Morphological identification of the ryegrass hybrid *Lolium multiflorum x Lolium perenne* and isolation of the pathogen *Fusarium pseudograminearum* in the Western Cape

Ferreira, MI[1*], CF Reinhardt[2], SC Lamprecht[3], M Sinclair[4], L MacKenzie[5] and G van Coller[6]

[1*, 6]*Plant Science, Western Cape Department of Agriculture, Elsenburg, South Africa*

[2]*Department of Plant Production and Soil Science, University of Pretoria, Pretoria, South Africa*

[3]*Agricultural Research Council-Plant Protection Research Institute, Stellenbosch, South Africa*

[4]*Veterinary Services, Western Cape Department of Agriculture, Elsenburg, South Africa*

[5]*Directorate Research Support Services, Western Cape Department of Agriculture, Elsenburg, South Africa*

**Corresponding author*

Abstract

Weed resistance to herbicides present one of the greatest current economic challenges to agriculture. Herbicide resistant ryegrass (*Lolium* spp.) is a serious problem in Western Cape grain producing areas. Morphological and pathogenic analyses were performed on ryegrass samples. Morphologically, 50% of specimens were classified as rigid ryegrass, 48% as a hybrid, namely *L. multiflorum x L. perenne* and 2% as perennial ryegrass. *Fusarium pseudograminearum* (cause of *Fusarium* crown rot) was isolated from six localities. Pathogencity tests confirmed that *F. pseudograminearum* isolates obtained from ryegrass and wheat are pathogenic on both crops, indicating that ryegrass can act as an alternative host and a source of inoculum of this important soilborne pathogen. Grass weed infestation can favour the disease, and grass weed control is therefore recommended as part of an integrated strategy to manage crown rot. Knowledge on morphological differences among ryegrass may be important to guide differential weed management of ryegrass. Smother cropping as part of conservation farming, should receive more prominence to suppress herbicide resistant ryegrass and simultaneously reduce the occurrence of crown rot.

Keywords: integrated weed management, morphological and pathogenic analyses; smother crops

E-mail address: mikefe@elsenburg.com

Introduction

Economically, there is no doubt that herbicides and herbicide-resistant crops have drastically improved agricultural efficiency and yields. However, the reliance on herbicides did not consider the effects which might result due to the continuous use of herbicides, namely the ability of living organisms to adapt to adverse environmental conditions (Gressel 1991). This became evident in the continuous application over many years and/or sometimes the misuse of herbicides with the same active ingredient in the same field which resulted in herbicide resistance (Lorraine-Colwill et al. 2001). Herbicide resistance refers to the capacity of the weed to grow and reproduce after being exposed to a normally lethal dose of a herbicide (Yuan et al. 2007). Weed resistance to herbicides has serious economic implications for agriculture (Baucom 2009) with more than 346 biotypes of weed known to be resistant to herbicides (Heap 2010). Through the process of mutation and selection, however, weeds develop resistance to herbicides after repeated use (Tranel and Trucco 2009). Rigid ryegrass (*Lolium rigidum* Gaud.) was regarded by Tranel and Trucco (2009) to be the most important in terms of developing resistance to multiple herbicides as it exhibits high levels of genetic diversity.

According to O'Hanlon et al. (2000), weed species with higher levels of genetic diversity will exhibit considerable potential for adaptation and may be able to reduce the effectiveness of weed control. Weeds contain inherent genetic traits that give them remarkable plasticity, allowing them to adapt, regenerate, survive, and thrive in a multitude of ecosystems (Chao et al. 2005). The most important requirements for the development of herbicide resistance are the occurrence of heritable variation for the genetic trait and natural selection (Jasieniuk and Maxwell 2001).

Perennial ryegrass (*Lolium perenne* L.) (Charmet and Balfourier 1994) is native to most of Europe and part of the Mediterranean and Middle Eastern areas, whereas rigid ryegrass 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). The genetic diversity of outbreeding *Lolium* spp. has been studied in relation to the characterisation of genetic resources of Italian ryegrass (*Lolium multiflorum* Lam.) (Charmet and 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 ryegrasses are wind-pollinated, self-incompatible outbreeding species (Balfourier et al. 2000). Balfourier et al. (2000) reported on significant patterns of geographical variation with respect to diversity indices and allele frequencies that have been observed in perennial ryegrass. In contrast, no spatial organisation of diversity has been detected in rigid ryegrass (Balfourier et al. 2000).

The first reported cases of herbicide resistance in South Africa was by Cairns and Laubscher (1986) who reported on resistance to diclofop-methyl in wild oats *(Avena fatua)* in the Western Cape province. In 1993, Botes and Van Biljon recorded multiple resistance by *L. rigidum* to ACCase (Acetyl-CoA carboxylase) and ALS synthase-inhibiting herbicides (Heap 2010). This was confirmed by Smit and De Villiers (1998) and Kellermann (2002) for *L. rigidum* and *Lolium* spp. The first case of ryegrass resistance to a non-selective herbicide (glyphosate) was reported by Cairns and Eksteen in 2001 (Heap 2010). In 2003 Cairns (Heap 2010) discovered multiple non-selective herbicide resistance in ryegrass which was confirmed by Eksteen (2007). Smit and Cairns (2001) reported on herbicide resistance of wild radish (*Raphanus raphanistrum*) to chlorsulfuron, while McDermott and Pieterse (Heap 2010) confirmed this for several other ALS synthase-inhibiting herbicides. Pieterse (2010) also recorded a biotype of *Conyza canadensis* Cronquist as resistant to paraquat.

Herbicide resistant ryegrass is a serious problem in Western Cape grain producing areas as it is threatening more than 100 000 ha of productive grain fields (Ferreira and Reinhardt 2010). If left unchecked, economic grain production in certain areas will be impossible in the foreseeable future, leading to huge production losses. Knowledge about the morphological constitution of *Lolium* spp. and its populations is increasingly becoming crucial, particularly with the extent of herbicide-resistance within the Western Cape. Eksteen (2007) described ryegrass as by far the most serious herbicide resistant weed in both annual and perennial crops in South Africa. Therefore, data in this regard will in turn further enhance our understanding of the evolution of herbicide-resistant ryegrass. Descriptive studies of patterns of diversity in weedy populations can be extremely important in weeds to minimise the emergence of resistance to herbicides (Madhou et al. 2005).

Crown rot, caused by *Fusarium pseudograminearum* Aoki and O'Donnell, 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 (Lamprecht et al. 2006). The disease can be significantly reduced by crop rotation with non-susceptible crops such as canola, lupine, annual *Medicago* spp. (medic) and clover in this area (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 therefore to determine the botanical classification of ryegrass samples by morphological analyses and to assess the presence of the crown rot pathogen of barley and wheat on ryegrass.

Material and methods

Four agricultural production areas of the Western Cape Province indicated in Figures 1 were included in this study. These areas were Malmesbury-Moorreesburg (Swartland, area A), Worcester-Robertson (Breede River Valley, area B), Stellenbosch-Paarl (Winelands, area C), and Caledon-Swellendam (Overberg, area D). Ryegrass was sampled in these areas from August until October 2008 at 10 localities in each area. Two more localities with known resistant and susceptible populations of ryegrass (PJ Pieterse, University of Stellenbosch, pers. Comm. 2008) 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 applied to ensure representative sample collection. The Random Geographic Coordinate Sampling function of the software program Survey Toolbox© (AusVet Animal Health services, Toowoomba, Australia;

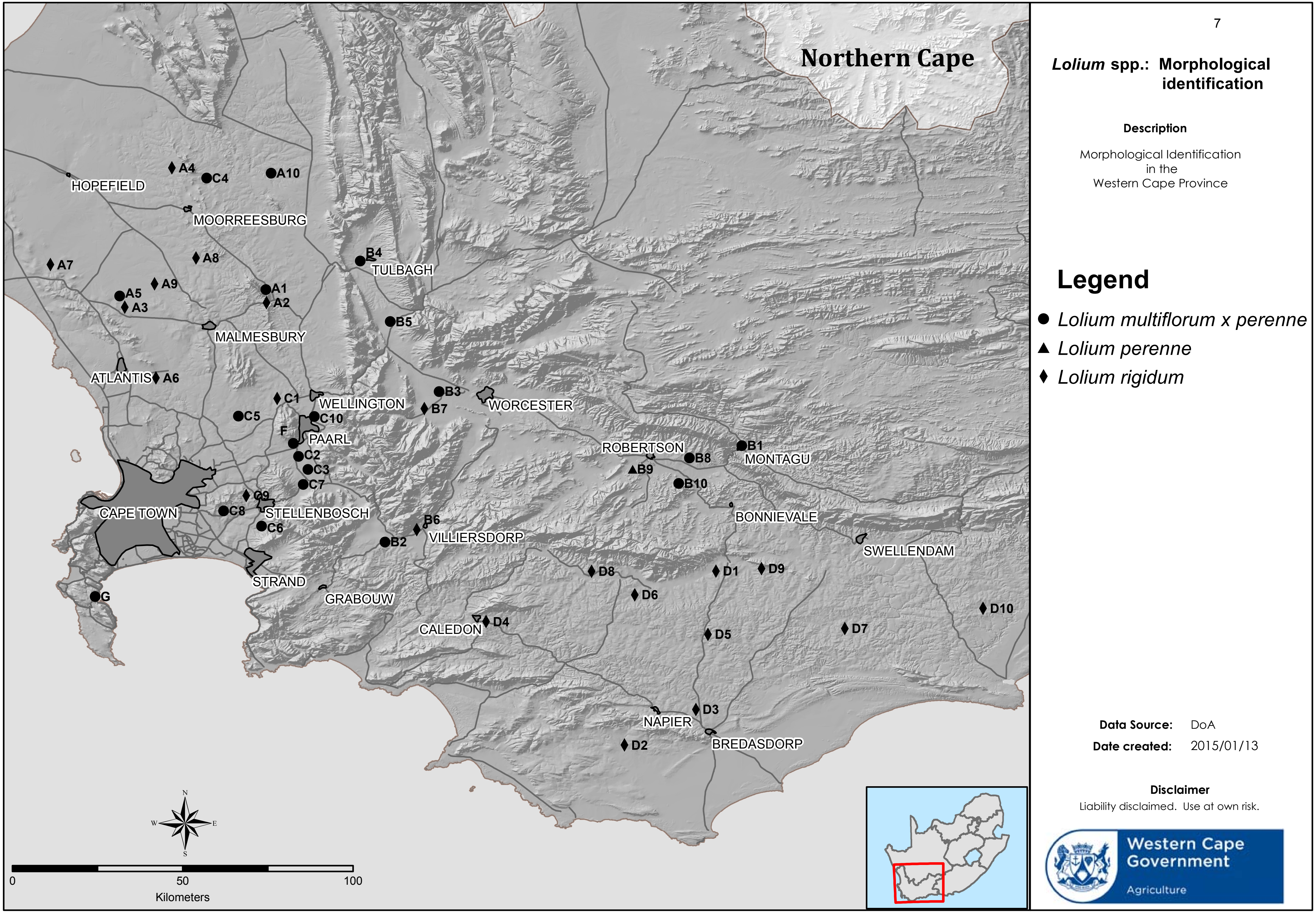

Figure 1: Distribution of Lolium spp. in four agricultural production areas, designated A, B, C and D, of the Western Cape province

http://ausvet.com.aucontent.php?page=software#st) 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 (RockWare, Inc., Golden, CO, USA) was used for GIS manipulation of these collection points for easy reference during collection. A Magellan® SporTrak GPS system (with 3 m accuracy) was utilised in the location of these randomly selected collection points.

Morphological analyses

The first specimen of each sample, which included all aboveground plant parts in all instances, was collected and taxonomically analysed by botanists at the Compton Herbarium, Kirstenbosch National Botanical Gardens, Cape Town, in order to identify the different species or hybrids. Taxonomy of ryegrass samples included the study of plant inflorescence (ryegrass head), endosperm within the seed and fruit (ryegrass seed morphology), and comparing these characteristics with existing herbarium specimens.

Pathogenic analyses

The second specimen of each sample was collected and analysed for the soilborne pathogen *F. pseudograminearum* 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. Isolates of the fungus were identified using morphological characteristics (Leslie and Summerell 2006). These identifications were confirmed by PCR using known species-specific primers for *F. pseudograminearum* (FPG-F and FPG-R) (Williams et al. 2002). The identity of the reference isolate (obtained from wheat in a separate study) that was used to compare the isolates obtained in this study was confirmed previously by Dr T.J. Ward (Bacterial Foodborne Pathogens and Mycology Research Unit, Agricultural Research Service, United States Department of Agriculture, USA) using a multilocus genotyping assay (MLGT) (Ward et al. 2008). Polymerase chain reaction assays were conducted using 2 µL template DNA (20 ng μL^{-1}) in a total volume of 25 µL containing 2.5 µL reaction buffer (1X), 1.25 µL $MgCl_2$ (2.5 mM), 1.25 µL bovine serum albumin (1mg mL^{-1}), 1 µL dNTPs (0.4 mM), 0.625 µL of the primers FPG-F/FPG-R (125 nM) and 1.5 U Taq DNA polymerase. Amplifications were carried out with an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s, with a final extension step at 72 °C for 5 min. The resulting amplicons were separated by gel electrophoresis on 1% agarose gels stained with ethidium bromide and visualised under UV lights.

The pathogenicity and virulence of the ryegrass isolates of *F. pseudograminearum* were compared with isolates of the fungus with known virulence to wheat. Four representative isolates of *F. pseudograminearum* (M7456G, M7458E, M7458F, M7468C) from ryegrass and four isolates (2-45 [Porterville], 2-62 [Porterville], 2-369 [Riversdale], 2-420 [Bredasdorp]) from wheat were selected to compare pathogenicity and virulence on ryegrass hybrid type and wheat under glasshouse conditions. Ryegrass hybrid type was selected because it is the most dominant grass weed in this area (Ferreira and Reinhardt 2010). Since four wheat isolates were included in this study, four ryegrass isolates were also included and these were representative of the isolates obtained from the ryegrass. Millet inoculum of each isolate was prepared according to the method described by Strauss and Labuschagne (1995). The inoculum was incubated for 11 days at 22°C without being directly exposed to light, and shaken every fourth day to ensure even growth of the mycelium throughout the medium. Inoculum was added to a planting medium at a concentration of 0.05% inoculum/planting medium (w/w). The planting medium was made up of equal amounts of soil, perlite and sand, which was pasteurised (30 min at 83 °C) and left for 3 days before being mixed with the inoculum. After mixing, pots were watered and left to stand overnight in the glasshouse (25 °C day and 15 °C night) before being planted with 50 seeds per pot (five seeds/hole and 10 holes/pot). There were three pots per treatment (isolate) per crop and pots were arranged in a randomised block design. Survival and growth of seedlings were recorded 14 days after planting.

Statistical analysis

Data were subjected to an appropriate analysis of variance using SAS version 9.2 statistical software (SAS Institute Inc., Cary, NC, USA) and the standardised residuals were tested for deviation from normality using the Shapiro-Wilk test (Shapiro and Wilk 1965). Student's protected *t*-least significant differences (LSD) were calculated at the 5% significance level to compare means for significant effects.

Results and discussion

Morphological analyses

Morphologically, 50% of the total number of specimens was classified as rigid ryegrass, 48% as ryegrass hybrid type, namely *L. multiflorum x L. perenne* and 2% as perennial ryegrass. Both the proven herbicide resistant (F) and susceptible specimens (G) were identified as ryegrass hybrid type (Figure 2).

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 ryegrass and 7% as ryegrass hybrid type. Morphological analyses identified 40.5% of specimens sampled in areas B and C as ryegrass hybrid type and 10% as rigid ryegrass. Only one specimen (2%) which occurred in area B (Breede River valley) was classified as *L. perenne.* Ryegrass hybrid type displayed forked ears, indicating prolific seed production, in 8% of specimens. These results are in agreement with those published by Treier et al. (2009), that taxonomists have identified at least two forms of the allelopathic weed, *Centaurea maculosa,* from weeds in their native region of origin. In addition to multiple flowering in a year, the tetraploid of this weed is capable of producing multiple flowering stems with up to 15 capitula each, whereas the diploid produces only one stem (Broz and Vivanco

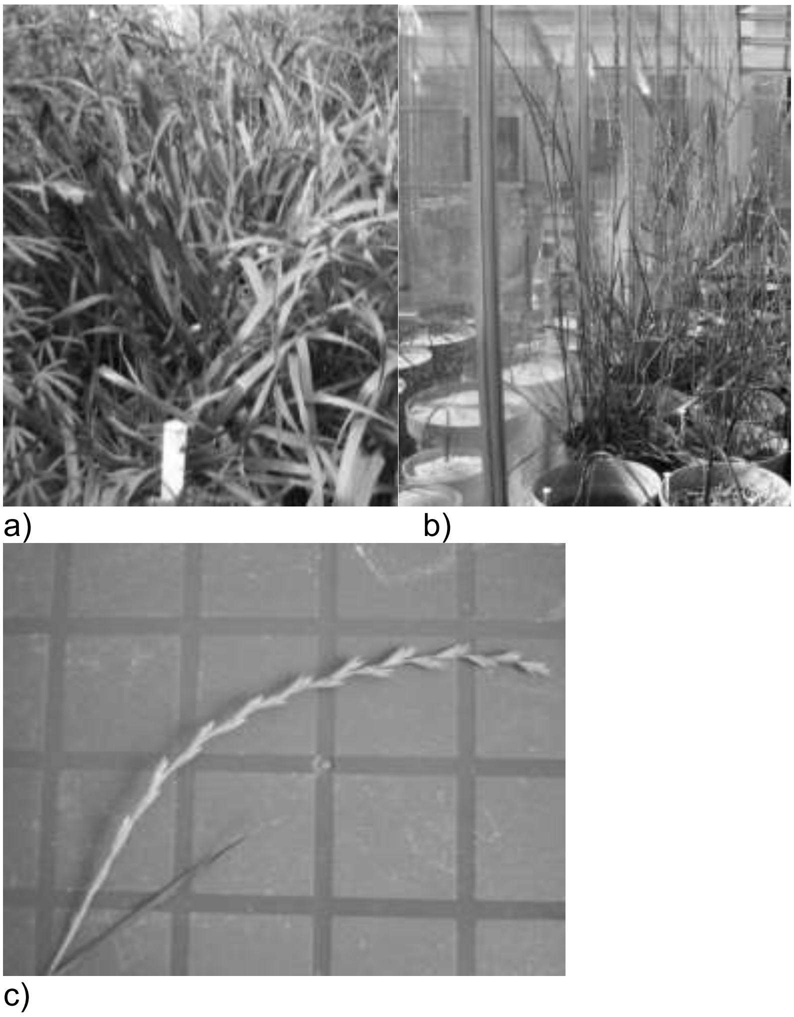

Figure 2 Images of ryegrass hybrid type (*Lolium multiflorum x perenne*) at four (a) and twelve (b) weeks after emergence and mature head (c)

2009). Furthermore, a case in point is the important and well-recognised component in the evolutionary history of *Amaranthus* spp. of interspecific hybridisation (Tranel and Trucco 2009). A clear example of this possibility is herbicide-resistance evolution which is also evident in the case of ryegrass. 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 and 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 (Tranel and Trucco 2009).

Pathogenic analyses

Morphological characters of all eight fungal isolates corresponded well to descriptions of *F. pseudograminearum* made by Leslie and Summerell (2006). The identity of the eight isolates was also confirmed with the PCR methodology.

Fusarium pseudograminearum was isolated from six localities namely, A2 (33.4008° S, 18.6734° E,) on rigid ryegrass, A4 (33.0443° S, 18.6236° E,) on rigid ryegrass, A7 (33.3001° S, 18.3026° E) on rigid ryegrass, B5 (33.4502° S, 19.2001° E,) on ryegrass hybrid type, C9 (33.9101° S, 18.8200° E) on rigid ryegrass and D10 (34°2078° S, 20.7653° E) on rigid ryegrass (Figure 3). The fungus was isolated from brown discoloured stem bases and crowns, which are typical of the symptoms of the pathogen on wheat as described by Burgess et al. (2001).

Data on the survival and growth of ryegrass and wheat seedlings grown in soil infested with *F. pseudograminearum* are given in Table 1. There were no significant crop x isolate interactions for seedling survival and growth indicating that the two crops reacted similarly to the *F. pseudograminearum* isolates. Isolates 2-62, 2-369, 2-420, M7458E and M7468C significantly reduced survival and the same isolates and isolate 2-45 significantly reduced

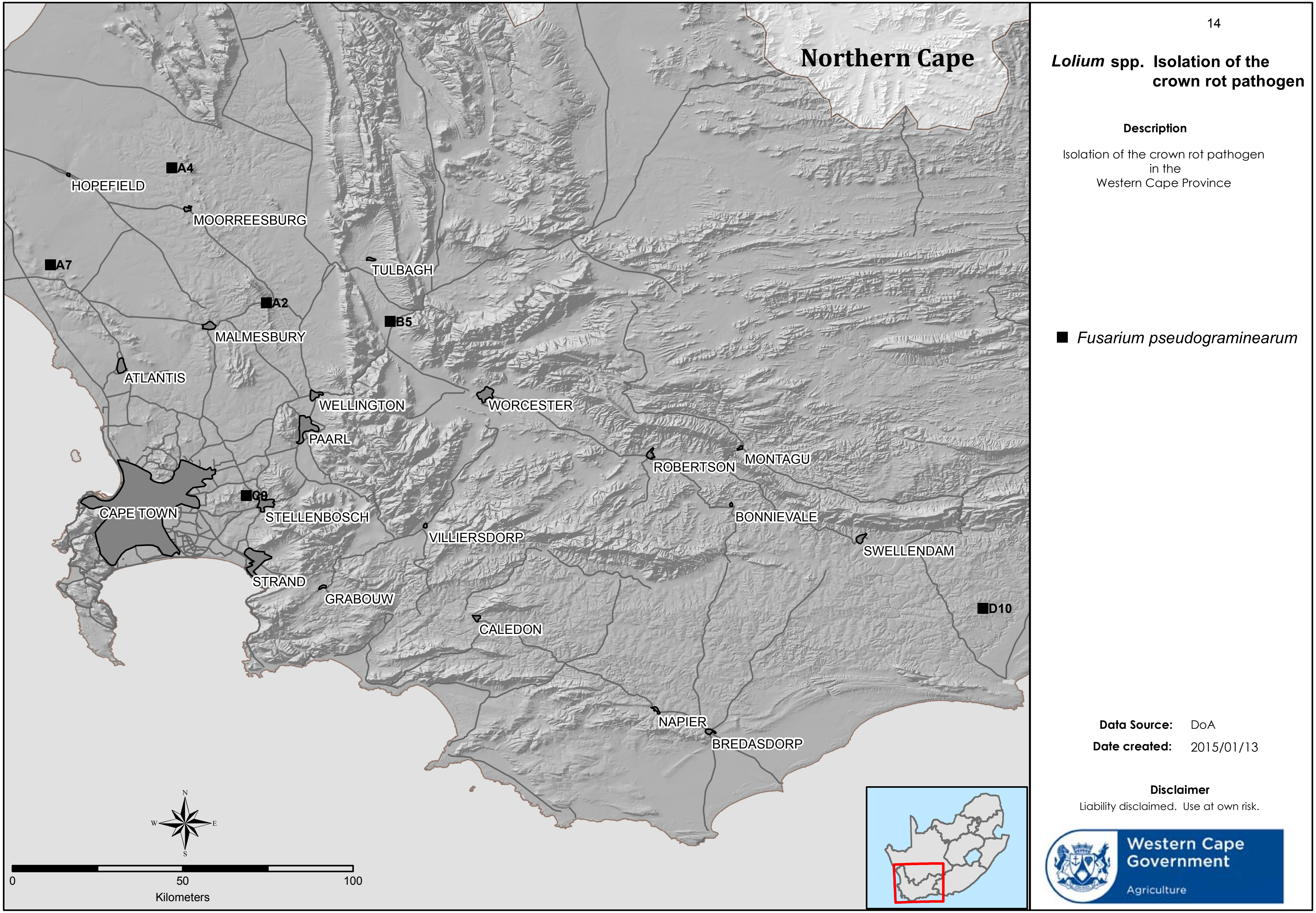

Figure 3: Collection points where crown rot was isolated from Lolium spp. in the Western Cape province

seedling growth of wheat. Isolates 2-62, M7458E and M7468C significantly reduced survival of ryegrass and all the isolates that significantly reduced growth of wheat also reduced growth of ryegrass. The low survival of the ryegrass control is unusual and difficult to explain. Crown rot was recorded on both wheat and ryegrass seedlings in pots inoculated with the fungus. To our knowledge and according to literature searches, this data on *F. pseudograminearum* has not been published previously, and this is the first report of it as a pathogen on ryegrass in South Africa.

Table 1 Survival and growth of ryegrass hybrid type and wheat seedlings grown in soil infested with *Fusarium pseudograminearum* under glasshouse conditions during pathogenic analyses

Isolate	Survival (%)[a]		Plant length (mm)[a]	
	Ryegrass	Wheat	Ryegrass	Wheat
2-45	39.3 ab	69.3 ab	122.5 bc	208.0 c
2-62	26.7 b	34.0 c	97.9 c	209.5 bc
2-369	41.3 ab	48.7 bc	125.5 bc	209.4 bc
2-420	35.3 ab	44.7 c	122.4 bc	174.1 c
M7456G	44.7 a	75.3 a	140.2 ab	227.2 ab
M7458E	27.3 b	44.0 c	99.0 c	174.5 c
M7458F	39.3 ab	75.3 a	147.5 ab	249.0 ab
M7468C	34.7 ab	46.0 c	100.9 c	174.0 c
Control	46.7 a	82.0 a	162.8 a	262.4 a

[*]Means in a column followed by the same letter do not differ significantly at P = 0.05

Ryegrass can therefore act as an alternative host and 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 ryegrass may lead to a higher incidence of *Fusarium* crown rot on wheat and barley due to higher disease pressure.

For the development of effective procedures to control invasive plants, it is necessary to learn about their mechanism of spread. Similar to morphological data from this study, it is highly likely that there are distinct genetic groups within weedy ryegrass populations of the Western Cape. 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 and Maxwell 2001). Knowledge of genetic and morphological diversity may be important to guide the development of differential management of ryegrass as opposed to a singular blanket approach for all ryegrass biotypes, which might increase resistance due to bad non-specific practices. Knowledge of the differentiation of ryegrass could aid research on ryegrass resistance and integrated control methods. Results from this study will further enhance our understanding of the evolution of herbicide-resistant weeds.

Although an initial attempt to do a genetic study was undertaken (data not presented), it was abandoned as simple sequence repeats (SSRs), using an appropriate selection of published primer pairs, have been published only for Italian, perennial or rigid ryegrass (Jones et al. 2002; Saha et al. 2004; Mian et al. 2005; Saha et al. 2005; Senda et al. 2005; Hirata et al. 2006; Studer et al. 2006). Therefore, no SSRs have been characterised and published for hybrids among *Lolium* species, such as *L. multiflorum x L. perenne,* rendering all efforts to have samples genetically analysed useless.

Nevertheless, Dinelli et al. (2002) reported high variability in Italian populations of ryegrass and hybrid banding profiles from electrophoretic data with up to 24% of individuals placed in an intermediate position between rigid ryegrass and Italian ryegrass. By contrast, Australian populations were more homogeneous with 88% of individuals showing an ordination closely related to rigid ryegrass. Furthermore, Italian populations of ryegrass were heterogeneous, consisting of several genetically unique individuals that can readily hybridise (Dinelli et al. 2002). The evolution of herbicide resistance often forces dramatic changes in weed management practices (Tranel and Trucco 2009). In view of the above, it is clear that further genetic analyses of *L. multiflorum x L. perenne* are needed locally. Nevertheless, with the herbicide resistance crisis at hand, Western Cape Province grain producers have been following integrated weed management programmes (Pieterse 2010). This might help to reduce reliance on particular herbicide types (Llewellyn et al. 2007) and might be achieved, as Kohli et al. (2006) suggested, with cultural control methods which include crop rotation, use of smother and green manure crops, crop residues, crop genotypes with better competitive and allelopathic ability, manipulation of sowing or planting date, crop density and crop pattern. Of these cultural methods, the use of smother cropping (FAO 2008) should receive more prominence.

Using a smother crop could suppress weeds, including herbicide resistant types, and provide other beneficial effects in sustainable agricultural systems such as increased organic matter and reduced occurrence of crown rot. The term smother crop refers to a dense and fast growing crop that suppresses or stops the growth of weeds and provides successful long-term weed management which, according to Storkey and Lutman (2008), requires a shift from simply controlling problem weeds with in-crop herbicides to agricultural production systems that are redesigned to manage weeds at all stages of their life cycle. Such systems should restrict weed emergence, reduce weed growth and reproduction, and minimise weed competition with crops.

Research has also clearly indicated that the effectiveness and consistency of these non-herbicide weed management practices greatly increases when three or more of these practices are simultaneously employed (Storkey and Lutman, 2008). Once these integrated weed management systems are implemented, herbicides can be used in a more targeted and sustainable manner, preserving their usefulness for decades to come as they are non-renewable resources (Pieterse 2010).

Conclusions

The wide morphological variation observed in this study is interpreted on the basis of high genotypic plasticity and hybridisation of *Lolium* spp. to produce a hybrid such as *L. multiflorum x L. perenne*. In addition, ryegrass can act as an alternative host and a source of inoculum of the important soilborne pathogen, *F. pseudograminearum* of barley and wheat. Effective localised control methods for the various species and hybrids in this genus should be prioritised to curb herbicide resistance. Smother cropping should receive more prominence to suppress herbicide-resistant ryegrass and simultaneously reduce the occurrence of crown rot.

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