 10% as rigid rye grass and 49.5% as Italian rye grass. Only one specimen (2%) which occurred in area B (Breede River valley) was classified as perennial rye grass. *L. multiflorum x perenne* displayed forked ears, indicating prolific seed production, in 8% of specimens. This characteristic could promote the proliferation of this weed to the extent that it may be a factor contributing to it becoming dominant in weed communities. Treier *et al.* (2009) reported that taxonomists have identified at least two forms of the allelopathic weed, *Centaurea maculosa,* in its region of origin. In addition to multiple flowering in a particular year, the tetraploid form of this weed is capable of producing multiple flowering stems with up to fifteen capitula each, whereas the diploid produces only one stem (Broz & Vivanco, 2009).

Figure 2 Distribution of rye grass based on morphological classification in the Western Cape

Comparing genetic and morphological maps

A comparison between the genetic and morphological maps revealed that 90% of specimens genetically classified as Italian rye grass were morphologically identified as the hybrid *L. multiflorum x perenne.* In three instances (7.5%), specimens genetically classified as perennial rye grass, were morphologically also analysed as *L. multiflorum x perenne* namely at B1 (20°1283'E, 33°7780'S), B8 (19°9901'E, 33°8102'S) and C7 (18°9705'E, 33°8803'S). One specimen (2.5%) collected from B9 (19°8401'E, 33°8402'S) was genetically analysed as Italian rye grass, while it was morphologically classified as rigid rye grass (Appendix A, Tables A2-A5). As no published primer pairs exist for *L. multiflorum x perenne,* no identifiable alleles associated with herbicide resistance could be observed.

A case in point is the important and well-recognised component in the evolutionary history of *Amaranthus* spp of inter-specific hybridisation (Tranel & Trucco, 2009). Hybridisation has been proposed as a critical stimulus for invasiveness and is perhaps aiding in the evolution of adaptations critical for the success of *Amaranthus* spp as weeds. Although species interbreeding is most often maladaptive, it might represent an important route for the evolution of genotypes favoured under the intense selection pressure found in agricultural habitats (Tranel & Trucco, 2009). A clear example of this possibility is herbicide resistance evolution. A resistant individual resulting from a hybridisation event may be lacking in health, vigour, and fertility, but may represent the only viable genotype upon herbicide treatment (Tranel & Trucco, 2009). From a weed management perspective, however, the most significant aspect of invasiveness is the ability of a species to modify a given attribute over time and in response to selection. The evolution of herbicide resistance often forces dramatic changes in weed management practices (Tranel & Trucco, 2009).

Results from the current study on the variability and occurrence of hybrids in rye grass populations from the study area, is in accordance with reports by

Dinelli *et al.* (2002). This author reported high variability in Italian populations of rye grass and hybrid banding profiles from electrophoretic data with up to 24% of individuals which were placed in an intermediate position between rigid rye grass and Italian rye grass. In contrast, Australian populations were more homogeneous with 88% of individuals showing an ordination closely related to rigid rye grass. Furthermore, Italian populations of rye grass were heterogeneous, consisting of several genetically unique individuals which can readily hybridise (Dinelli *et al.,* 2002).

It should also be noted that darnel (*L. temulentum*) was not detected at any collection point, though it was supposed to occur in the sampling area. There could be a few reasons for this: a) not enough sampling points in the study area; b) it was replaced by the more aggressively invasive *L. multiflorum x perenne* and/or rigid rye grass and c) as crop production practices changed substantially over the last two decades in the study area, it was eradicated, because according to Spahillari *et al.* (1999), it cannot survive without the agricultural practices to which it has become adapted. These crop production practices include type of soil disturbance, seed drilling as opposed to sowing, shorter growth season crop cultivars allowing less time for the weed to reproduce, different times of nitrogen application and weed control with herbicides containing active ingredients from different chemical groups, effectively eradicating darnel.

Soil analyses

From Table 1 it is clear that the soils preferred by rye grass cover a wide range of fertility below and above optimum ranges for wheat. Soils range from very acidic pH to high pH or alkaline soils (with the mean being a little above optimum for wheat). The high upper value in the range for sodium values indicates very saline soils. On average the phosphorus content is high, indicating a preference for agricultural fields. Soil analyses emphasises the wide adaptability of rye grass which contributes to their success as invasive weeds.

Table 1 Properties of soils sampled at rye grass collection points

Soil Property	All soils (n = 42)		Optimum
	Mean	Range	range for wheat
pH (KCl)	6.0	4.2-7.9	4.5-5.5
Resistance Ω	740	40-3450	> 400
Calcium (cmol/kg)	8.7	1.17-61.45	1-10
Magnesium (cmol/kg)	2.3	0.27-5.99	0.3-3
Sodium (mg/kg)	128	10-1564	< 100
Potassium (mg/kg)	229	64-790	60-150
Total bases (cmol/kg)	12.5	2.56 – 65.98	2-20
Phosphorus (mg/kg)	104	12-518	60-100
Copper (mg/kg)	3.4	0.42-21.49	0.5-1
Zinc (mg/kg)	7.9	1.05-50.06	0.5-1
Manganese (mg/kg)	60.2	4.4-269.2	> 5
Boron (mg/kg)	0.4	0.07-1.3	0.3-0.8
Sulphur (mg/kg)	10.4	1.12-130	> 7
Carbon (%)	1.9	0.6-4.84	2-3

The only distinction in soil preference among rye grass samples could be drawn on clay content among the different soil samples analysed. In the Swartland (area A) and Overberg (area D), where rigid rye grass mainly occurred, the soil samples contained an average of 21% clay. Italian rye grass was predominant in the Breede River Valley (area B) and Winelands (area C), where the average clay content was 16%. However, since the latter two areas has a high incidence of irrigation, this could have contributed to the wide spread occurrence of Italian rye grass.

Pathogenic analyses

Crown rot was isolated from six localities namely, A2 (18°6734'E, 33°4008'S) on rigid rye grass A4 (18°6236'E, 33°0443'S) on rigid rye grass, A7 (18°3026'E, 33°3001'S) on rigid rye grass, B5 (19°2001'E, 33°4502'S) on *L. multiflorum x perenne*, C9 (18°8200'E, 33°9101'S) on rigid rye grass and D10 (20°7653'E, 34°2078'S) on rigid rye grass (Figure 3). To our knowledge and according to literature searches, this data on crown rot had not been published before, and this is the first report of crown rot on rye grass in South Africa.
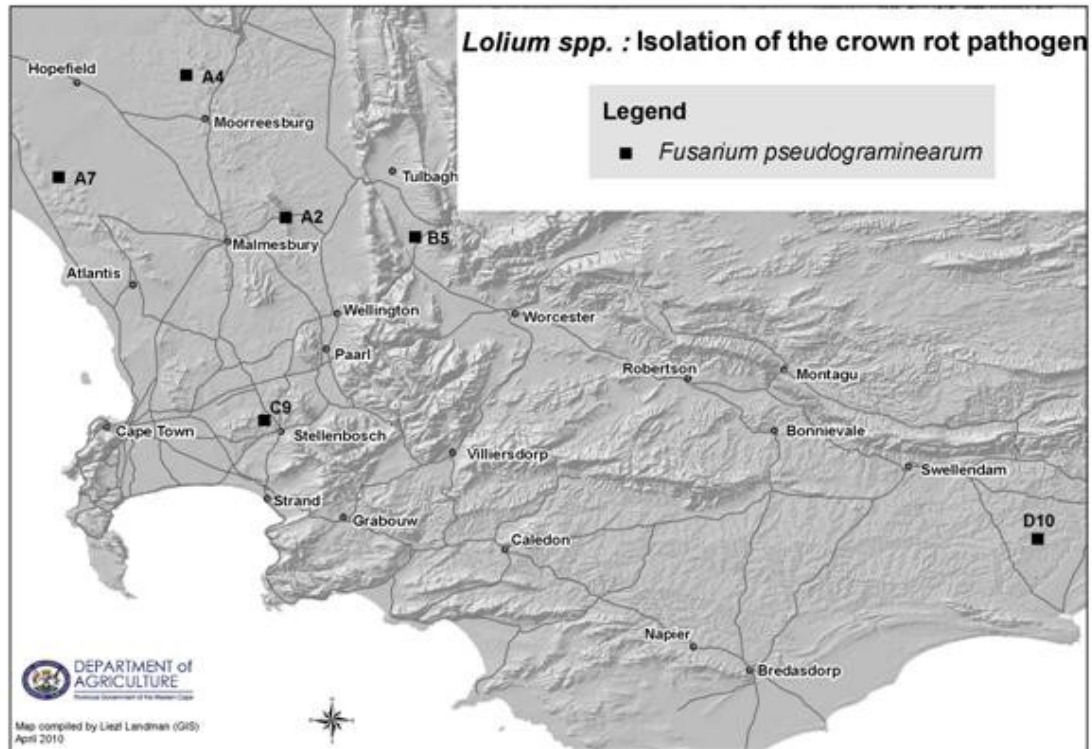
Figure 3 Collection points where *F. pseudograminearum* was isolated from rye grass in the Western Cape

Rye grass can therefore act as alternative hosts and as a source of inoculum of this important soilborne pathogen of barley and wheat in the Western Cape Province. This further complicates sustainable dry land crop production, since the build-up of herbicide resistant rye grass may lead to a higher incidence of crown rot on wheat and barley due to a higher disease pressure. However, it could also point to a possible biological control agent for rye grass.

Implications for invasive weed control

There is some evidence to suggest that environmental adaptation and evolution play an important role in the success of invasive weed species and ecological hypotheses of plant invasion have been developed based on this evidence (Callaway & Aschehoug, 2000). Furthermore, strong evidence points to the fact that allelopathy plays a role in the invasive success of many plant invaders and weeds (Ridenhour & Callaway, 2001; Bertin *et al.,* 2003;

Hierro & Callaway, 2003). Callaway and Aschehoug (2000) suggested that plants come to the new environment equipped with chemical or biochemical weapons that have a greater negative effect against plants in the invaded range than similar species in the native range. These weapons (allelochemicals) give the invader an advantage in the new environment, because they have putative strong phytotoxic effects on other plants. If plant invaders gain a competitive advantage through the use of novel weapons in the invaded range they will evolve to have greater concentrations of these weapons than populations in the native range (Callaway & Aschehoug, 2000). For the high population densities of rye grass occurring in the Western Cape Province, this implies that the weed gained a competitive advantage, not only by being better competitors than other plant species, but also by exuding allelopathic substances. Allelopathy has long been suspected to be important in both agronomic and native ecosystems (Weston & Duke, 2003) and could account for the aggressive behaviour of weeds (Locken & Kelsey, 1987).

In contrast, Moodie *et al.* (1997) suggested that it is more likely that the variation detected between weed populations may be a result of herbicide treatments giving rise to variations in phenotypes, which may not be due completely to herbicide resistance. Ash *et al.* (2003) reported that genetic diversity studies give an indication of underlying genetic diversity and an indication of divergent evolution. As highly diverse populations may harbour differing resistances and so may be more difficult to control as it may result in differential reactions and prevent uniform plant responses. Increased understanding of the meaning of identity values could lead to important evidence related to differential tolerance to herbicides in field conditions and to development and spread of resistance (Frigo *et al.,* 2009).

For the development of effective procedures to control rye grass, it is necessary to learn about their mechanisms of spread, for which an understanding of the plant's genetic variation may be important. Data have indicated that there are distinct genetic groups within weedy rye grass populations of the Western Cape. Knowledge about this differentiation of rye grass could aid in the research approach on rye grass resistance and

integrated control methods. In fact, knowledge of both genetic and morphological diversity may be important to guide the development of differential management of rye grass. Results from this study will further enhance our understanding of the genetics and evolution of herbicide-resistant rye grass and may lead to the development of specific and differential management strategies for weed control in each population.

In the Literature Review, Chapter 2 Exp 2 and in Chapter 5 the difference in responses by cultivars from the same crop is emphasized. As rigid, Italian, perennial and weed hybrid rye grass occurred in the study area, it could be speculated that their responses to herbicides may display plant differential effects. Practically speaking and with herbicides registered for grass control (graminicides) grouped as controlling either annual or perennial grass species, this means that the rye grass weed hybrid may have characteristics enabling it to be non-susceptible to herbicides registered as annual graminicides. In that case it cannot be regarded as weed resistance to herbicides but rather as non-susceptibility, because the weed has perennial characteristics. The implication of this is that different control strategies should be devised according to the prevalent species occurring in a particular field.

CONCLUSION

The wide genetic and morphological variation detected in rye grass is interpreted on the basis of high genotypic plasticity and hybridisation for producing *Lolium multiflorum x perenne*. High levels of heterozygosity would indicate that rye grass plant populations probably have substantial amounts of adaptive genetic variations to escape the effects of a control agent. It may also be the result of the differential selection pressure or of the heterogeneity of environmental factors. Effective localised control methods for the various species and hybrids in this genus should be prioritised to curb further development of herbicide resistance. Soils preferred by rye grass cover a wide range of fertility below and above optimum ranges for wheat and emphasises its wide adaptability and success as an invasive genus. Furthermore, rye grass can act as alternative hosts and a source of inoculum

of the important soilborne pathogen crown rot of barley and wheat, underlining its importance as a production constraint in crop production.