Busseola fusca and *Chilo partellus* survival on Bt and stacked-gene maize varieties

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ABSTRACT

Genetically modified Bt maize expressing Cry proteins are used to control lepidopteran pests of maize. Damage caused by these stemborer species *Busseola fusca* (Lepidoptera: Noctuidae) and *Chilo partellus* (Lepidoptera: Crambidae) may result in economically important yield losses. Effective control of insect pests on maize is therefore of utmost importance. However, *B. fusca* has been reported to be resistant to Bt maize in South Africa. The main reason for resistance evolution was thought to be poor compliance to the high dose/refuge strategy requirements, but later studies showed that other factors could also have played a role. Therefore the possible effect of stacking of insecticidal (Bt) and glyphosate resistance (GR) traits as well as application of glyphosate to plants of stacked events (Bt/GR) on the efficacy of Bt needed to be investigated. The aim of the study was to determine if the combination of Bt and GR traits as well as the application of glyphosate have an effect on the efficacy of Bt maize against stem borers and whether the Bt expression levels in plants may be effected when stacked with the GR trait or if glyphosate is applied. The survival and larval development of two stem borer species were evaluated on single gene (Bt and GR) and stacked gene (Bt/GR) maize cultivars. Laboratory bioassays were conducted over three consecutive seasons and in greenhouse trials (2014/15). These six treatments were: 1) non-Bt control, 2) Bt-maize, 3) GR maize, 4)Bt/GR stacked maize, 5) GR maize sprayed with glyphosate and 6) Bt/GR stacked maize sprayed with glyphosate. All Bt gene varieties expressing the same Bt gene (MON810) were used. Plants were inoculated either with *B. fusca or C. partellus* in a greenhouse trial whereas plant tissue was used in the laboratory trials. Although not significantly different, larval survival recorded from plant tissue of the GR treatment and GR maize onto which glyphosate was applied tended to be the highest in six out of the seven trials for *B*. *fusca* and five out of seven for *C*. *partellus*. Results indicated that the GR trait together with the application of glyphosate does not have an adverse effect on larval development, and that it may have a positive effect on larval survival and growth. Larval development as the only effect indicators of stacked traits and glyphosate application on Bt expression levels cannot be used solely to make a final conclusion. Results from ELISA's that were conducted using leaf tissue of plants of all treatments showed large variation in the Bt expression levels in these plants. Indications were however that glyphosate application or the presence of the GR trait in a stacked event with Bt trait, did not affect Cry protein expression in plants.

Key words: *Busseola fusca,* ELISA, Bt expression, stacked traits, stem borers, glyphosate.

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Chapter 1 - Introduction and literature review

1.1 What is genetically modified (GM) crops?

Crops were originally genetically modified to produce insecticidal Cry proteins, which are also produced by the soil bacterium, *Bacillus thuringiensis* (Bt). GM crops are also modified to tolerate application of the broad spectrum herbicide, glyphosate, without damaging the crop. Genetic modification of crops enables farmers to minimise the reliance on a diversity of herbicides and also to simplify pest management (Tabashnik, 2010). Genetic modification of crops provides farmers with increased productivity at farm level despite the fact that farmers need to pay more for seed. This improvement results from a combination of cost reduction, time saving and improved pest management benefits which also limit the adverse environmental and health impacts associated with application of pesticides (Dent, 2000; Gouse, 2005; Duke and Powles, 2009; Brookes *et al.,* 2010).

Through planting genetically modified (GM) Bt crops, farmers move towards advanced levels of integrated pest management (IPM), thereby limiting negative effects on the environment and non-target species (Cerdeira and Duke, 2006; Brookes and Barfoot, 2008). Prior to the introduction of GM crops farmers largely depended on cultural control and conventional chemical control methods in order to control major pests (Hyde *et al*., 2000).

Currently both maize and cotton have either stand-alone or a combination of glyphosate resistant (GR) or insect resistant traits in a single cultivar (Duke and Powles, 2009). In existing literature, such combinations are referred to “stacked” or “pyramided” traits or events (Taverniers *et al.,* 2008). Stacking of multiple traits provides farmers the benefit of multiple pest and weed control practices all at once (Dill *et al.*, 2008).

The four major GM crops planted worldwide is soybean, cotton, maize and canola which was planted on a total area of 368 million hectares, of which 49% was genetically modified. The adoption of GM crops has increased since 1996 from 1.7 million hectares to over 181 million hectares in 2014 (Fig. 1.1). Currently, South Africa is ranked ninth out of 27 countries, based on surface area planted to GM crops, reaching 2.7 million hectares in 2014 (James, 2014). GM crops with stacked traits were planted on 43.7 million hectares in 20 countries in 2012 whereas in 2013, 47 million hectares of stacked gene crop varieties were planted. Stacked traits occupied 28% of the 181 million hectares of biotech crops planted in 2014, which shows a steady and growing trend expect to continue in the future (James, 2014).

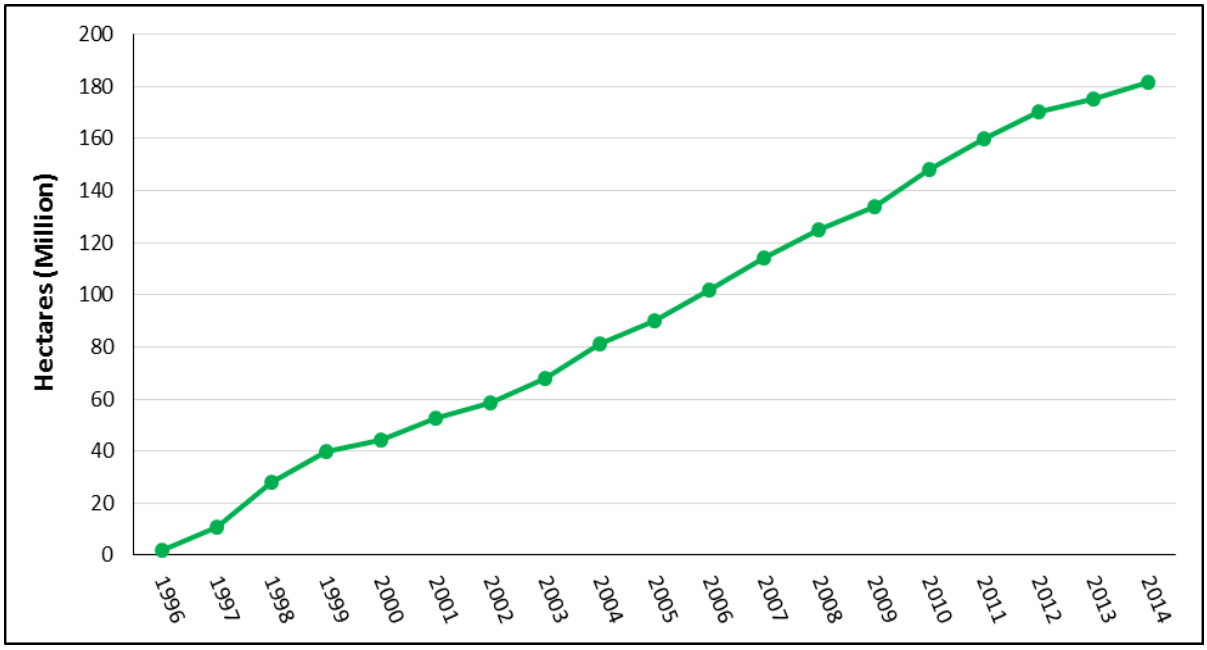

Figure 1.1 The total area of GM maize planted globally since 1996 (James, 2014).

1.2 Glyphosate resistant crops

1.2.1 The use of glyphosate resistant (GR) crops globally and in South Africa

Weeds affect crop yields when competing for water, sunlight, space and nutrients. The critical period for weed control in crops is crucial in order to protect the crop yield. This critical period of weed control depends on several factors such as the weed species present, weed densities, the specific crop, weed emergence in relation to crop emergence, environmental conditions and production practices used (Hall *et al.,* 1992).

Weeds have traditionally been a significant problem in soybean, canola and maize production systems. Farmers successfully controlled weeds in the past based on repeated applications of broad-spectrum herbicide mixtures. Glyphosate resistant crops have changed weed management practices over the past few decades (Foresman and Glasgow, 2008).

1.2.2 What is Glyphosate?

Glyphosate is the active ingredient in the commonly used herbicide Roundup®. Glyphosate is a foliar-applied, broad spectrum systemic herbicide used to control annual, perennial and biennial herbaceous grass, sedge and broadleaf weeds in cropping systems and is labelled that it can control over 300 weed species (Green and Owen, 2011; Kannan & Chinnagouder,

2013). This is a popular non-selective herbicide that is translocated to both above- and below-ground meristems of plants (Duke, 2005; Funke *et al.,* 2006). It is a post-emergence herbicide, as it has no residual activity in the soil. Before GR crops were commercialized, glyphosate was generally used in non-crop situations (Duke *et al.,* 2003b). Since GR crops were developed farmers started to apply glyphosate in a whole new way (Shah *et al*., 1990).

Currently, GR maize provides farmers with an effective, economical, safe and easier weed control strategy which delivers a "higher yield" to farmers (Brookes *et al.,* 2010). Glyphosate resistant crops also require less tillage in cropping systems and it does not restrict crop rotation programs (Foresman and Glasgow 2008; Gustafson, 2008).

The adoption of GR crops contributes to the practice of conservation agriculture (CA). However, resistance development is a threat when glyphosate alone is applied over wide areas on highly viable and prolific weeds (Duke and Powles, 2009; Green and Owen, 2011). The continuous development of GR weeds also holds a threat to the sustainable use of glyphosate (Heap, 2010). Furthermore, the presence of GR weeds increases the cost of weed management and thereby reduces the benefits that glyphosate-based weed management systems deliver (Green and Owen, 2011).

1.2.3 Commercialization of Glyphosate resistant crops

Glyphosate-resistant soybean and canola was commercialized during 1996, 22 years after the development of glyphosate (Monsanto, 2005; Owen and Zelaya, 2005). Since then the pattern of glyphosate use has changed dramatically. Herbicide resistance became a reality where the selection of herbicides is most persistent (Duke and Powles, 2009).

The adoption rate of genetically modified (GM) crops has shown a gradual increase worldwide over the past few years since its first release (James, 2013a; 2013b). A total area of 2.9 million hectares of GM crops was planted in South Africa in 2013. A total of 2.36 million hectares of genetically modified maize plantings was reached in 2013, of which 18.2% contained the GR gene. Of the total soybean plantings, 478 000 hectares (92%) were GR. Cotton production decreased over the past few years to 8 000 hectares planted in 2013. All of the total cotton production was genetically modified, with only 5% of these serving as the refuge and therefore having only the GR trait (Fig. 1.3) (James, 2013a; 2013b).

1.2.4 The use of glyphosate in general

The usage of glyphosate have increased with the development of GR crops and led to the misuse of glyphosate since then. The reasons for this can ascribed to farmers who now only rely on glyphosate, whereas in the past, farmers used different herbicides with different modes of action (James, 2013a). Shortly after the introduction of GR crops, stacked events also became a reality. Stacked event crops now occupy 27% out of the 175 million hectares of GM crops that were planted during 2013 (James, 2013a). This resulted in an increase in the usage of glyphosate over the years since then. The reason for this could be due to the fact that glyphosate is a broad spectrum herbicide, it is cheap, easy and safe to use and, most importantly, glyphosate is easily accessible.

When glyphosate is applied to plants, it is absorbed through the leaves and translocated to the growing points of shoots and roots of plants. The shikimate pathway, enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), is important for the survival of plants. Glyphosate inhibits the enzyme EPSPS which is critical in the shikimate acid pathway responsible for biosynthesis of aromatic amino acids in plants. Crops that are genetically modified to withstand glyphosate application, carries a glyphosate-insensitive form of the gene coding of this enzyme, derived from the *Agrobacterium* sp. strain CP4. Structural similarities to phosphoenol pyruvate enable glyphosate to bind to the substrate binding site of the EPSPS. Glyphosate then inhibits/interfere with the enzyme production of certain amino acids that are essential for plant growth, and blocks the import thereof into the chloroplast. As a result, the growth and the development of the plant are stunted, and it dies off. Herbicide tolerant maize produces an enzyme that have the same functions as EPSPS synthase, but it is not inhibited by glyphosate (Yamada *et al.,* 2009; Zobiole *et al.*, 2010a).

1.2.5 Effects of Glyphosate

Farmers have reported that some GR soybean varieties show injury such as “yellow flashing” or yellowing of the upper leaves after glyphosate application (Zablotowicz and Reddy, 2007). These symptoms also affect the nutritional status of the crop (Zobiole *et al.,* 2010a). Other studies showed that the application of glyphosate affects some photosynthetic parameters of soybean. These parameters that can be affected may include net photosynthesis, transpiration rate and stomatal conductance of GR in different maturity group cultivars (Zobiole *et al.,* 2010b). Traces of glyphosate can be found in plants from the time of application up to physiological maturity of maize (Arregui *et al.,* 2003; Duke *et al.,* 2003a).

Reports also indicate that GR soybeans are sensitive to water stress after glyphosate application (Zablotowicz and Reddy, 2007) and it was reported that soybean plants at a younger stage (V4 growth stage) are more sensitive to glyphosate effects than older plants (V7 growth stage) (Zobiole *et al.,* 2010c). Studies done by Zobiole *et al.* (2010c) revealed that decreases in the photosynthetic rates are directly linked to the effect of glyphosate on chlorophyll content in the plant, which may relate to chlorosis symptoms observed in GR soybean plants after glyphosate application.

Glyphosate application may have the following effects on GR soybeans:

- Glyphosate reduces the availability of nutrients to the plant (Zobiole *et al.,* 2010b).
- Direct damage to chlorophyll which may in turn result in lower chlorophyll content (Reddy *et al.,* 2000).
- Immobilization of the nutrients which is compulsory for chlorophyll production and function (Mg and Mn) (Taiz and Zeiger, 1998).
- AMPA, which is the main metabolite of glyphosate, may also contribute to chlorosis (Reddy *et al.,* 2000; Duke *et al.,* 2003b).
- Glyphosate or its metabolites may have long-term physiological impacts on soybean plants (Zobiole *et al.,* 2010b).

1.3 Introduction to Bt maize

1.3.1 Background information on Bt insecticides and Bt maize

Bt maize is genetically engineered to produce an insecticidal protein (Cry1Ab) that is derived from the entomopathogenic bacterium, *Bacillus thuringiensis* (Bt). The Bt gene encodes for endotoxins selectively lethal to economically important stem borer species, *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), *Chilo partellus* (Swinhoe) (Lepidoptera: Crambidae) (Van Rensburg, 1999; Gray, 2010) and *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae) (Van Wyk *et al*., 2009). These Cry proteins are introduced into several commercially grown crops, including maize, to protect it against pests such as the African stem borer, *B. fusca.* Bt Cry proteins cause perforations in the mid-gut of target insects, through which infections are caused, resulting in larval death (Gray, 2010).

Although first-generation Bt crops produced only a single Bt toxin, some second-generation Bt crops produce two distinct Bt toxins that are active against the same pest (Tabashnik *et al*., 2009).

Busseola fusca and *C. partellus* are two of the most important insect pests of maize and grain sorghum in South Africa and provides one of the most important insecticide markets considering all field crops in the country. These species may occur in single or mixed populations (Van den Berg *et al*., 1991) in the same crop. The maize stem borer causes an average annual yield loss of 10 % in commercial farming systems which can contribute to an annual yield loss of R 2.6 billion if the 13 million tonnes of 2014 is taken into account (James, 2014). Yield losses due to stem borers are common and the level of loss depends on the agro-ecological zone, pest population density and crop age at infestation stage. Control measures are costly and epidemics pose a constant threat to food security (Kfir *et al.*, 2002).

Insecticide applications can be used to control these pests, but it is crucial for the application to be timed correctly in order for it to be effective. A problem with chemical control arises when older larvae enter stalks where they are difficult to reach with contact insecticides (Slabbert and Van den Berg, 2009).

Bt maize served to alleviate problems regarding insecticide applications to a large extent. However, during the 2004/05 season Bt hybrids were severely damaged in some irrigation as well as dry land areas in South Africa. Shortly thereafter the first field resistance was reported in 2006 (Van Rensburg, 2007; Kruger *et al.,* 2009).

Successful deployment of Bt technology has led to reduction in insecticide application and an effective control practice for stem borers (Hellmich and Hellmich, 2012). Bt maize is currently planted on more than 80% of the maize production area of South Africa (James, 2013b).

In 1996, Bt maize containing the Cry1 transgene was introduced in commercial farming in the USA. Bt maize was initially commercialized to control *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Ostilie *et al*., 1997) and *Diatraea grandiosella* (Dyer) (Lepidoptera: Crambidae) in North America (Archer *et al*., 2001). Subsequently Bt maize was introduced in South Africa, during 1998 growing season, to control *B. fusca* and *C. partellus* (Van Rensburg, 1998).

1.3.2 Advantages of Bt maize

Bt maize provides farmers with various benefits but farmers can only benefit from planting Bt maize if target pests are present in high infestation levels (Van den Berg *et al*., 2013). Bt maize provides farmers with reduced input costs, season-long protection irrespective of weather conditions, effective control of stem borers which may be difficult to reach with insecticide sprays (Kruger *et al.,* 2009).

1.3.3 The development of resistance against Bt maize

Before the commercialization of Bt crops, there has been concerns with regard to the development of resistance to the insecticidal protein (Gould, 1998; Tabashnik *et al*., 2003; Zhao *et al*., 2003). Although measures have been set in place to counter and delay resistance development, Van Rensburg (2007) reported that *B. fusca* has attained some levels of resistance to Bt maize in South Africa. Resistance can be seen as a development of a strain of the pest capable of surviving a dose lethal to a majority of individuals in a normal population (Van den Berg *et al.,* 2013). Resistance can also be defined as reduced susceptibility of a population to a toxin caused by field exposure (Tabashnik, 1994). Resistant populations of *B. fusca* are now widespread within the maize production region based on surveys and studies done by Kruger *et al.* (2012). Van den Berg *et al*. (2013) indicated that there are no management strategies in place to limit the spread and further development of resistant populations. During 2011, another Bt event, MON89034, was commercially released in South Africa, to control MON810-resistant larvae of *B. fusca*. The MON810 Bt event expresses one Cry protein (Cry1Ab), while MON89034 expresses two different Cry proteins (Cry1A.105 and Cry2Ab). Up until now, event MON89034 controls *B. fusca* effectively (Van den Berg *et al*., 2013). *Bacillus thuringiensis* was used as an insecticidal spray without substantial resistance developing in field populations until 1994 (Tabashnik, 1994).

1.3.4 Insect resistance management (IRM)

Evolution of resistance by pests threatens the continued efficacy of GM crops (Tabashnik *et al*., 2009). The high-dose/refuge strategy was set in place in order to delay resistance development in insects. The main aim is to produce a high proportion of susceptible moths compared to resistant moths (Gould, 1998; Tabashnik, 2010).

This strategy is aimed at killing individuals with incomplete dominant resistance or heterozygous resistance (Gould, 2000; Bourguet *et al.,* 2005; Tiwari and Young, 2011). This strategy entails two components: Bt maize that expresses a high dose of the Bt Cry protein and planting a refuge of non-Bt maize near the Bt maize. The high dose refers to a high dose of the Cry protein expressed by the plant, lethal to all susceptible and heterozygous individuals (Gould, 2000; Tabashnik, 2010; Tiwari and Youngman, 2011). The purpose of the high dose strategy is to kill all susceptible homozygous individuals, including heterozygous genotypes that carry one resistant allele (Gould, 1998). If the high dose of Bt toxin does not eradicate all larvae with one resistant allele, the larvae may survive to adulthood, after which moths d mate

with other resistant moths, and the offspring from these mating's would be resistant to Bt maize (Gould, 1998; 2000).

Poor refuge compliance by farmers is one of the major reasons that contributed to the selection pressure for resistance development to Bt crops (Kruger *et al*., 2009). If refuge requirements are not adhered to, the risk of resistance development of target species may be elevated. Sub-lethal dosage expression levels of the Cry protein in maize plants could also play a role together with farmers that do not scout and monitor their fields for pests as they assumed the technology controlled the pests effectively (Kruger *et al*., 2009). Resistance could also be a result of continuous exposure of larvae of the second seasonal moth flight to sub-lethal dosages of the Cry-toxin at late growth stages of maize plants. (Van Rensburg, 2007).

Resistance development could possibly also have resulted from general late planting dates, following high stem borer infestations and variance in time of planting, thus providing continuous supply of moths (Kruger *et al*., 2009). An insect is able to develop resistance based on ecological, biochemical and genetic principles if the insect population has a high development rate of the immature stages and a quick succession of generations, while being exposed to a sub-lethal dose of a toxin. Studies done by Van Rensburg (2007) showed that moths may give preference to irrigated maize, which could also have contributed to the development of resistance to the Bt-toxin. Previous studies confirmed that the Cry1Ab expression levels in the plants differ between young and older maize plants and environmental conditions do influence Bt expression levels in transgenic maize (Dutton *et al.,* 2009).

The principle of the refuge strategy is based on the theory of population genetics. The purpose is to promote random mating, thus providing a source of stem borers, not exposed to Bt maize or Bt insecticides that could mate with potentially resistant moths emerging from nearby Bt maize and thereby reducing the number of resistant alleles and prolonging the susceptibility of the pest population (Gould, 1998; 2000; Tiwari and Youngman, 2010).

There are a few concerns with regard to the high dose refuge strategy that may limit its success. One factor that may interfere with random mating between susceptible and resistant populations is asynchrony in moth emergence. It is necessary for the development period of the resistant and susceptible individuals to overlap, to ensure mating between these different populations.

1.3.5 Factors that may influence the expression of Cry- toxin (Cry1Ab) in maize

The efficacy of insecticidal proteins in crops may be associated and/or influenced by numerous factors and environmental conditions. One of the main reasons for the “failure” or reduced efficacy of Bt cotton against target insect-pests may be ascribed variation in Cry-toxin levels in the crop (Benedict *et al.,* 1996).

Observations have shown that the Cry-toxin levels in cotton decreased significantly under extreme temperature (Chen *et al.,* 2005), deficient nitrogen (Coviella *et al.,* 2002) and NaCl stress (Jiang *et al*., 2006). All of these issues are associated with the failure of the insect-resistant efficacy (Zhen *et al*., 2008; Shen *et al*., 2010). The efficacy of Bt cotton shows a discrepancy under environmental stresses with plant structure or age (Dong and Li, 2007). Studies done by Bruns and Abel (2003) indicated that the Cry toxin in maize is positively affected by the increase in N-fertility during reproductive growth stages. Several other studies showed that the Bt-concentration may vary over the growing season and within the different parts of the plant (Dutton *et al.,* 2009).

1.4 Genetically modified maize in South Africa

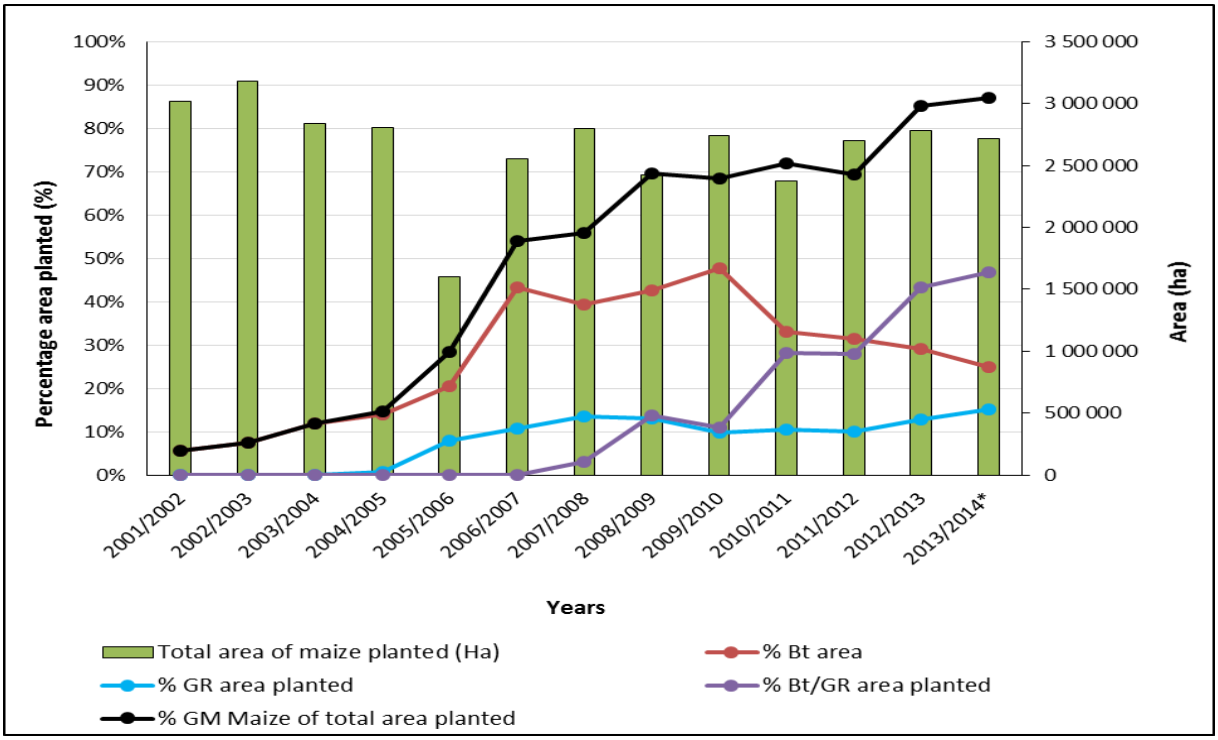

Figure 2.2 The total area and percentage GM maize planted each year in SA (Grain SA, 2014).

The adoption of GM maize in South Africa over the last decade is provided in Figure 2.2 South Africa was the first country in Africa to approve and produce GM crops in 1997 (James, 2013b; 2014). Bt maize was commercialized in South Africa during the 1998 growing season and Glyphosate resistant (GR) maize in 2005. Six years ago Bt maize stacked with GR traits was commercialized. Of the total maize area that was planted in 2014, 86.9% was GM maize (Fig. 2.3), with 25% the single Bt gene, 18.2% GR maize and 46.9% Bt stacked with GR genes. Since 2009 the stacked maize event increased gradually each year, but in 2013 it increased drastically from 28% to over 43% (James, 2014).

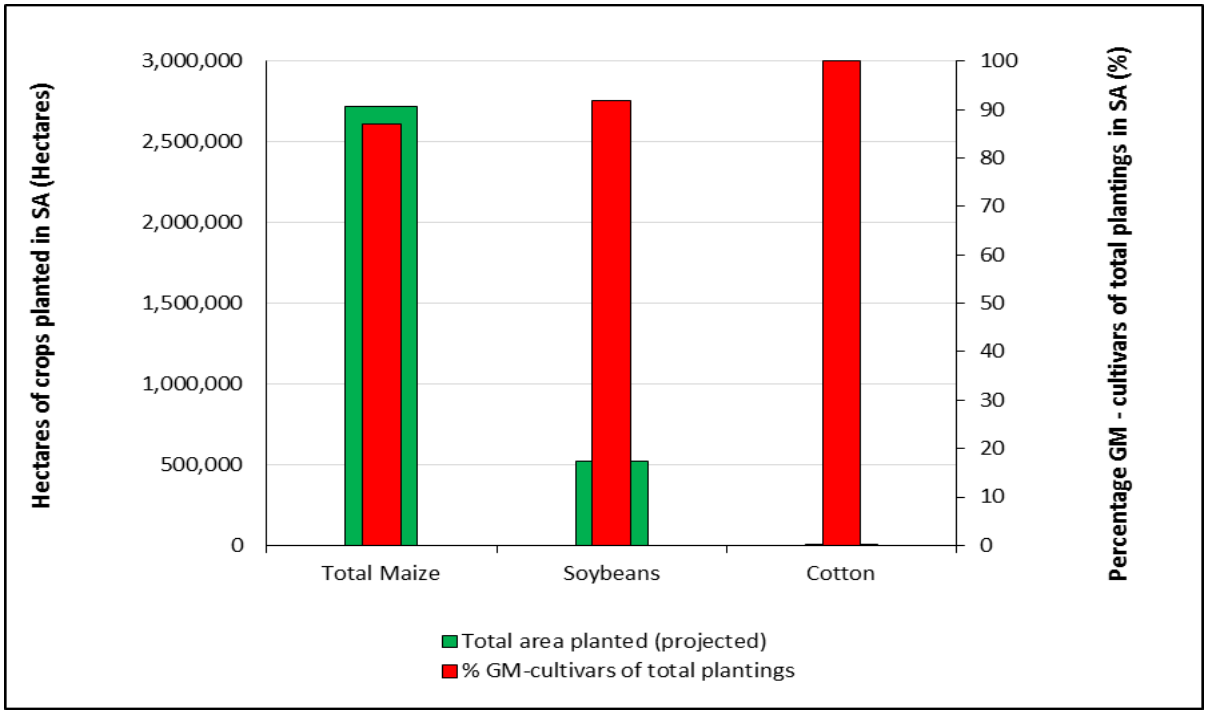

Figure 2.3 The total area (projected) of crops planted during 2014 (y-axis) and the total percentage of GM-cultivars, of the total plantings, in South Africa during 2014 (z-axis) (James, 2014).

Thus questions arise with the adoption of the stacked event, whether the application of glyphosate influences the expression levels of the Cry toxin in the stacked event. Could this have contributed to the reported increased resistance development in *B*. *fusca*?

The misuse of glyphosate could have possibly contributed to resistance development in weeds, which could in turn have affected the expression of the Cry toxin when stacked in maize.

Table 1.1 Commercialization of GM crops over the years (James, 2014).

Crop	Resistance trait	First sales
Cotton	Glyphosate resistance	1997
	Bacillus thuringiensis	1997
	Bacillus thuringiensis and Glyphosate resistance	2007
Maize	Glyphosate resistance	2005
	Bacillus thuringiensis	1998
	Bacillus thuringiensis and Glyphosate resistance	2007
Soybean	Glyphosate resistance	1996

1.5 The importance of this study

Reports from literature above verified that the Bt concentration in crops may be influenced positively or adversely by several factors. Studies also showed that GR crops may be affected by glyphosate applications. The unanswered question therefore still remains, if there is an indirect effect on larval development with the application of glyphosate on stacked events?

To ensure the success of the insect resistance management strategies used for Bt maize, the expression levels of the Cry-toxin (Cry1Ab) in the stacked events containing both Bt and herbicide tolerant (GR) traits needed to be assessed. This will determine whether the efficacy of the Bt expression levels in the stacked event will be influenced with glyphosate spray applications. Although soybeans are genetically engineered to tolerate glyphosate application, Zobiole *et al*. (2010a) have shown that transgenic soybeans are still affected by the application of glyphosate. As mentioned above, glyphosate affects the availability of nutrients and chlorophyll content in the plant, therefore glyphosate may have physiological impacts in soybean plants.

Bt expression levels in plants are influenced by abiotic factors and the expression levels may decrease with the maturity of the plant (Coviella *et al.,* 2002; Chen *et al.,* 2005; Jiang *et al*., Dutton *et al.,* 2009).

Therefore the question remains if the application of glyphosate has an effect on the efficacy of the stack event to control stem borers and if this could have contributed to the development of resistance to the Cry toxin.

1.6 Aims and objectives of study

The main reason for resistance evolution was thought to be poor compliance to the high dose/refuge IRM strategy. Later studies, however, indicated that other factors could also have played a role (Kruger *et al*., 2009; Kruger *et al*., 2011; Van den Berg *et al.,* 2013). The possible effect that stacking of insecticidal and herbicide tolerant traits could have on stem borer resistance evolution has, however, never been investigated. No information exits on the effect of herbicide application on efficacy of Cry protein expression and subsequent stem borer larval mortalities on Bt maize when treated with glyphosate. This study will contribute to the understanding of the mechanisms that drive resistance evolution to Bt maize.

The aim of the study was to determine if the combination of Bt and GR resistant traits, as well as the application of glyphosate have an effect on the efficacy of Bt maize against stem borers.

The specific objectives were to determine if:

- stem borer larval development and survival on single-gene and stacked maize hybrids over time.
- if Bt protein expression levels in Bt and Bt/GR stacked maize plants are similar.
- if glyphosate application has an effect on the Bt protein concentration in maize plants with stacked traits.

The chapter division is as follows:

- Chapter 2 - Larval development of *Busseola fusca* (Lepidoptera: Noctuidae) and *Chilo partellus* (Lepidoptera: Crambidae) on Bt- and Bt/GR maize cultivars.
- Chapter 3 - Determining the effect of Bt/GR stacking and glyphosate application on the expression levels of Bt Cry toxin in maize leaves.
- Chapter 4 - Conclusions.

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Chapter 2 - Larval development of *Busseola fusca* (Lepidoptera: Noctuidae) and *Chilo partellus* (Lepidoptera: Crambidae) on Bt- and Bt/GR maize cultivars

2.1 Abstract

Genetically modified (GM) maize with multiple traits such as resistance to lepidopteran insects and glyphosate resistance (GR) are widely planted in South Africa. By 2012 more than 80% of maize planted in South Africa, was genetically modified. Bt maize is commercialized in South Africa to control *Busseola fusca* (Lepidoptera: Noctuidae) and *Chilo partellus* (Lepidoptera: Crambidae) whereas GR maize is tolerant to the broad spectrum herbicide, glyphosate. The aim of the study was to determine if the combination of Bt and GR traits as well as the application of glyphosate have an effect on the efficacy of Bt maize against stem borers. The survival and larval development of the above mentioned stem borer species were evaluated on single gene (Bt and GR) and stack gene (Bt/GR) maize cultivars. Laboratory bioassays were conducted over three consecutive seasons and in a greenhouse trial (2014/15). In the first growing season (2012/13) only one cultivar was used (Monsanto). In the second (2013/14) and third (2014/15) season three different cultivars from three different seed companies (Monsanto, Pannar and Pioneer) with the same six treatments were used. The six treatments were: 1) non-Bt control, 2) Bt-maize, 3) GR maize, 4) Bt/GR stacked maize, 5) GR maize sprayed with glyphosate and 6) Bt/GR stack maize sprayed with glyphosate. All Bt gene maize varieties express the same Bt gene (MON810). All trials were inoculated with *B. fusca* and *C. partellus*, respectively. Although not significantly different, larval survival recorded from plant tissue of the GR and GR treatment with the spray application tended to be the highest in six out of the seven trials for *B*. *fusca* and five out of seven for *C*. *partellus*. Therefore it can be concluded that the GR trait or glyphosate application does not have an adverse effect on larval development. In order to obtain conclusive results in regard to the possible effect of glyphosate application on the Cry protein content in Bt/GR plants, ELISA's need to be included. Larval development as the only effect indicators of glyphosate and stacking traits on Bt expression levels cannot be used singly to make a final conclusion. Continued research, including the use of ELISA's to determine the effect of glyphosate on Cry protein expression levels is also needed.

2.2 Introduction

The maize stem borers, *Busseola fusca* (Lepidoptera: Noctuidae) and *Chilo partellus* (Lepidoptera: Crambidae) are the most important insect pests of maize in South Africa and provide one of the most important insecticide markets considering all field crops (Van Rensburg and Van den Berg, 1992; Van den Berg, 1997; Van Rensburg, 1999a; Van Rensburg, 2000; Padmaja *et al*., 2012; Calatayud *et al*., 2014). Yield losses due to stem borers occur regularly and at unacceptably high levels. Control measures are costly and epidemics pose a constant threat to food security. Successful deployment of Bt technology has led to reduction in insecticide application and effective control of stem borers (Van Rensburg, 1999b; Van Rensburg, 2001). If stem borers cause an average annual yield loss of 10% in commercial farming systems it can contribute to an annual yield loss of R 2.6 billion if the 13 million tonnes of maize produced during the 2014/15 season is taken as a guideline. Bt maize provides an effective control method for stem borers and alleviates the problems regarding insecticide applications (Hellmich and Hellmich, 2012).

During the 2004/05 season in South Africa Bt hybrids were severely damaged by *B*. *fusca* in some irrigation as well as dry land areas (Van Wyk *et al*., 2008). Shortly thereafter, d. Even before the commercialization of Bt crops, concern has been raised with regard to the development of resistance to insecticidal proteins (Gould, 1998; Tabashnik *et al*., 2003; Zhao *et al*., 2003). Although measures have been set in place to counter and delay resistance development, Van Rensburg (2007) reported that *B. fusca* was resistant to Bt maize in South Africa. Resistant populations of *B*. *fusca* are now widespread within the maize production region (Kruger *et al.*, 2012). Van den Berg *et al*. (2013) indicated that there are no management strategies in place to limit the spread and further development of resistant populations. Event MON89034 (expressing Cry1A.105 and Cry2Ab2) was however introduced into South Africa and effectively controls *B. fusca* larvae that are resistant to MON810 (Van den Berg *et al*., 2013).

Evolution of resistance by pests threatens the continued efficacy of genetically modified (GM) crops (Tabashnik *et al*., 2009). The high-dose/refuge strategy was developed to delay resistance development in pest populations. The aim of this strategy is to produce a high number of susceptible moths in the non-Bt refuge area compared to resistant moths, and to kill as many of the offspring by means of the high dose of Bt expressed by the Bt crop (Gould, 1998; Tabashnik, 2010). If Bt expression levels are too low and larvae are not killed by the Bt protein this can contribute to resistance development. Therefore, any factor that might affect Bt expression to such an extent that it results in a low-dose expression will result in increased larval survival on Bt maize and subsequent more rapid evolution of resistance.

The efficacy of insecticidal proteins in crops may be associated and/or influenced by numerous factors and environmental conditions, as described by Dutton *et al*. (2009). Target pests are able to develop resistance based on ecological, biochemical and genetic principles. Characteristics of pests that would evolve resistance more rapidly are high development rates of the immature stages and a quick succession of generations, while being exposed to a sub-lethal dose of a toxin.

Previous studies confirmed that the Cry1Ab expression levels in maize differ between young and older plants and that environmental conditions influence Bt expression levels in transgenic maize (Dutton *et al*., 2009). Several reports indicated that the Bt concentration level in crops may be positively or adversely influenced by biotic and/or abiotic factors (Greenplate, 1999; Coviella *et al*., 2000; Dutton *et al*., 2004). Observations have shown that the Cry-toxin levels in cotton decreased significantly under extreme temperature conditions (Chen *et al.,* 2005), deficient nitrogen levels (Coviella *et al.,* 2002) and NaCl stress (Jiang *et al*., 2006) and that these issues were associated with the failure in efficacy of insect-resistant crops (Zhen *et al*., 2008; Shen *et al*., 2010). The efficacy of Bt cotton shows a discrepancy with plant structure or age (Dong and Li, 2007). Studies done by Bruns and Abel (2003) showed that the Cry toxin in maize is positively affected by the increase in N-fertility during reproductive growth stages. Several other studies showed that the Bt concentration may vary over growing season, since leaves of young plants contain a higher Bt concentration compared to leaves of older plants (Greenplate, 1999; Coviella *et al*., 2000; Olsen and Daly, 2000; Dutton *et al.,* 2004). If Bt expression levels do also change in maize as described above, these changes, if they result in increased survival of stem borer larvae, will contribute further to resistance evolution.

If plant stress factors such as those described above influence protein expression levels in Bt plants, the possibility exists that application of agro-chemicals, could, under certain conditions also stress plant growth. In such cases the possibility exists that such applications may then influence Bt protein expression in plants with stacked traits such as Bt maize with Glyphosate Resistant (GR) traits. The Bt/GR stack event has the ability to control stem borers and at the same time, the plant can tolerate application of glyphosate, a broad-spectrum systemic herbicide (Taverniers *et al*., 2008). Glyphosate usage increased dramatically with the introduction of GR crops (James, 2013). These GR crops can tolerate glyphosate applications (Taverniers *et al*., 2008) which facilitate easier weed control. The popularity of stacked events with Bt and GR traits in South Africa have also increased drastically over the past five years (James, 2014).

The application of glyphosate on GR plants may cause stresses by affecting nutritional status (Zablotowicz and Reddy, 2007; Zobiole *et al*., 2010a), transpiration rate, photosynthesis and stomatal conductance (Zobiole *et al*., 2010b). Increased sensitivity of younger GR plants (4

weeks old) to glyphosate (Zobiole *et al*., 2010c) and long-term physiological impacts (Zobiole *et al*., 2010c) have also been reported.

The question therefore arises if glyphosate application has adverse effects on plants of stack maize events and if it influences Bt expression in plants. If this adverse effect does exist this might have contribute to resistance development. In this chapter the survival and larval development of two stem borer species were evaluated on single gene (Bt and GR) and stack gene (Bt/GR) maize cultivars. The aim of the study was to determine if the combination of Bt and GR traits as well as the application of glyphosate have an effect on the efficacy of Bt maize against stem borers.

2.3 Material and methods

In this study 4 laboratory trials (Experiments 1 and 2 - season 2012/13; Experiments 3 and 4: season 2013/14 and 2014/15) and two greenhouse trials (season 2014/15) was conducted. Experiments 1 and 3, and 2 and 4 were conducted with *B. fusca* and *C. partellus*, respectively.

All trials were conducted at the Agricultural Research Council - Grain Crops Institute, Potchefstroom (46°43'S, 27°06'E) in the North-West province. In the first growing season (2012/2013) only one cultivar was used with six treatments (Table 2.1). In the second (2013/2014) and third (2014/15) seasons three different cultivars from three different seed companies (Monsanto, Pannar and Pioneer) with the same six treatments were used (Table 2.1).

To allow for enough plant tissue for bioassays to be conducted, seed of the different cultivars were panted in field plots. Glyphosate was mixed according to the label rate of 2 ℓ/ha and applied to designated GR treatments before the V7 growth stage of maize plants, approximately three weeks after seedling emergence (Fig. 2.1).

Table 2.1 The six treatments and different cultivars used in this study (GR = Glyphosate resistant; Bt or B = stem borer resistant).

Treatments	**Cultivars**		
	Monsanto	**Pioneer**	**Pannar**
1	Non-Bt control (cultivar: DKC 80 – 10)	Non-Bt control (cultivar: 32 B 07)	Non-Bt control (cultivar: BG 3792)
2	Bt (MON810) (cultivar: DKC 80 – 12 B)	Bt (MON810) (cultivar: 32 B 06 B)	Bt (MON810) (cultivar: BG 3592 B)
3	GR (cultivar: DKC 80 – 30 R)	GR (cultivar: 32 B 05 R)	GR (cultivar: BG 3292 R)
4	Bt/GR (cultivar: DKC 80 – 40 BR)	Bt/GR (cultivar: 32 B 10 BR)	Bt/GR (cultivar: BG 3492 BR)
5	GR with glyphosate application (cultivar: DKC 80 – 30 R)	GR with glyphosate application (cultivar: 32 B 05 R)	GR with glyphosate application (cultivar: BG 3292 R)
6	Bt/GR with glyphosate application (cultivar: DKC 80 – 40 BR)	Bt/GR with glyphosate application (cultivar: 32 B 10 BR)	Bt/GR with glyphosate application (cultivar: BG 3492 BR)

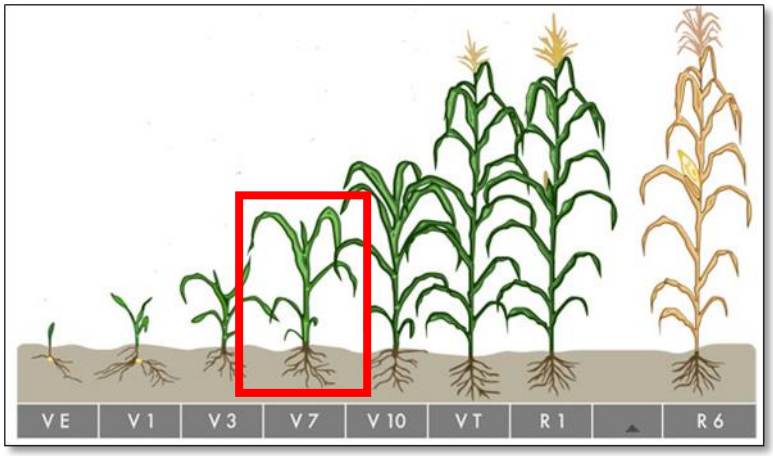

Figure 2.1 The different growth stages of a maize plant. The red square indicates the relative size before which growth stage glyphosate was applied. Glyphosate was applied three weeks after plant emergence (Cunningham, 2011).

2.3.1 Laboratory trials

Stem borer larvae were reared on plant tissue of the different treatments under laboratory conditions. The whorls of 4 - 6 week old plants of each treatment were collected from the field and used in the laboratory. The tissue used in the feeding bioassay were cut from the bottom part of the plant whorl and consisted of very tightly rolled sections of leaves 3 - 4 cm long. Larvae were kept in 100 ml rearing containers. The whorl tissue was placed in the rearing container together with filter paper to absorb excess moisture. The containers were customized with stainless steel mesh on the lid to prevent larvae from escaping. All rearing containers were kept in a completely randomized design inside walk-in incubators (24±2 °C, photoperiod of 14L: 10D and a 40 - 60% relative humidity). Larvae fed on the maize treatments for the entire larval stage. Fresh food was provided at 3- and 4 day intervals.

2.3.1.1 Laboratory trials, experiments 1 and 2 (2012/13)

The first two trials were conducted over one growing season (2012/13). Whorl tissue inside the containers was inoculated with five neonate larvae of the respective species per treatment. Each treatment was replicated 20 times. Larval survival and development was monitored twice a week, determining larval mass and survival for a period of 28 days.

The larvae that were used in this experiment were F1 larvae derived from diapause larvae collected from maize fields in the Ventersdorp area (26°20'15.9"S 26°45'21.7"E), North-West province during the winter months of 2012. *Busseola fusca* sampled from this locality has already shown some levels of resistance to Bt (MON810) maize in a previous study (Marais, 2014). The *C*. *partellus* population used was provided by the ARC-GCI and was collected at the ARC-GCI experimental farm in Potchefstroom (26°43'44.5"S 27°04'52.9"E).

2.3.1.2 Laboratory trials, experiments 3 (2013/14) and 4 (2014/15)

These experiments were conducted over two consecutive seasons with both *B*. *fusca* and *C*. *partellus*. The same procedure described above was followed except that each treatment was replicated 25 times.

The *B*. *fusca* larvae used in this experiment were the F1 generation derived from diapause larvae collected from Bt maize in the Lichtenburg area (25°57'52.4"S 26°34'04.4"E) in the North-West Province. The same *C*. *partellus* population mentioned above were used in the

experiments. Larval development was monitored over a period of 28 days, by determining the larval survival and mean mass twice a week.

2.3.2 Greenhouse trials (2014/15)

The study was conducted in the greenhouse to prevent natural infestation of larvae and also to protect the leaves from hail damage. Two trials were conducted, one with *B. fusca* and one with *C. partellus*. Maize was planted in a commercial greenhouse with a completely randomized block design used as layout. Each treatment was replicated four times. Each replicate consisted of four rows with 10 plants per row. The maize plants were inoculated with 10 - 15 first instar larvae of *B*. *fusca* and *C*. *partellus*, respectively into plant whorls, one week after glyphosate application. At 14 and 21 days respectively, 40 plants of each replicate were dissected. Larvae were collected to record larval mass and survival for the different treatments.

2.3.3 Data analyses

Data on larval survival and mass over time were analysed using repeated measurements ANOVA. One-way ANOVAs were used to analyse and compare data between treatments on day 28, the final day of the experiments. GenStat 17th Edition (VSN International) was used for all analyses. Data were presented as means with an associated least significant difference (LSD, at P = 0.05).

2.4 Results

2.4.1 Laboratory trials

Results are discussed separately for cultivars of the different companies, firstly for *B. fusca* and then for *C. partellus*.

2.4.1.1 Laboratory trials: *Busseola fusca* survival and mass

Season 2012/13: Monsanto cultivar

Repeated measures ANOVA showed that larval survival was the highest (65.30% - mean percentage survival over time) when feeding on the GR sprayed treatment and the lowest survival (22.80% - mean percentage survival over time) was observed on the Bt treatment and differed significantly over time (($F_{(5;192)}$ = 119.91; P < 0.001). The larval survival of *B*. *fusca* on the three Bt treatments (Bt, Bt/GR and Bt/GR sprayed) was significantly lower than on the non-Bt treatments (Control, GR and GR sprayed) (($F_{(35;192)}$ = 0.97; P = 0.526) (Fig. 2.2 (a)) in the 2012/13 growing season.

The mean mass of the larvae over time that fed on the Bt treatments was slightly lower than the non-Bt treatments. No significant difference were observed in mean larval mass between any of the treatments ($F_{(35;192)}$ = 1.08; P = 0.361) (Fig. 2.2 (a)). The larvae that fed on the Bt/GR maize sprayed with glyphosate had a mean mass (over time) of 117.5 mg which was lower when compared to the mean mass (over time) of the larvae that fed on the single Bt-gene treatment (127.10 mg), but did not differ significantly. When the mean mass of the larvae that fed on the single Bt-gene plants was compared to that of larvae on stacked maize, there was a difference of 31 mg, but no significant difference was observed. Although not significant, in both cases the larvae that fed on the stacked gene maize had a greater mass.

Comparison between treatments on the final day of the experiment showed that *B*. *fusca* had the highest mortality on the Bt treatment, but survival did not differ significantly from that on Bt/GR and Bt/GR sprayed treatments ($F_{(5;24)}$ = 24.4; P < 0.001) (Table 2.2). A significant difference in survival was observed between the treatments which contained Bt and the treatments without the Bt trait. Although not significant, on day 28 the larvae that fed on the Bt/GR treatment and Bt/GR sprayed treatment had a higher larval survival than the single Bt-gene treatment. Survival on the non-Bt treatment as well as on the GR treatment were almost the same and therefore did not differ significantly from each other on day 28.

No significant difference was observed in the mean mass of the larvae although the larvae on the Bt treatment had the lowest mean mass on day 28 ($F_{(5;24)}$ = 1.53; P = 0.217) (Table 2.2). No significant differences in larval mass were observed between treatments on day 28.

Season 2013/14: Monsanto cultivar

Repeated measures ANOVA showed that there was no significant difference in larval survival between treatments over time ($F_{(30;126)} = 0.28$; $P = 1.000$) (Fig. 2.2 (b)). Larval survival was the highest on the GR treatment. The Bt/GR treatment appeared to have controlled the larvae the best when compared to the Bt/GR sprayed with glyphosate treatment. On day 28 the Bt/GR treatment had a larval survival of 35%. Larval survival on the treatment in which GR maize was sprayed with glyphosate did not differ significantly compared to that of the non-Bt (control) treatment on day 28.

The larval mass gain was the greatest on the single Bt gene treatment and lowest on the GR treatment sprayed with glyphosate ($F_{(30;126)} = 2.33$; $P < 0.001$) (Fig. 2.2 (b)).

Comparison between treatments on the final day of the experiment showed significantly lower survival was recorded on the Bt/GR treatment compared to the GR treatment ($F_{(5;18)} = 2.85$; $P = 0.046$) (Table 2.2).

At the end of the trial the *B. fusca* larvae had the greatest mean mass on the Bt treatment ($F_{(5;18)} = 4.61$; $P = 0.007$) (Table 2.2). The larvae that fed on the Bt (268.9 mg) and the non-Bt (259.2 mg) treatments had a significantly higher mean mass than the larvae that fed on the Bt/GR treatment that was sprayed with glyphosate (191.2 mg) (Table 2.2).

Season 2014/15 Monsanto cultivar

Larvae that fed on the Bt treatment had a higher survival compared to the other treatments, however no significant difference was reported ($F_{(30;126)} = 0.50$; $P = 0.985$) (Fig. 2.2 (c)). Repeated measures ANOVA showed that larval survival was the highest (63.57% - mean percentage survival over time) when feeding on the Single Bt treatment significantly from the other five over time ($F_{(5;126)} = 21.15$; $P < 0.001$). The mean mass of the larvae that fed on the six different treatments followed the same tendency in mass increase with no significant differences between these treatments ($F_{(30;124)} = 1.45$; $P = 0.084$).

Comparison between treatments by means of ANOVA on the final day of the experiment showed that survival of larvae that fed on the Bt treatment had a significantly higher larval survival than some other treatments and that survival was similar to that observed on te control treatment, indicating large variation in data. The Bt/GR treatment had 2% higher survival than the Bt/GR treatment sprayed with glyphosate which also did not differ significantly between these two treatments (Table 2.2).

The mean mass of the larvae did not differ significantly between treatments ($F_{(5;17)} = 1.96$; $P = 0.138$) (Table 2.2). Although not significant, when the mean larval mass of the three different treatments containing Bt were compared on the final day of the trial, it showed that the larvae that fed on the Bt/GR treatment had the greatest mean mass (311 mg), Bt/GR sprayed with glyphosate had a slightly lower mean mass (269 mg) and the single Bt treatment the lowest mean mass (227 mg) of all treatments (Fig. 2.2 (c)) (Table 2.2).

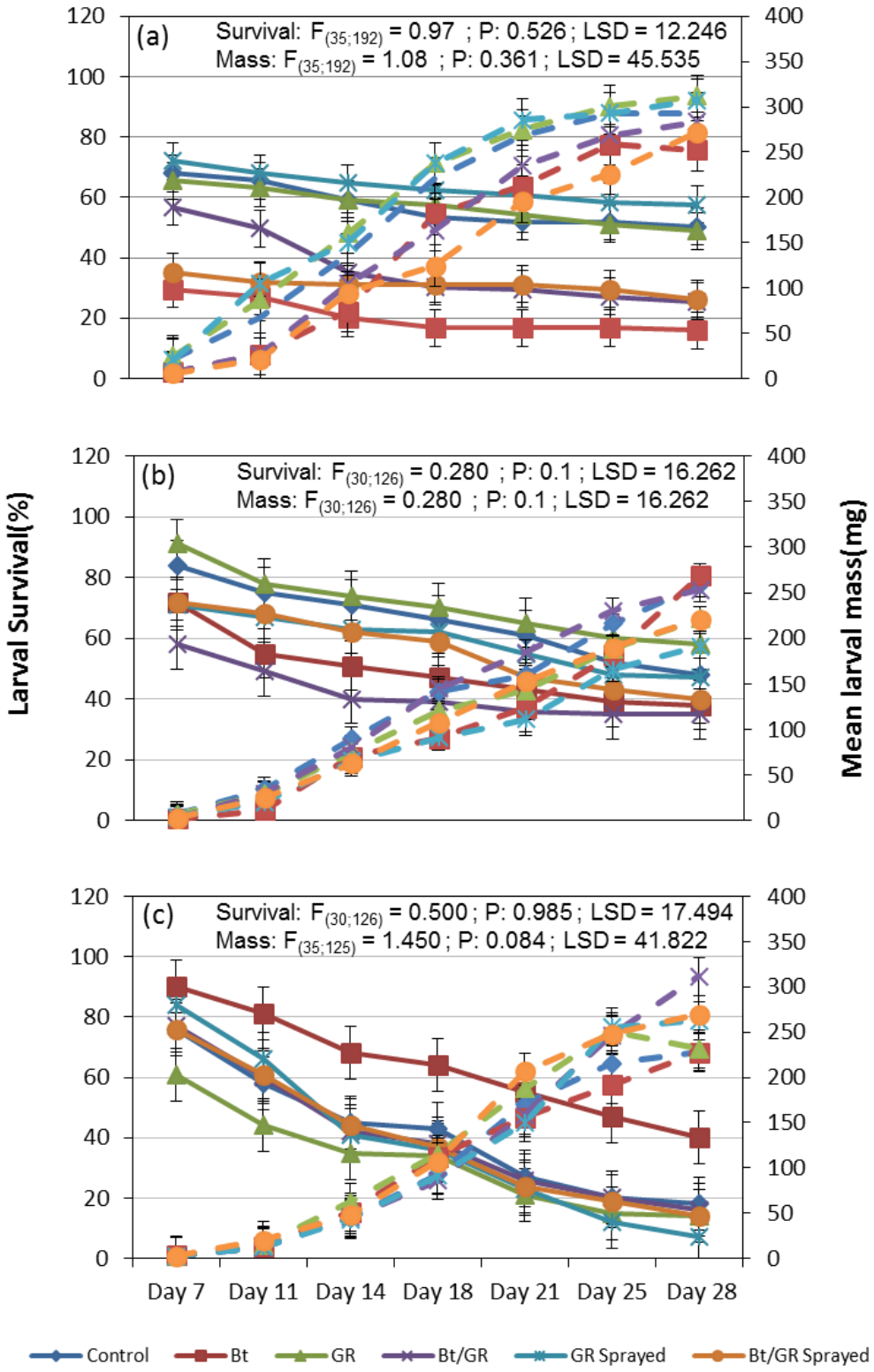

Figure 2.2 Percentage survival (%) and mean larval mass (mg) of *Busseola fusca* over time on maize whorls on the different treatments of the Monsanto cultivar: (a) season 2012/13, (b) season 2013/14 and (c) season 2014/15. Error bars indicates LSD value. Solid lines indicate larval survival (%) (y-axis), while dotted lines indicate mean larval mass (mg) (z-axis).

Season 2013/14: Pioneer cultivar

The highest larval survival over time was recorded on the non-Bt (control) treatment but it did not differ from that on other treatments ($F_{(30;126)}$ = 0.78; P = 0.786) (Fig. 2.3 (a)). The mean larval mass did not differ between any of the treatments over time ($F_{(30;126)}$ = 1.02; P = 0.450) (Fig. 2.3 (a)).

Comparison between treatments by means of ANOVA on the final day of the experiment results showed significantly lower survival on the Bt maize treatment compared to the control and GR maize ($F_{(5;18)}$ = 4.44; P = 0.008) (Table 2.2). The Bt treatment showed a survival of 32%. No differences were however observed between the Bt/GR and Bt/GR sprayed (Table 2.2). The mean mass of the larvae did not differ significantly between treatments ($F_{(5;18)}$ = 1.5; P = 0.24).

Season 2014/15: Pioneer cultivar

Repeated measures ANOVA showed that the highest larval survival was recorded on the GR treatment followed by the Bt/GR treatment. Repeated measures ANOVA showed no significant differences in larval mass between treatments over time ($F_{(30;123)}$ = 1.06; P = 0.399) (Fig. 2.3 (b)).

Comparison between treatments by means of ANOVA on the final day of the experiment showed significant differences between treatments where a survival of 13% on Bt/GR maize sprayed with glyphosate and and 41% on GR maize was recorded ($F_{(5;18)}$ = 3.21; P = 0.030) (Table 2.2). However, no differences were observed between larval survival on Bt/GR and the Bt/GR sprayed treatments.

No significant differences were observed in the mean mass between the three Bt treatments and the non-Bt treatments ($F_{(5;17)}$ = 1.16; P = 0.367) (Table 2.2).

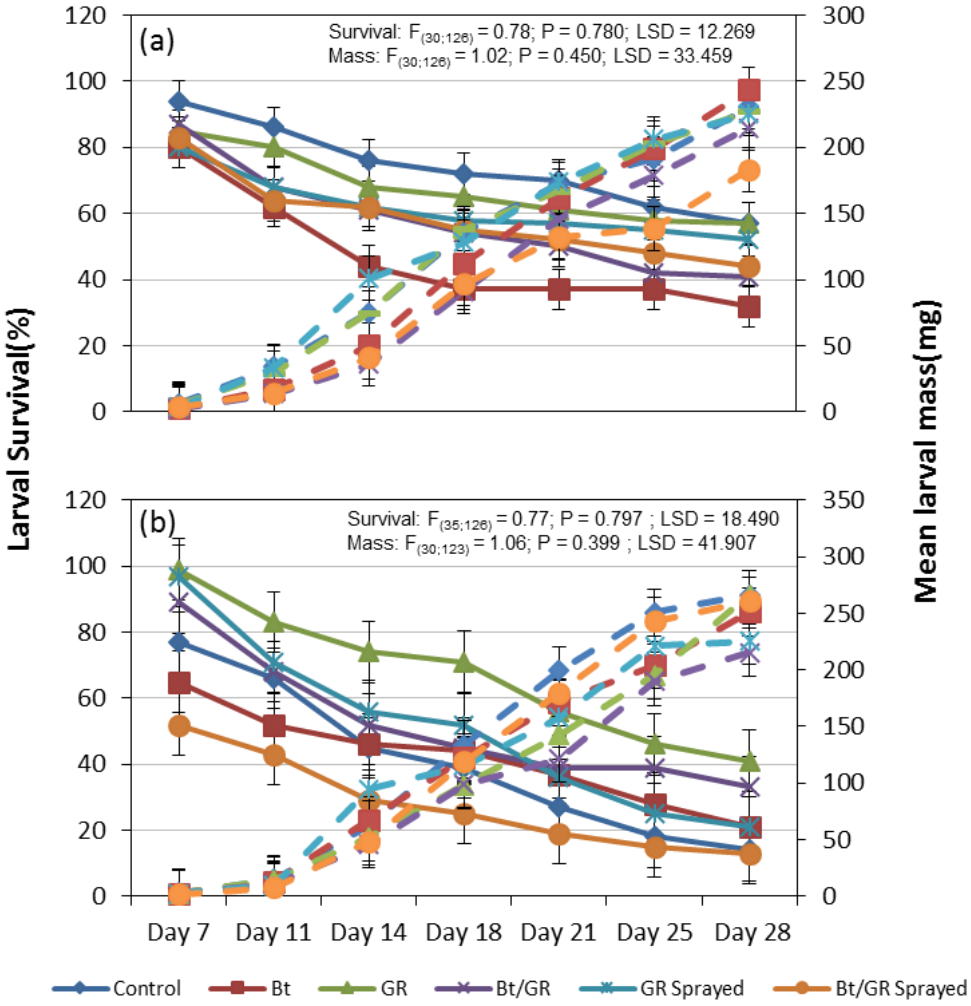

Figure 2.3 Percentage survival (%) and mean larval mass (mg) of *Busseola fusca* over time on maize whorls on the different treatments of the Pioneer cultivar: (a) season 2013/14 and (b) season 2014/15. Error bars indicates LSD value. Solid lines indicate larval survival (%) (y-axis), while dotted lines indicate mean larval mass (mg) (z-axis).

Season 2013/14: Pannar cultivar

The larval survival over time for all of the treatments with the Bt gene (Bt, Bt/GR and Bt/GR sprayed with glyphosate) had the same tendency over time and there was no significant differences ($F_{(30;126)}$ = 0.32; P = 1.000) between these three treatments (Fig. 2.4 (a)). Larvae on the Bt/GR treatment showed slightly higher survival than those on Bt/GR treated with glyphosate, but did not differ significantly from other treatments.

All the treatments showed a gradual increase in larval mean mass with the same tendency over time, with no significant difference ($F_{(30;126)} = 1.17$; $P = 0.270$) (Fig. 2.4 (a)).

However, ANOVA on survival data on day 28 showed that the Bt treatment had the highest percentage larval survival (40%), followed by the Bt/GR treatment (36%) when Bt treatments were compared. Larvae feeding on plant tissue of the control and GR treatment had a significantly higher survival compared to the other treatments ($F_{(5;18)} = 14.43$; $P < 0.001$) (Table 2.2).

No significant differences were observed in mean larval mass between treatments ($F_{(5;18)} = 1.86$; $P = 0.152$) (Table 2.2).

Season 2014/15: Pannar cultivar

During the second season on this cultivar no significant differences were observed between larval survival over time on plant tissue of the different treatments ($F_{(30;126)} = 1.00$; $P = 0.474$) (Fig. 2.4 (b)).

Significant differences in the mean mass over time were observed between treatments with larvae that fed on the Bt treatment having the greatest increase in mass ($F_{(30;123)} = 1.630$; $P = 0.034$) (Fig. 2.4 (b)). The mass gain of the larvae that fed on the stacked and stacked maize sprayed with glyphosate had the same tendency over time and did not differ significantly between treatments.

There were no significant differences in the survival of larvae on the six different treatments on the final day of the experiment ($F_{(5;18)} = 1.38$; $P = 0.279$) (Table 2.2). Although not significantly different, when larvae fed on the Bt/GR treatment that was sprayed with glyphosate a total of 21% of the larvae survived followed by Bt/GR treatment with 14% survival and only 8% survived on the Bt treatment.

The larvae that fed on the Bt/GR treatment had a mean mass of 239 mg, compared to the larvae fed on the Bt/GR treatment sprayed with glyphosate which had a mean mass of 211 mg. However, no significant differences were observed between larval mass on the different treatments ($F_{(5;17)} = 2.11$; $P = 0.114$) (Table 2.2).

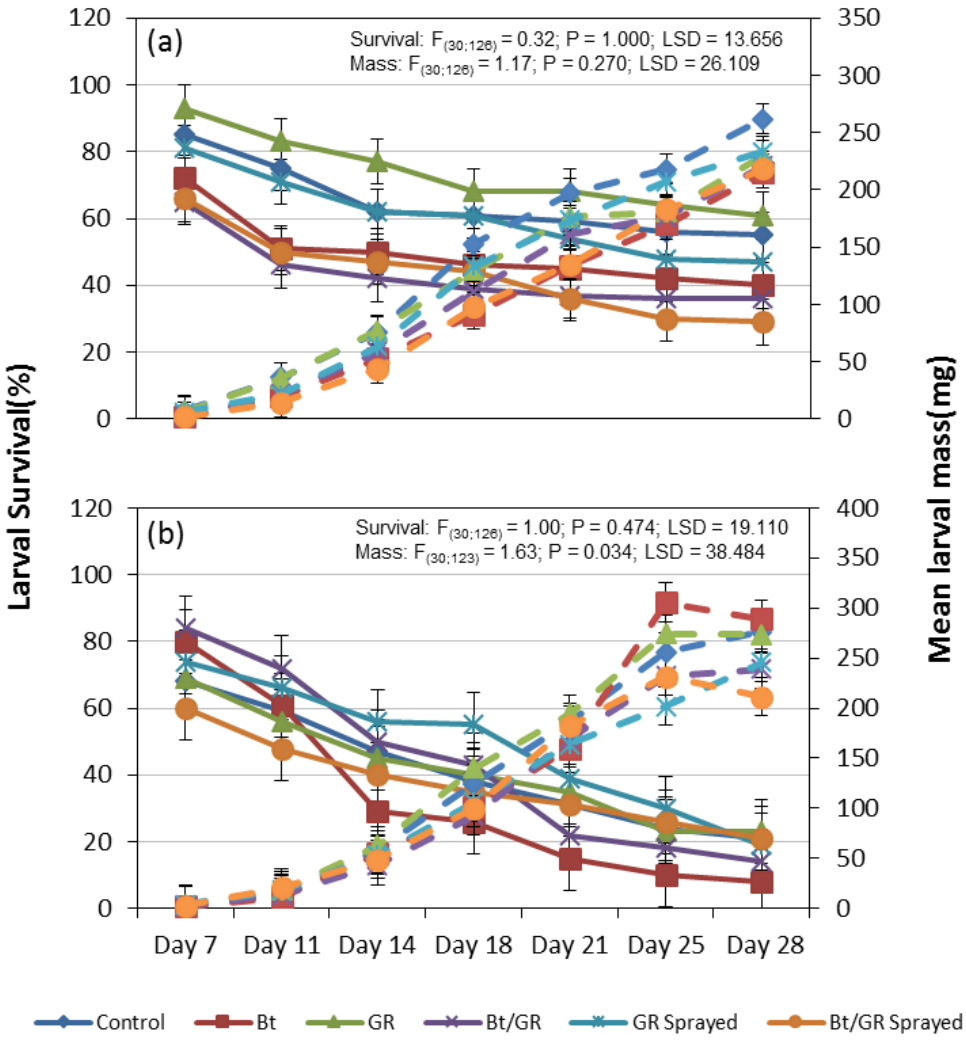

Figure 2.4 Percentage survival (%) and mean larval mass (mg) of *Busseola fusca* over time on maize whorls on the different treatments of the Pannar cultivar: (a) season 2013/14 and (b) season 2014/15. Error bars indicates LSD value. Solid lines indicate larval survival (%) (y-axis), while dotted lines indicate mean larval mass (mg) (z-axis).

Table 2.2 Survival (%) and mean larval mass (mg) of *Busseola fusca* on maize plants on the different treatments after 28 days.

Season	Treatment	Laboratory trial - Day 28 *Busseola fusca*					
		Survival (%)			Mean mass (mg)		
		Monsanto	Pioneer	Pannar	Monsanto	Pioneer	Pannar
2012/13	Control	50 b			292.8 a		
	Bt	16 a			216.0 a		
	GR	48 b			311.7 a		
	Bt/GR	25 a			283.3 a		
	GR Sprayed	57 b			307.3 a		
	Bt/GR Sprayed	26 a			271.8 a		
	F-value	24.4			1.53		
	P-value	**<0.001**			0.217		
	LSD	9.980			62.197		
2013/14	Control	48 ab	57 b	55 c	259.2 b	230.3 a	261.7 a
	Bt	38 ab	32 a	40 ab	268.9 b	243.7 a	215.0 a
	GR	58 b	57 b	61 c	221.2 ab	227.5 a	229.6 a
	Bt/GR	35 a	41 ab	36 ab	252.9 ab	215.0 a	220.9 a
	GR Sprayed	47 ab	52 ab	47 bc	220.8 ab	225.4 a	233.2 a
	Bt/GR Sprayed	40 ab	44 ab	29 a	191.2 a	183.3 a	218.4 a
	F-value	2.85	4.44	14.43	4.61	1.50	1.86
	P-value	**0.046**	**0.008**	**<0.001**	**0.007**	0.240	0.152
	LSD	14.790	14.020	9.400	40.905	49.991	37.232
2014/15	Control	18 ab	14 ab	21 a	228.3 a	266.3 a	276.0 a
	Bt	40 b	21 ab	8 a	227.6 a	251.4 a	289.1 a
	GR	14 a	41 b	23 a	231.8 a	266.4 a	273.8 a
	Bt/GR	16 ab	33 ab	14 a	311.3 a	215.5 a	239.1 a
	GR Sprayed	7 a	21 ab	19 a	262.9 a	225.6 a	245.5 a
	Bt/GR Sprayed	14 a	13 a	21 a	269.3 a	260.9 a	211.2 a
	F-value	4.13	3.21	1.38	1.96	1.16	2.11
	P-value	**0.001**	**0.030**	0.279	0.138	0.367	0.114
	LSD	16.560	18.300	14.280	70.381	60.698	59.659

* Significant difference at P = 0.005 and indicated in red.
* Means within columns followed by different letters differ significantly at the indicated level.

2.4.1.2 Laboratory trials: *Chilo partellus* survival and mass

Season 2012/13: Monsanto cultivar

Repeated measures ANOVA showed no significant differences observed in larval survival between non-Bt treatments ($F_{(12;84)}$ = 0.07; P = 1.000) (Fig. 2.5 (a)). No larval survival was recorded on any of the treatments containing the Bt trait, therefore no mass could be recorded on any of the Bt treatments ($F_{(12;85)}$ = 1.33; P = 0.219) (Fig. 2.5 (a)).

Comparison between treatments by means of ANOVA on the final day of the experiment showed that larvae did not survive on any of the Bt treatments and that there were no significant differences between survival and mass on the control and the two GR treatments ($F_{(2;12)}$ = 2.61; P = 0.115) (Table 2.3). Although no significant difference was observed, the larvae that fed on the GR maize that was sprayed with glyphosate had the highest larval survival (39% on day 28).

Season 2013/14: Monsanto cultivar

The results obtained showed that the larvae that fed on the three treatments containing Bt (Bt; Bt/GR; Bt/GR sprayed with glyphosate) all died within the first seven days. No survival was recorded on Bt treatments ($F_{(12;63)}$ = 0.16; P = 0.999) (Fig. 2.5 (b)). Although not significant, the larvae that fed on the control (non-Bt) treatment had the highest larval survival, with an average survival of 66 % over time, followed by GR treatments (54%) and the GR treated with glyphosate had a significantly lower average survival of less than 35% (Figure 2.5 (b)).

No mass was recorded on any of the Bt treatments due to 100% mortality within 7 days after inoculation (Fig. 2.5 (b)).

The larvae that fed on the GR sprayed treatment had a significantly lower larval survival on the last day of the trial ($F_{(2;9)}$ = 18.44; P < 0.001) (Table 2.3). Larval mass was similar between the treatments on which larvae survived $F_{(2;9)}$ = 1.13; P = 0.364).

Season 2014/15 Monsanto cultivar

A gradual increase in mortality was observed in all three non-Bt treatments, with no survival on any of the three treatments that contained Bt. Higher numbers of larvae survived when

larvae fed on the GR treatment than on the GR treatment that was sprayed with glyphosate but did not differ significantly ($F_{(12;63)} = 0.42$; $P = 0.950$) (Fig. 2.5 (c)).

There was a significant difference in the mass of larvae between the three non-Bt treatments. The larvae that fed on the control treatment, had a significantly lower mean mass while larvae that fed on plants of the GR treatment had the greatest mean mass of all the non-Bt treatments ($F_{(12;63)} = 5.66$; $P < 0.001$) (Fig. 2.5 (c)) over time.

There was minor differences in the survival rate on the three non-Bt treatments, but did not differ significantly in the ANOVA ($F_{(2;9)} = 0.93$; $P = 0.431$) (Table 2.3) on day 28. The mass of larvae also did not differ between treatments on which larvae survived ($F_{(2;9)} = 2.7$; $P = 0.120$) (Table 2.3) when day 28 was reached.

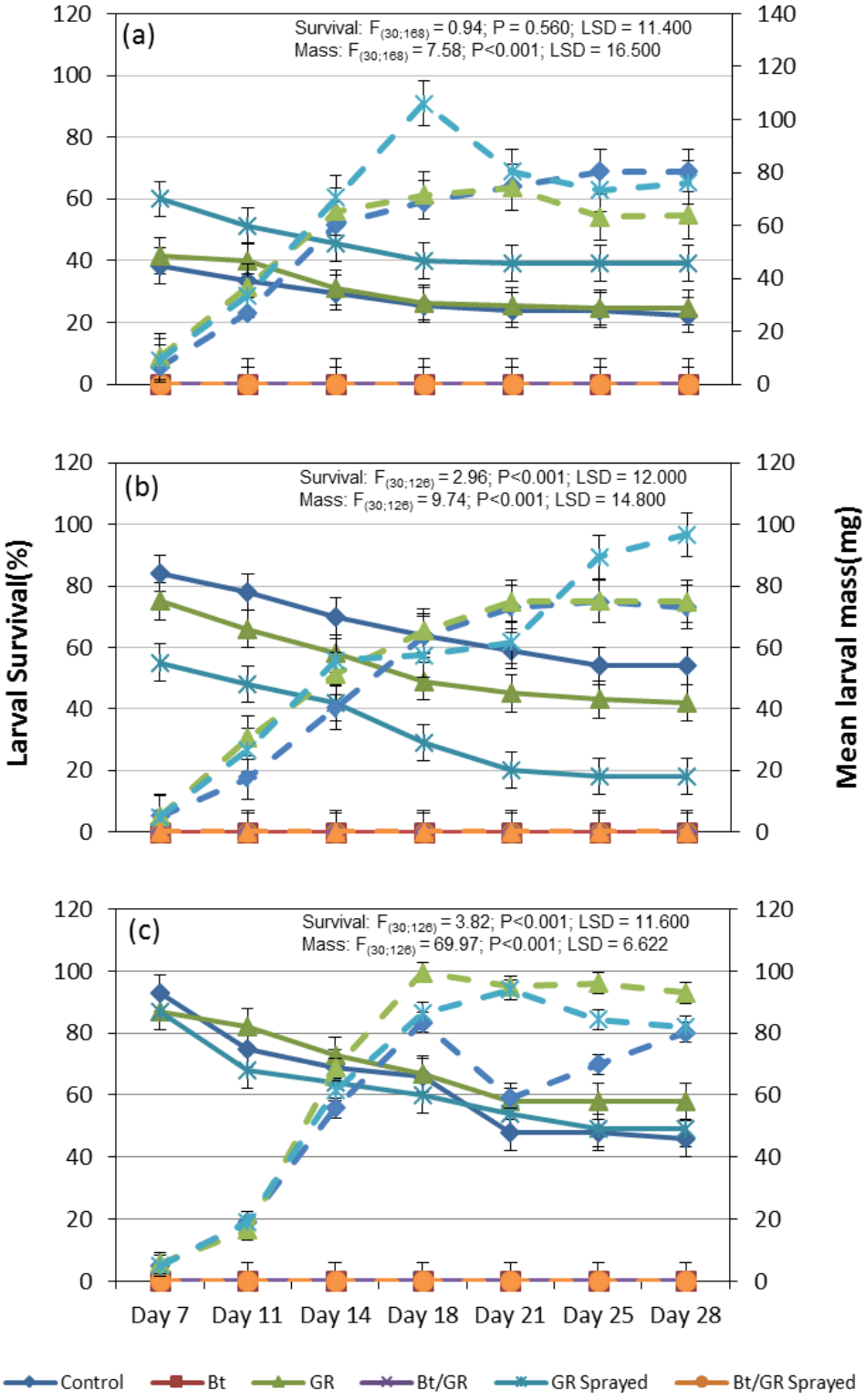

Figure 2.5 Percentage survival (%) and mean larval mass (mg) of *Chilo partellus* over time on maize whorls on the different treatments of the Monsanto cultivar: (a) season 2012/13, (b) season 2013/14 and (c) season 2014/15. Error bars indicates LSD value. Solid lines indicate larval survival (%) (y-axis), while dotted lines indicate mean larval mass (mg) (z-axis).

Season 2013/14: Pioneer cultivar

There was no survival of *C*. *partellus* recorded on any of the three Bt treatments ($F_{(12;63)}$ = 1.17; P = 0.320) (Fig. 2.6 (a)). Due to no larval survival on any of the Bt treatments, no larval mass was recorded on these treatments. Although repeated measures ANOVA indicated no significant differences between larval mass on the non-Bt treatments for the first 18 days, from day 21 onwards, the mean mass of the larvae that fed on the GR maize sprayed with glyphosate was greater than the mean larval mass of the control and GR treatments ($F_{(12;63)}$ = 0.35; P = 0.977) (Fig. 2.6 (a)).

Comparison between treatments by means of ANOVA on the final day showed no significant differences in survival ($F_{(2;9)}$ = 0.62; P = 0.558) (Table 2.3) and mass ($F_{(2;9)}$ = 0.45; P = 0.649) (Table 2.3) of larvae on plant tissue of the three treatments which did not involve Bt maize. Although not significantly different, the highest larval mass was recorded when larvae fed on the GR treatment sprayed with glyphosate.

Season 2014/15: Pioneer cultivar

Within seven days after inoculation a 100% larval mortality was observed on the three treatments that contained the Bt gene ($F_{(12;63)}$ = 0.63; P = 0.805) and therefore no mass was recorded on the Bt treatments (Fig. 2.6 (b)).

No significant difference was found in mass gain of the larvae when feeding on the three non-Bt treatments ($F_{(12;63)}$ = 1.53; P = 0.136) (Fig. 2.6 (b)).

All survival rates averaged below 50% for the non-Bt treatments but did no differ significantly ($F_{(2;9)}$ = 0.25; P = 0.784) (Table 2.3) on the final day of the trial. The mean mass of larvae that fed on the GR treatment sprayed with glyphosate weighed 88.5 mg and was 4 mg heavier than the larvae that fed on the other two non-Bt treatments, but no significance was recorded between these treatments ($F_{(2;9)}$ = 0.43; P = 0.664) (Table 2.3).

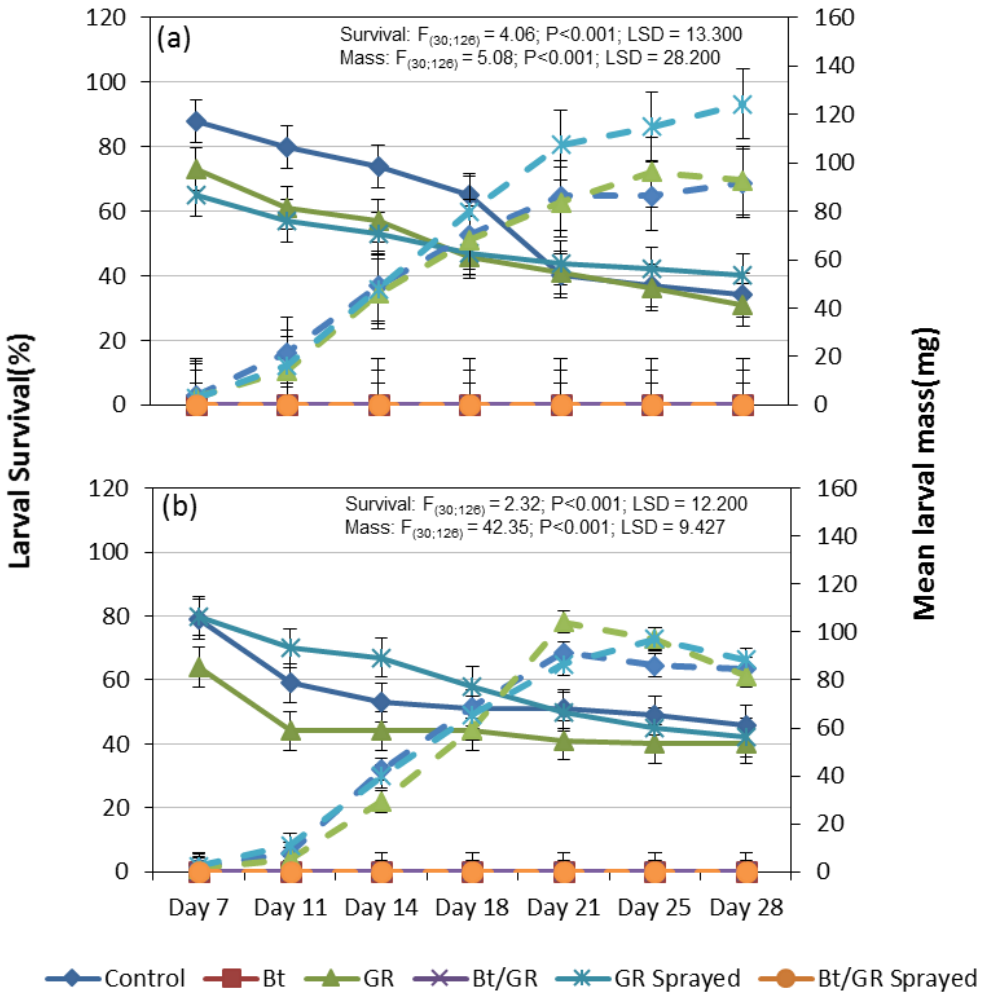

Figure 2.6 Percentage survival (%) and mean larval mass (mg) of *Chilo partellus* over time on maize whorls on the different treatments of the Pioneer cultivar: (a) season 2013/14 and (b) season 2014/15. Error bars indicates LSD value. Solid lines indicate larval survival (%) (y-axis), while dotted lines indicate mean larval mass (mg) (z-axis).

Season 2013/14: Pannar cultivar

Repeated measures ANOVA showed a slight difference between the larval survival on the GR treatment and the GR maize sprayed with glyphosate, but it differed significantly from the control treatment ($F_{(12;63)}$ = 2.48 ; P = 0.010) (Fig. 2.7 (a)).

The mean mass of the larvae that fed on the GR treatment and the GR maize was significantly lower than the mean mass of the larvae that fed on the control ($F_{(12;63)}$ = 2.06; P = 0.033) (Fig.

2.7 (a)). The mortality rate and the mass gain of the larvae on the GR and GR treatment sprayed with glyphosate had the same tendency over time.

The results for *C. partellus* also showed to have a significant susceptibility to maize plants containing the MON810 event, as no larvae survived ($F_{(2;9)}$ = 2.88; P = 0.108) (Table 2.3).

Although more larvae survived on the GR maize sprayed with glyphosate, the larvae had the lowest mean mass when feeding on GR maize sprayed with glyphosate compared to the other treatments ($F_{(2;9)}$ = 2.66; P = 0.124) (Table 2.3) on the final day of the trial. Since *C. partellus* remains highly susceptible to Bt maize, no larvae were recovered on any Bt treatment.

Season 2014/15: Pannar cultivar

Chilo partellus larvae had the same reaction to Bt as in the previous trials and once again the larvae were highly susceptible to the Bt toxin ($F_{(12;63)}$ = 0.46; P = 0.932) (Fig. 2.7 (b)). Repeated measures ANOVA showed no significant differences, but a mass increase over time was recorded on the three treatments which did not contain the Bt trait ($F_{(12;63)}$ = 1.27; P = 0.260) (Fig. 2.7 (b)).

Results recorded on the final day had no significant difference between the non-Bt treatments in terms of survival ($F_{(2;9)}$ = 1.23; P = 0.337) (Table 2.3). The results for *C. partellus* showed that the GR maize sprayed with glyphosate resulted in higher levels of survival than on unsprayed GR maize. When larvae fed on the GR maize sprayed with glyphosate larvae gained 89 mg after 28 days, whereas larvae that fed on the GR maize only gained 73 mg ($F_{(2;9)}$ = 1.23; P = 0.336) (Table 2.3).

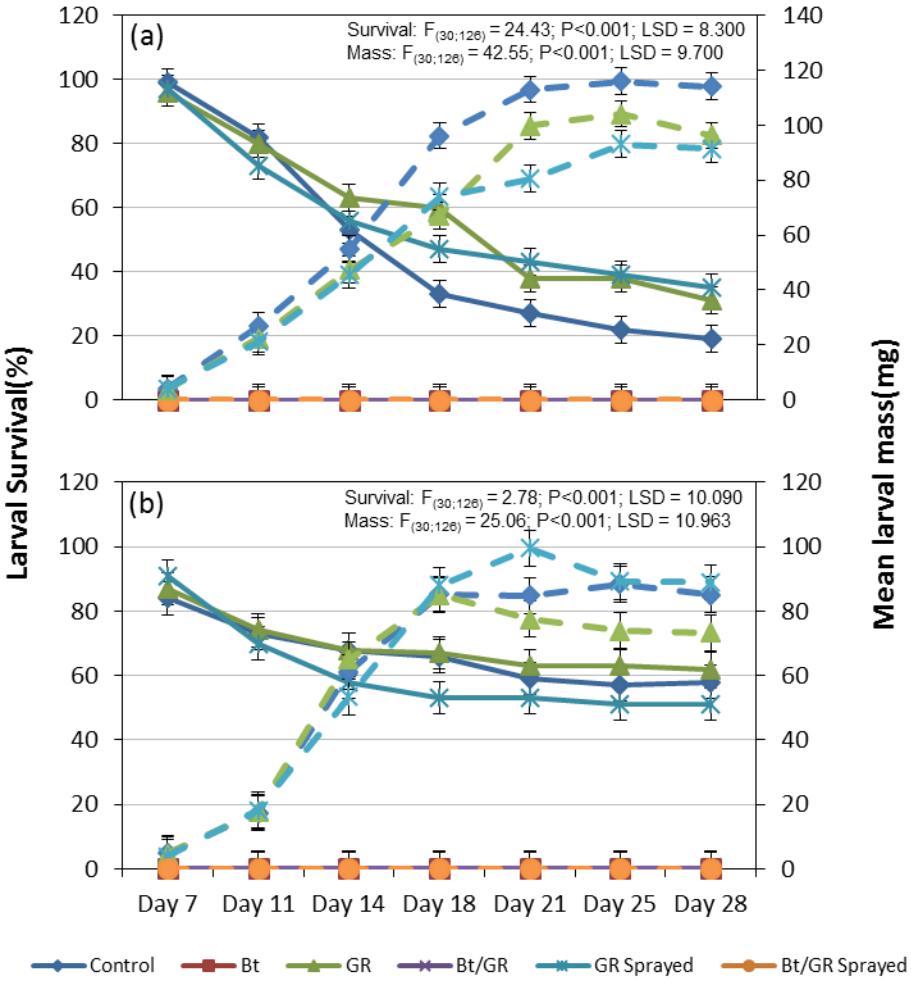

Figure 2.7 Percentage survival (%) and mean larval mass (mg) of *Chilo partellus* over time on maize whorls on the different treatments of the Pannar cultivar: (a) season 2013/14 and (b) season 2014/15. Error bars indicates LSD value. Solid lines indicate larval survival (%) (y-axis), while dotted lines indicate mean larval mass (mg) (z-axis).

Table 2.3 Survival (%) and mean larval mass (mg) of *Chilo partellus* on maize plants on the different treatments after 28 days.

Season	Treatment	Laboratory trial - Day 28 *Chilo partellus*					
		Survival (%)			Mean mass (mg)		
		Monsanto	Pioneer	Pannar	Monsanto	Pioneer	Pannar
2012/13	Control	22 a			80.3 a		
	Bt	-			-		
	GR	24 a			63.8 a		
	Bt/GR	-			-		
	GR Sprayed	39 a			76.1 a		
	Bt/GR Sprayed	-			-		
	F-value	2.61			1.4		
	P-value	0.115			0.283		
	LSD	17.34			30.813		
2013/14	Control	54 b	34 a	19 a	73.1 a	91.5 a	114.1 a
	Bt	-	-	-	-	-	-
	GR	42 b	31 a	31 a	74.9 a	92.8 a	96.3 a
	Bt/GR	-	-	-	-	-	-
	GR Sprayed	18 a	40 a	35 a	96.6 a	124.3 a	91.3 a
	Bt/GR Sprayed	-	-	-	-	-	-
	F-value	18.44	0.62	2.88	1.13	0.45	2.66
	P-value	**< 0.001**	0.558	0.108	0.364	0.649	0.124
	LSD	13.66	18.56	15.71	39.45	88.64	120.45
2014/15	Control	46 a	46 a	58 a	80.3 a	84.7 a	85.1 a
	Bt	-	-	-	-	-	-
	GR	58 a	40 a	62 a	93 a	81.7 a	73.2 a
	Bt/GR	-	-	-	-	-	-
	GR Sprayed	49 a	42 a	51 a	82 a	88.5 a	89 a
	Bt/GR Sprayed	-	-	-	-	-	-
	F-value	0.93	0.25	1.23	2.7	0.43	1.23
	P-value	0.431	0.784	0.337	0.120	0.664	0.336
	LSD	20.8	19.5	16.1	13.43	16.75	23.66

* Significant difference at P = 0.005 and indicated in red.
* Means within columns followed by different letters differ significantly at the indicated level.

2.4.2 Greenhouse trials

2.4.2.1 Greenhouse trial with *Busseola fusca*

Monsanto cultivar

At 14 days after inoculation, there was only 17% of the larvae that survived on the Bt/GR treatment that was sprayed with glyphosate, which was significantly lower than the survival on the Bt treatment (50%) and the GR treatment (58%) ($F_{(5;12)}$ = 7.25; P = 0.002) (Table 2.4). There was no significant difference in mass of the larvae that fed on any of the six treatments ($F_{(5;12)}$ = 3.20; P = 0.046) (Table 2.4).

The survival on all six treatments ranged between 32% and 37% after 21 days with no significant differences between treatments ($F_{(5;12)}$ = 0.09; P = 0.992) (Table 2.4). After 21 days, the larvae that was collected from the Bt/GR treatment had the lowest mean mass of all six treatments and differed significantly from the non-Bt treatment ($F_{(5;12)}$ = 3.31; P = 0.041) (Table 2.4).

Pioneer cultivar

After 14 days only 23% of the larvae survived the Bt/GR treatment that was sprayed with glyphosate, which differed significantly from the survival from the GR treatment (73%). Larval survival on the Bt/GR treatment sprayed with glyphosate was the lowest compared to the Bt treatment (45%) and the Bt/GR treatment (57%) ($F_{(5;12)}$ = 3.40; P = 0.038) (Table 2.4). There was no significant mass increase observed, however the larvae that fed on the Bt/GR treatment sprayed with glyphosate had a slightly lower mean mass than the larvae that fed on the other two treatments containing Bt ($F_{(5;12)}$ = 1.80; P = 0.18) (Table 2.4).

After 21 days of feeding slightly lower survival was observed on the maize that contained the single Bt gene (29%) than on the Bt/GR treatment sprayed with glyphosate (36%) and on the Bt/GR treatment (40%) ($F_{(5;12)}$ = 0.87; P = 0.528) (Table 2.4). Larvae on two of the Bt treatments (Bt and Bt/GR) had a minor mass increase and did not differ significantly, whereas the larvae that fed on the Bt/GR treatment sprayed with glyphosate had a greater mass than the larvae that fed on the Bt treatment, but did not differ significantly ($F_{(5;12)}$ = 3.55; P = 0.034) (Table 2.4).

Pannar cultivar

The lowest larval survival after 14 days was observed on the Bt/GR treatment that was sprayed with glyphosate (22%) and the highest survival on the non-Bt treatment (44%). However, no significant difference was observed between the treatments ($F_{(5;12)}$ = 1.35; P = 0.309) (Table 2.4). Results also showed no differences in mean larval mass between treatments ($F_{(5;12)}$ = 0.760; P = 0.595) (Table 2.4).

No significant differences in larval survival were observed between different treatments after 21 days ($F_{(5;12)}$ = 2.24; P = 0.118) (Table 2.4). Mean larval mass were largely similar between treatments with the exception that larvae that fed on the Bt weighed less than those recovered from GR maize ($F_{(5;12)}$ = 3.45; P = 0.037) (Table 2.4).

Table 2.4 The results of day 14 and day 21 for the greenhouse trial (Season 2014/15). Survival (%) and mean larval mass (mg) of *Busseola fusca* on maize plants on the different treatments.

Season	Greenhouse trial - *Busseola fusca*							
	Monsanto							
		Day 14				Day 21		
	Treatment	**Survival (%)**		**Mass (mg)**		**Survival (%)**		**Mass (mg)**	
2014/15	Control	45	abc	4.65	a	37	a	42.55	b
	Bt	50	bc	3.64	a	33	a	20.79	ab
	GR	58	c	3.36	a	33	a	30.03	ab
	Bt/GR	35	abc	2.44	a	36	a	11.70	a
	GR Sprayed	24	ab	2.51	a	36	a	32.64	ab
	Bt/GR Sprayed	17	a	2.33	a	32	a	26.34	ab
	F-value	7.25		3.2		0.09		3.31	
	P-value	**0.002**		**0.046**		0.992		**0.041**	
	LSD	18.000		1.567		18.900		17.838	
	Pioneer								
		Day 14				Day 21			
	Treatment	**Survival (%)**		**Mass (mg)**		**Survival (%)**		**Mass (mg)**	
	Control	65	ab	6.85	a	37	a	35.65	ab
	Bt	45	ab	5.30	a	29	a	16.91	a
	GR	73	b	9.57	a	56	a	29.71	ab
	Bt/GR	57	ab	5.33	a	37	a	19.12	a
	GR Sprayed	42	ab	8.01	a	36	a	59.75	b
	Bt/GR Sprayed	23	a	4.14	a	36	a	40.75	ab
	F-value	3.4		1.8		0.87		3.55	
	P-value	0.38		0.187		0.528		**0.034**	
	LSD	30.100		4.613		29.500		25.800	
	Pannar								
		Day 14				Day 21			
	Treatment	**Survival (%)**		**Mass (mg)**		**Survival (%)**		**Mass (mg)**	
	Control	44	a	7.63	a	40	a	34.12	ab
	Bt	30	a	2.58	a	26	a	20.49	a
	GR	42	a	4.23	a	40	a	45.72	b
	Bt/GR	41	a	10.03	a	30	a	24.48	ab
	GR Sprayed	26	a	4.19	a	20	a	32.80	ab
	Bt/GR Sprayed	22	a	2.01	a	30	a	27.37	ab
	F-value	1.35		0.76		2.24		3.45	
	P-value	0.309		0.595		0.118		**0.037**	
	LSD	25.300		10.976		16.800		14.755	

* Significant difference at P = 0.005 and indicated in red.
* Means within columns followed by different letters differ significantly at the indicated level.

2.4.2.2 Greenhouse trial with *Chilo partellus*

Monsanto cultivar

No larvae survived on any of the Bt treatments, therefore the results showed no significant difference in the survival ($F_{(2;6)}$ = 0.01; P = 0.991) (Table 2.5) and the mass ($F_{(2;6)}$ = 0.13; P = 0.878) (Table 2.5) after 14 days of inoculation. After 14 days of feeding on the GR treatment the larvae gained less mass than the larvae that fed on the control and the GR maize sprayed with glyphosate.

After 21 days only 10% of the larvae on the GR maize sprayed with glyphosate was recovered, 8% less than the control ($F_{(2;6)}$ = 2.44; P = 0.168) (Table 2.5) and a 0% survival rate was recorded on the Bt treatments.

Larvae that fed for 21 days on the GR maize that was sprayed with glyphosate showed the greatest mass gain when compared to the other treatments ($F_{(2;6)}$ = 0.71; P = 0.529) (Table 2.5).

Pioneer cultivar

A survival rate of 15% was recorded on the non-Bt treatment and 20% on the GR treatment, after 14 days which differed significantly from the Bt maize treatments with a 0% survival ($F_{(2;6)}$ = 2.41; P = 0.171) (Table 2.5). No significant differences were observed in larval mass between the three non-Bt treatments after 14 days ($F_{(2;6)}$ = 0.34; P = 0.722) (Table 2.5).

Although no significant results were recorded, after 21 days *C. partellus* larvae indicated that there was only a survival rate of 18% on the GR maize sprayed with glyphosate ($F_{(2;6)}$ = 0.49; P = 0.634) (Table 2.5). Larvae that fed on GR maize that was sprayed with glyphosate were heavier than larvae of some of the other treatments but did not differ statistically ($F_{(2;6)}$ = 0.32; P = 0.736) (Table 2.5).

Pannar cultivar

The highest survival rate after 14 days was recorded on the GR treatment but did not differ statistically from the other non-Bt treatments ($F_{(2;6)}$ = 1.62; P = 0.274) (Table 2.5). Although no significant difference was observed in the mean mass of the larvae between the non-Bt treatments, the larvae had the lowest mass gain on the GR treatment sprayed with glyphosate,

with no differences between the other two non-Bt treatments ($F_{(2;6)}$ = 0.32; P = 0.735) (Table 2.5).

No significant differences were observed in the survival of non-Bt treatments and no larvae survived on any of the Bt treatments after 21 days ($F_{(2;6)}$ = 0.61; P = 0.576) (Table 2.5). The larvae that fed on the GR treatment that was sprayed with glyphosate had the greatest mass after 21 days ($F_{(2;6)}$ = 1.47; P = 0.302) (Table 2.5).

Table 2.5 The results of day 14 and day 21 for the greenhouse trial (Season 2014/15). Survival (%) and mean larval mass (mg) of *Chilo partellus* on maize plants on the different treatments.

Season	Greenhouse trial - *Chilo partellus*				
	Monsanto				
		Day 14		Day 21	
	Treatment	**Survival (%)**	**Mass (mg)**	**Survival (%)**	**Mass (mg)**
2014/2015	Control	17 b	9.62 b	18 b	7.4 ab
	Bt	-	-	-	-
	GR	17 b	7.54 b	17 b	9.84 ab
	Bt/GR	-	-	-	-
	GR Sprayed	18 b	7.83 b	10 b	13.68 b
	Bt/GR Sprayed	-	-	-	-
	F-value	0.01	0.13	2.44	0.71
	P-value	0.991	0.878	0.168	0.529
	LSD	19.4	10.643	9.1	13
	Pioneer				
		Day 14		Day 21	
	Treatment	**Survival (%)**	**Mass (mg)**	**Survival (%)**	**Mass (mg)**
	Control	15 b	10.5 b	21 b	17.31 b
	Bt	-	-	-	-
	GR	20 b	7.98 ab	13 ab	15.09 b
	Bt/GR	-	-	-	-
	GR Sprayed	10 ab	7.32 ab	18 ab	19.78 b
	Bt/GR Sprayed	-	-	-	-
	F-value	2.41	0.34	0.49	0.32
	P-value	0.171	0.722	0.634	0.736
	LSD	11.4	9.886	20.6	14.276
	Pannar				
		Day 14		Day 21	
	Treatment	**Survival (%)**	**Mass (mg)**	**Survival (%)**	**Mass (mg)**
	Control	22 ab	12.05 b	11 ab	11.96 ab
	Bt	-	-	-	-
	GR	29 b	10.69 ab	10 ab	18.87 b
	Bt/GR	-	-	-	-
	GR Sprayed	10 ab	8.22 ab	15 b	22.08 b
	Bt/GR Sprayed	-	-	-	-
	F-value	1.62	0.32	0.61	1.47
	P-value	0.274	0.735	0.576	0.302
	LSD	25.6	11.803	13.6	14.752

* Significant difference at P = 0.005 and indicated in red.
* Means within columns followed by different letters differ significantly at the indicated level.

2.5 Discussion

2.5.1 Laboratory trials

2.5.1.1 Laboratory trials: *Busseola fusca* survival and mass

Monsanto cultivar

The survival of larvae on treatments with Bt maize in all three seasons indicated that the *B*. *fusca* populations used in this study were tolerant to Bt maize. Since there were largely no significant differences or tendencies regarding mean mass of *B*. *fusca* larvae on different treatments, it can be concluded that, as expected, larvae developed equally well on plants of all treatments and that application of glyphosate on Bt/GR plants did not affect larval survival and development on the Monsanto cultivar used in this study (Fig. 2.2 a - c; Table 2.2).

Pioneer cultivar

As with the above mentioned cultivar, survival and mean larval mass during both seasons did largely not differ between any of the treatments, indicating good development on the GR cultivar and that application of glyphosate on the Bt/GR stack had no influence on larval survival and development (Fig. 2.3 a and b; Table 2.2).

Pannar cultivar

The similar levels of larval survival and lack of differences in larval mass on the non-Bt control treatments, as well as the GR and GR sprayed treatments indicate that this hybrid and different treatments allowed normal larval development to take place (Fig. 2.4 a and b; Table 2.2).

2.5.1.2 Laboratory trials: *Chilo partellus* survival and mass

Monsanto cultivar

Data on larval survival during all seasons indicate that *C*. *partellus* is highly susceptible to Bt maize (Event MON810) and that the GR trait or glyphosate application had no effect on development of larvae (Fig. 2.5 a - c; Table 2.3).

Pioneer cultivar

Data on larval survival during all seasons indicate that *C*. *partellus* is highly susceptible to Bt maize (Event MON810) and that the GR trait or glyphosate application had no effect on development of larvae (Fig. 2.6 a and b; Table 2.3).

Pannar cultivar

Chilo partellus larvae responded similarly to all treatments than reported above for other cultivars and it can be concluded that the GR trait or glyphosate application had no effect on development of larvae (Fig. 2.7 a and b; Table 2.3).

2.5.2 Greenhouse trials

2.5.2.1 Greenhouse trial: *Busseola fusca* survival and mass

Monsanto cultivar

The lack of differences between treatments in terms of larval survival and mean larval mass indicated that the population of *B. fusca* used was tolerant to Bt maize and that the application of glyphosate did not have an effect on larval development (Table 2.4).

Pioneer cultivar

Stem borer larvae developed satisfactorily on plant tissue of all treatments indicating that the application of glyphosate did not have an effect on larval development (Table 2.4).

Pannar cultivar

The lack of differences between treatments in terms of larval survival and mean larval mass indicated that the application of glyphosate did not have an effect on larval development (Table 2.4).

2.5.2.2 *Greenhouse trial:* Chilo partellus *survival and mass*

Monsanto cultivar

Similar to observations made during laboratory trials, results from greenhouse trials showed that *C*. *partellus* was highly susceptible to Bt maize and that no larvae survived for seven days on any Bt treatment. Application of glyphosate on GR maize did not influence larval development (Table 2.5).

Pioneer cultivar

The lack of significant differences between treatments in terms of mean larval mass and survival indicate no effect of the GR trait or spray application on the development of *C*. *partellus* larvae (Table 2.5).

Pannar cultivar

Stem borer larvae developed satisfactorily on plant tissue of all treatments indicating that the application of glyphosate did not have an effect on larval development (Table 2.5).

2.6 Conclusions

Although not significantly different, larval survival recorded from plant tissue of the GR and GR treatment with the spray application tended to be the highest in six out of the seven trials with *B*. *fusca* and five out of seven for *C*. *partellus*. It can therefore be concluded that the GR trait or glyphosate application does not have an adverse effect on larval development.

In order to obtain conclusive results on the possible effect of glyphosate application on expression levels of Cry proteins in Bt/GR plants, ELISA's need to be done. Using larval survival and mass as indicators of possible effects of glyphosate on expression levels of insecticidal proteins is not conclusive.

Interpreting results from this study was complicated by the fact that the *B. fusca* populations used were all tolerant to Bt maize used in these trials. *Chilo partellus* has previously been reported to be much more susceptible to Bt maize than *B. fusca.* The MON810 Bt maize event was also reported to be a low dose event against *B. fusca* (Tabashnik *et al*., 2009). For that

reason, a similar study to the one described above may yield different results if a Cry1Ab susceptible population of *B. fusca* is used. Continued research, including the use of ELISA's to determine the effect of glyphosate on Cry protein expression levels is needed.

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Chapter 3 - Determining the effect of Bt/GR stacking and glyphosate application on the expression levels of Bt Cry toxin in maize leaves

3.1 Abstract

Stem borers are serious pests of maize in sub-Saharan Africa. These pests cause damage to maize which may result in yield losses or it may lead to secondary disease infections The effective control of insect pests on maize is therefore of utmost importance. Bt maize that express Cry1Ab insecticidal protein, is toxic to lepidopteran stem borer larvae. During 2006 the first report of *B*. *fusca* field resistance against Bt maize (MON810) was made in South Africa. The main reason for resistance evolution was thought to be poor compliance to refuge requirements but studies on soybean and cotton indicated that other factors could also influence expression levels of Cry proteins in plants. The possible effect of Bt/GR stacking and the application of glyphosate on Bt expression therefore needs to be investigated. If stacking and glyphosate application have an effect on Bt expression and high-dose requirements are not met, it may contribute to resistance development. The aim of the study was to determine whether Bt expression levels are affected when Bt is stacked with the glyphosate resistant (GR) trait or if glyphosate is applied. Results indicated that the Bt concentration in the leaves decreases as the maize plant ages. Large variation was observed in the Bt protein concentration of various. It cannot be concluded that glyphosate application or stacking of Bt/GR traits affects the Bt expression levels in the maize leaves.

3.2 Introduction

Genetically engineered insect-resistant maize that express the insecticidal protein derived from *Bacillus thuringiensis* (Bt) have become an important component in integrated pest management worldwide (Kennedy, 2008). Bt maize expressing Cry1Ab insecticidal proteins was introduced for the control of two stemborer species in South Africa, *Busseola fusca* and *Chilo partellus,* after its development for the control of other stem borer species in North America (Archer *et al*., 2001).

Bt maize is currently planted on more than 80% of the maize production area in South Africa (James, 2013). Of the total area of maize that was planted in 2014, 86.9% were GM maize, with 25% the Bt gene, 18.2% glyphosate resistant GR maize and 46.9% Bt stacked with GR genes. Since 2009 the adoption of the stacked maize event increased gradually each year, but in 2013 it increased drastically from 28% to over 43% (James, 2014).

The adoption rate of Bt maize increased rapidly in South Africa and has until recently been very effective against the target pest *B. fusca*. However, the first report of field resistance was made in 2006 by Van Rensburg (2007). Within one year thereafter another resistant population was recorded approximately 60 km from the initial site (Kruger *et al*., 2011).

According to Insect Resistant Management (IRM) requirements and in cases where events are considered as high-dose, the Bt concentration needs to be 25 times higher than the concentration needed to kill 99% of the target organism (Agbios, 2015). However, no information is available on the Cry toxin concentration in MON810 that is needed to fulfil this criterion (Then, 2007). Event MON810 has in the past been considered to be a low-dose event for *B. fusca* (Tabashnik *et al*., 2009).

Various factors may influence expression levels of insecticidal proteins in plants (Shen *et al*., 2010; Zhen *et al*., 2008). The efficacy of transgenic Bt cotton varies under the related age and structure of the plant, as well as under certain environmental stresses (Dong and Li, 2007; Rochester, 2006). Bt cotton is seen as one of the most effective and environmental friendly insect control method in cropping systems (Kranthi *et al*., 2005), but the inconsistency of the Cry toxin under field conditions still remains an unresolved issue (Adamczyk *et al*., 2001).

The inconsistency of Cry protein expression in maize plants may contribute to resistance development and the limited success of Bt technology in some localities in South Africa .Bt concentration may vary between neighbouring plants (Nguyen and Jehle, 2007), different plant individuals, between different plant parts (Greenplate, 1999), over seasons (Nguyen, 2004) and different localities (Greenplate, 1999). Another study concluded that a single leaf may produce different Bt levels in different parts of the leaf (Abel and Adamczyk, 2004). Several studies have been done to investigate the relationship of Bt toxins in plants under high temperature conditions. Under high temperatures a decrease in insecticidal protein in leaves is observed (Xia and Guo, 2004) and the Bt gene in cotton may switch off at earlier development stages (Xia and Guo, 2004). Protein content in Bt cotton was observed to decrease significantly during the boll filling stage (Chen *et al*., 2005).

A study done by Bruns and Abel (2003) on maize showed that the Cry1Ab concentration is positively affected (Chen *et al*., 2005) during the reproductive growth stage and with an increase in N-fertility of soils. Some reports also showed that the Cry1Ac protein levels are improved with the foliar application of a growth regulator (Oosterhuis and Brown, 2004).

The GM crop market has been dominated by single gene herbicide tolerant traits and Cry-toxin traits the first decade of GM crop production (Taverniers *et al.,* 2008). Glyphosate is the world's leading agrochemical since it is not selective in the plant species that it affects.

Glyphosate resistant plants were introduced in the USA during 1996, thereby allowing the post-emergence application of glyphosate without causing damage to the crop (Monsanto, 2005; Pollegioni *et al.,* 2011).

Farmers have reported that application of glyphosate onto GR crops shows stress symptoms and injury (Zablotowicz and Reddy, 2007; Zobiole *et al.,* 2010) and even effects on yield (Baylis, 2000). It was found that glyphosate causes damage to chlorophyll which may in turn result in lower chlorophyll content (Reddy *et al.,* 2000). Glyphosate application may also immobilize nutrients in the plant which is essential for chlorophyll production and function (Mg and Mn) (Taiz and Zeiger, 1998). The main metabolite of glyphosate (AMPA), may contribute to chlorosis (Duke *et al.,* 2003b; Reddy *et al.,* 2004) and glyphosate may have physiological impacts in soybean plants (Zobiole *et al.,* 2010). All of the above mentioned effects of glyphosate may result in plant stress which might influence biochemical processes in plants. It may also be possible that these stresses experienced by plants have an effect on expression levels of the Bt Cry protein in the plant. A study done on transgenic maize showed that when two genes are stacked into the genome of a single maize variety it may have an effect in the overall expression of endogenous genes (Agapito-Tenfen *et al.,* 2014). Furthermore, transgenic transcript accumulation levels showed a significant decrease when compared to a parental single event variety (Agapito-Tenfen *et al.,* 2014).

If Cry protein levels were adversely affected by application of glyphosate in South Africa, the high percentage of Bt/GR stacked maize planted in South Africa (James, 2014) could have contributed to resistance development. To ensure successful insect resistance management strategies for Bt maize, the expression levels of the Cry-toxin (Cry1Ab) in the stacked event needs to be assessed. This will determine whether the efficacy of the Bt in the stacked event is influenced by glyphosate spray applications. The relationship between the presence of transgenes (stacked traits) and the expression of Cry-toxin levels also needs to be explored in GM crops. Control over the expression of transgenes is important (Tabashnik *et al.*, 2013) since it is expected that transgenes in GM plants are fundamentally expressed in all plant tissue at high levels (Corrado and Karali, 2009). This is of importance with regard to target species developing resistance (Tabashnik *et al.*, 2013). Transgenes can be measured by mean of Reverse Transcription Polymerase Chain Reaction (RT-PCR), which is used to detect genes which are expressed in tissue. This can help to identify whether a transgene is active in a GM crop (Singh *et al.,* 2009).

ELISA (enzyme-linked immunosorbent assay), is a method for biochemical research. The assay allows the detection of all types of biological molecules at very low concentrations and quantities (Gan and Patel, 2013). Sometimes no Bt content expression is detected in Bt products (ELISA test), but it cannot be concluded that the Bt transgene is not present. It can

be that the Bt protein is expressed at extremely low levels, and therefore it is important that both analyses needs to be done when assessing Bt plants.

The aim of the study was to determine whether Cry protein expression levels in maize plants are influenced by the presence of a GR trait in the same cultivar and if glyphosate application influences Cry protein expression levels in Bt/GR plants.

3.3 Materials and methods

3.3.1 Determining the expression levels of Bt proteins in Bt maize plant tissue

A trial was conducted in in a greenhouse under controlled conditions. The study was conducted at the ARC - Grain Crops Institute, Potchefstroom (location: 46°43'S, 27°06'E) in the North-West Province, South Africa. The temperatures in the greenhouse ranged between 15°C and 26°C. The trial was carried out under natural daylight/night conditions over one season (2014/15). Glyphosate application (full rate - 2ℓ/ha) was performed three weeks after seedling emergence, since application guidelines stipulate application prior to the V7 growth stage. Cultivars from three different seed companies were used (Table 3.1). The six different treatments were: 1) non-Bt control, 2) Bt-maize, 3) GR maize, 4) Bt/GR stacked maize, 5) GR maize sprayed with glyphosate and 6) Bt/GR stacked maize sprayed with glyphosate. All treatments from all the hybrids that contain the Bt gene was from the MON810 event. The treatments with Bt and/or GR traits were iso-hybrids of the corresponding controls (Table 3.1). One of the treatments was non-transgenic maize which is further referred to the control treatment. The experimental design was a completely randomized block design with three replicates.

Leaf samples were collected from plants growing in the greenhouse at predetermined time intervals. These were T0 (one day before glyphosate application), T1 (1 day after application), T5, T9, T15, and T21 days after glyphosate application (DAA) for treatments GR sprayed and Bt/GR sprayed (Table 3.2). Although treatment Bt and Bt/GR were not sprayed, samples were collected at the corresponding time intervals. There were 10 plants in each plot row and leaf samples were taken from these plants. Three leaf samples were randomly collected from different plants at the different time intervals. Each leaf sample served as a replicate. The upper fully unfolded leaf from a plant was collected to ensure the leaves of the same developmental stage and physiological age were used. A leaf sample of 5 x 5 cm was collected from the middle part of the leaf avoiding the middle vein of the leaf. Leaf tissue samples were immediately frozen in liquid nitrogen and restored at -80°C until ELISA tests were performed.

Cry1Ab protein content in the leaves was determined using a QualiPlate^{Tm} kit for Cry1Ab/Cry1Ac (Envirologix) according to the manufacturer's instructions. All leaf tissue was freeze dried three days prior to the protein extraction period, in order to ensure that no water molecules were present in the leaf samples.

Approximately 0.005 g of the dry leaf tissue was grind to a very fine powder using acid purified sea sand. Subsequently, 1.5 ml extraction buffer was added to each sample in order to extract the protein. Contents were transferred into low protein binding Eppendorf tubes and centrifuged. Supernatants were diluted 1:100 with wash buffer for the immunological assay. Contents were filtered and transferred to 2 ml low protein-binding Eppendorf tubes. Samples were replicated three times and two standards were added in the 96-well microplate provided. Twelve Cry1Ab concentrations were used for the calibration curve at standard concentrations (Table 3.3).

Spectrophotometric measurements were conducted with a microplate reader (Berthold Technologies Mithras Multimode Microplate Reader Lb 940).

Table 3.1 The three different cultivars that were used in the trial and the six different treatments.

Treatments	Cultivars		
	Monsanto	**Pioneer**	**Pannar**
1	Non-Bt control (cultivar: DKC 80 – 10)	Non-Bt control (cultivar: 32 B 07)	Non-Bt control (cultivar: BG 3792)
2	Bt (MON810) (cultivar: DKC 80 – 12 B)	Bt (MON810) (cultivar: 32 B 06 B)	Bt (MON810) (cultivar: BG 3592 B)
3	GR (cultivar: DKC 80 – 30 R)	GR (cultivar: 32 B 05 R)	GR (cultivar: BG 3292 R)
4	Bt/GR (cultivar: DKC 80 – 40 BR)	Bt/GR (cultivar: 32 B 10 BR)	Bt/GR (cultivar: BG 3492 BR)
5	GR with glyphosate application (cultivar: DKC 80 – 30 R)	GR with glyphosate application (cultivar: 32 B 05 R)	GR with glyphosate application (cultivar: BG 3292 R)
6	Bt/GR with glyphosate application (cultivar: DKC 80 – 40 BR)	Bt/GR with glyphosate application (cultivar: 32 B 10 BR)	Bt/GR with glyphosate application (cultivar: BG 3492 BR)

Table 3.2 The number of readings done over time, days after glyphosate application and the corresponding time interval when leaf samples were collected.

Reading	**Days after glyphosate application (DAA)**	**Time intervals ***
1	One day before glyphosate application	T 0
2	1 DAA	T 1
3	5 DAA	T 5
4	9 DAA	T 9
5	15 DAA	T 15
6	21 DAA	T 21

* Time intervals when samples were collected to determine Cry protein content in the leaves

Table 3.3 The standard concentrations that were used as a calibrator.

Calibration curve	Cry (ng/ml)	Optical Density 450 nm
S1	0	0
S2	0.03	0.142
S3	0.06	0.201
S4	0.12	0.273
S5	0.24	0.446
S6	0.5	0.797
S7	1	1.42
S8	1.5	1.951
S9	2	2.426
S10	2.5	2.818
S11	3	3.082
S12	3.5	3.13

3.3.2 Data analysis

The data over time were analysed using repeated measurements ANOVA (Graphs). One-way ANOVAs were used to analyse and compared between treatments at each time interval (table). GenStat 17th Edition (VSN International) was used for all analyses. Data are presented as means with an associated least significant difference (LSD, at P = 0.05).

3.4 Results

3.4.1 Bt protein concentration measured over time (Repeated measurements) (Dry mass)

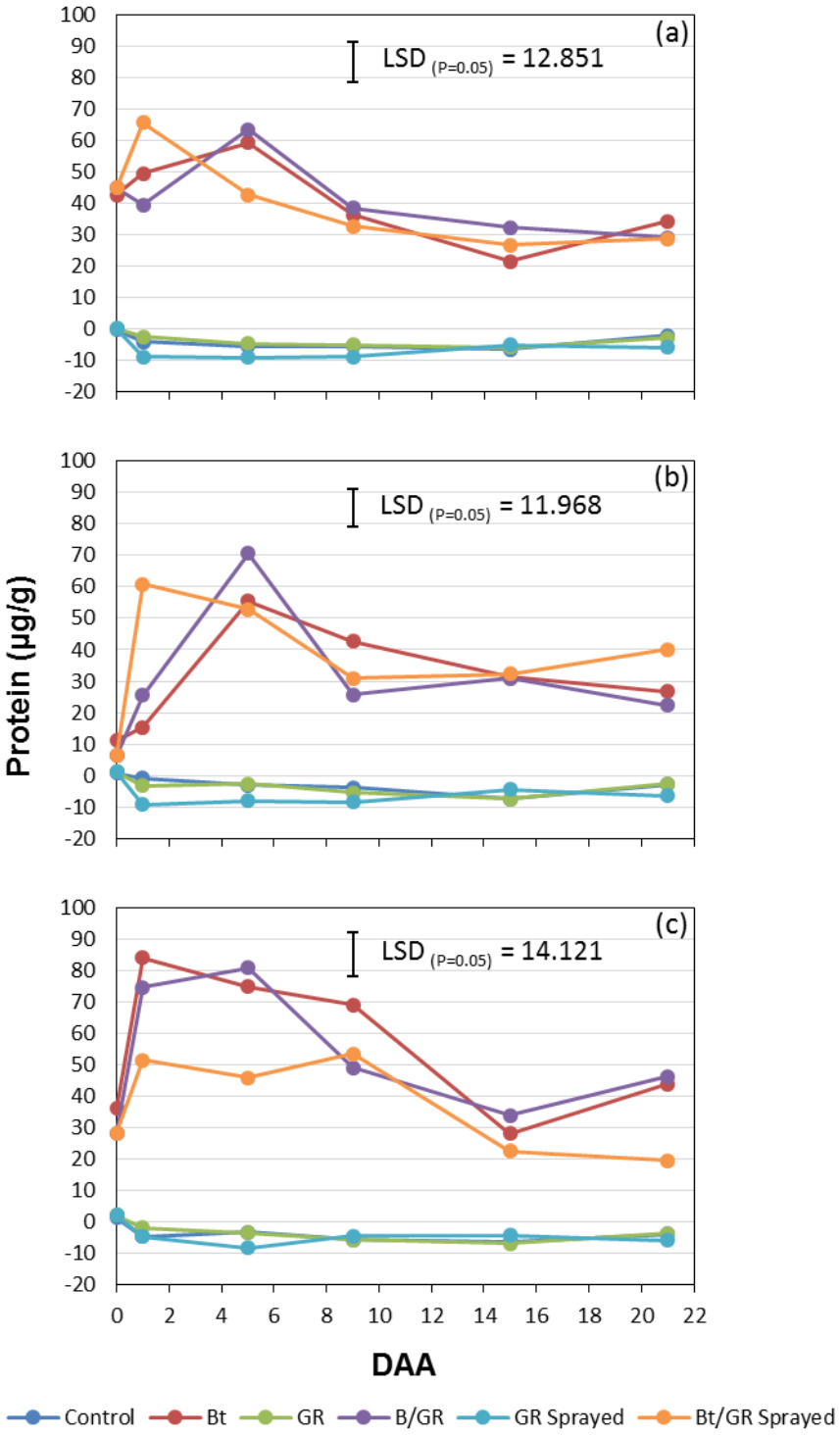

Figure 1. Mean Cry protein content (µg Cry/g leaf) measured at different time intervals in maize leaves of three different maize cultivars a) Monsanto ($F_{(10;31)}$ = 1.58; P = 0.160); b) Pioneer ($F_{(10;36)}$ = 4.36; P < 0.001) and c) Pannar ($F_{(10;36)}$ =1.62; P = 0.139). Leaf samples were collected one day before glyphosate application, there after 1, 5, 9, 15, 21 days after glyphosate application.

Monsanto cultivar

The Cry protein concentration in the leaves of plants in the Bt and Bt/GR treatment with no glyphosate application were similar over time ($F_{(10;31)}$ = 1.58; P = 0.160) (Fig. 1. (a)). Although not significantly different, the protein concentration in the leaves of Bt and the stacked (Bt/GR) treatment (Bt/GR) leaves decreased after T5. Cry protein content in the Bt/GR sprayed treatment, decreased after the first day glyphosate was applied at T1 but did not differ significantly. The insecticidal protein concentration of the Bt and Bt/GR treatments, decreased from T5 to T21. Whereas for Bt/GR sprayed treatment a decrease was observed from T1 to T21. The results indicated that the Bt concentration in the leaves gradually decreased with the age of the maize plant.

Pioneer cultivar

The Bt protein concentration in the Bt/GR sprayed treatment showed a sharp increase from T0 to T1. However a sudden increase for Bt and Bt/GR treatments were observed from T1 to T5. Cry protein concentration measured in Bt/GR sprayed treatment, showed a sudden decrease at T1 onwards ($F_{(10;36)}$ = 4.36; P < 0.001) (Fig. 1. (b)). The Pioneer cultivar had the same tendencies as in the case of the Monsanto cultivar, but with a higher spike from T0 to T5.

Pannar cultivar

The Pannar cultivar did not show the same tendencies as the previous two cultivars. Bt concentrations decreases for all three treatments containing Bt from T0 to T1. However with this cultivar the Bt concentration for the Bt and Bt/GR treatments were higher compared to the Bt/GR sprayed treatment The results showed that Bt and Bt/GR treatments express similar amounts of Cry protein over time and that concentrations did not differ significantly ($F_{(10;36)}$ =1.62; P = 0.139.) (Fig. 1. (c)). Results indicated that the Bt concentration in maize leaves increased in the young leaves until the maize plant reached a certain age. As the plant ages the Bt concentration decreases over time.

3.4.2 Bt protein concentration measured at specific time intervals (One-way ANOVA) (Dry mass)

Monsanto cultivar

A significantly higher protein content was measured in the Bt/GR sprayed treatment compared to the unsprayed Bt/GR and Bt treatments at T1. Results indicated that the protein content that was expressed in the Bt/GR sprayed treatment was 20 µg Cry protein per gram leaf higher than the other two treatments containing Bt at T1 (Table 3.4). The lack of differences between treatments in terms of protein concentration at the other time intervals indicated that the application of glyphosate onto plants in the Bt/GR sprayed treatment, did not have an effect on the protein expression in the maize leaves compared to the Bt and Bt/GR treatments. The highest protein expression levels for the Bt and Bt/GR treatments where no glyphosate was applied, was observed at T5, whereas the highest protein expression for the Bt/GR sprayed treatment was observed at T1. Results indicated that as a plant ages, the protein concentration decreases over time.

Pioneer cultivar

Comparison of the Cry protein expression levels of the three Bt treatments at T1, showed a significant difference in the protein content (Table 3.4). A significantly higher protein content was measured in the Bt/GR sprayed treatment than in the Bt and Bt/GR treatments at T1. There was also a significant difference between the Bt/GR sprayed treatment (40 µg/g leaf) and the unsprayed Bt/GR treatment (22 µg/g leaf) at T21. The Bt expression level for the Bt and Bt/GR treatments showed the highest expression at T5, compared to the Bt/GR sprayed treatment with the highest expression at T1. Results on Cry protein concentrations in plants of the non-sprayed treatment indicated that the protein content increased until the plant reached a certain age, after which the protein content decreased as plants mature.

Pannar cultivar

The Bt protein content was significantly higher in plants of the Bt treatment compared to those in the Bt/GR treatment at T9 (Table 3.4). Although the Bt protein concentration in the Bt and Bt/GR treatments were similar when compared over the different time intervals, the Bt content in the Bt/GR treatment tended to be slightly higher than that in the Bt treatment over most of the period. However, it was only significantly higher at T9, but the highest protein expression

level in plants of the Bt treatment was observed at T1, for the Bt/GR treatment at T5 and for the Bt/GR sprayed treatment at T9.

Table 3.4 Mean Cry protein content (µg Cry/g leaf) measured in maize leaves of cultivars at different time intervals. Data presented were analyzed by means of one-way ANOVA. Leaf samples were collected one day before glyphosate application, thereafter 1, 5, 9, 15, 21 days after glyphosate application (DAA) (Table 3.2)

Greenhouse trial (2014/15) Mean Cry protein concentration (µg/g) (Dry mass)												
Treatment	Monsanto											
	T0		T1		T5		T9		T15		T21	
Control	-		-		-		-		-		-	
Bt	42.79	a	49.27	a	59.40	a	37.13	a	21.50	a	35.15	a
GR	-		-		-		-		-		-	
Bt/GR	44.89	a	39.63	a	63.53	a	38.51	a	32.31	a	29.20	a
GR Sprayed	-		-		-		-		-		-	
Bt/GR Sprayed	44.89	a	65.62	b	42.67	a	32.68	a	26.55	a	28.47	a
F-value	0.07		22.28		1.72		0.12		0.64		0.34	
P-value	0.930		**0.003**		0.256		0.890		0.566		0.728	
LSD	15.507		10.119		29.114		32.094		24.609		24.551	
Treatment	Pioneer											
	T0		T1		T5		T9		T15		T21	
Control	-		-		-		-		-		-	
Bt	11.179	a	15.37	a	55.52	a	42.68	a	31.24	a	26.66	ab
GR	-		-		-		-		-		-	
Bt/GR	6.377	a	25.61	b	70.65	a	25.68	a	30.94	a	22.35	a
GR Sprayed	-		-		-		-		-		-	
Bt/GR Sprayed	6.377	a	60.76	c	52.94	a	30.85	a	32.21	a	40.07	b
F-value	2.78		151.91		1.79		1.99		0.00		6.17	
P-value	0.140		**< 0.001**		0.246		0.217		0.995		**0.035**	
LSD	5.758		6.685		24.769		21.376		33.227		12.874	
Treatment	Pannar											
	T0		T1		T5		T9		T15		T21	
Control	-		-		-		-		-		-	
Bt	36.02	a	83.88	a	74.75	b	69.02	b	28.1	a	43.83	a
GR	-		-		-		-		-		-	
Bt/GR	28.23	a	74.64	a	80.87	b	49.12	a	33.81	a	46.23	a
GR Sprayed	-		-		-		-		-		-	
Bt/GR Sprayed	28.23	a	51.5	a	45.9	a	53.46	ab	22.52	a	19.43	a
F-value	0.45		2.47		10.49		5.64		1.13		4.07	
P-value	0.657		0.165		**0.011**		**0.042**		0.382		0.076	
LSD	23.199		36.698		19.957		15.255		18.346		25.441	

* Significant difference at P = 0.005 and indicated in red.

3.5 Discussion

A similar tendency was observed in the Monsanto and Pioneer cultivars where the highest protein concentration for the Bt and Bt/GR treatment was observed at T5, and for the Bt/GR treatment sprayed with glyphosate at T1. The Bt protein concentration showed an increase for all three cultivars on the day immediately following glyphosate application to plants of the Bt/GR sprayed treatment. A possible explanation could be that the physiology of the maize plant may change after glyphosate application, but this phenomenon needs to be investigated with a more in depth study. Between the V3 and V5 growth stages the maize plant is the most tolerant to stresses. This is the optimal time for chemical weed control, because the growing point is still below ground (Ritchie *et al.,* 1993). This should be kept in mind in future reference studies. The effect of glyphosate on the Cry protein concentration should also be kept in mind.

In the Monsanto and Pioneer cultivars, the protein concentration measured in the Bt treatment and the Bt/GR treatment decreased after T5. The reason for this is unknown, as this study focused on the effect of gene stacking and on the effect of glyphosate on the Bt protein expression levels in the maize leaves. Dutton *et al.* (2004) also reported that Bt content in the leaves of three different Bt treatments decreased gradually as the plant aged. All graphs verified the results of Dutton *et al.* (2004)

At T1 a significantly higher Cry protein concentration was measured in the Bt/GR sprayed treatment than in the Bt and Bt/GR treatments for both the Monsanto and Pioneer cultivars. The Bt/GR sprayed treatment in the Pannar cultivar expressed a significantly lower Bt protein concentration than the other two Bt treatments at T1.

3.6 Conclusions

This study showed that different maize varieties, when grown under similar environmental conditions, express different concentration levels of Cry proteins. This could probably be ascribed to the genetic backgrounds of the different varieties (Agbios, 2015). When the Bt gene is evaluated, it is expressed in its homozygous state (inbred lines). Whereas with commercialization the gene is expressed in heterozygous state (hybrid). Therefore, we can conclude that the Bt gene expression seems to be affected by the genetic background and should be evaluated for this reasons before commercialization.

The results show that the Bt concentration in the leaves decreases with the age of the maize plant. The results showed that there is no effect on the Bt expression levels in the maize leaves

when the Bt and GR genes are stacked in a single maize variety. Although significant differences were observed at T1 when glyphosate was applied to the Bt/GR treatment in the Monsanto and Pioneer cultivar, it cannot be concluded that glyphosate affects the Bt toxin expression levels of Bt/GR maize varieties.

Due to high variation in the Bt concentration levels between plants grown under the same environmental conditions (Nguyen and Jehle, 2007), more studies with more replicates should be repeated over more than one season. Trtikova *et al.* (2015) suggested that the expression of transgenes should be measured when research on Bt plants are being conducted. Transgene expression is an alternative way to determine whether the Bt protein content is affected by environmental factors or by the plants' regulatory system and whether the transgene is absent or present (Trtikova *et al.,* 2015). RT-PCR is used to identify genes which are expressed in the tissue (Singh *et al.,* 2009).

Future studies need to give attention to why different parts of the plants produce different Bt concentration levels and how does the different Bt concentrations in different parts of the plant compare (Nguyen, 2004). If unexpectedly low levels of Bt content are expressed it can increase the development of resistance in target species. With this we can in future determine the concentration of Cry protein needed to fulfil the criterion of the high dose principle.

To conclude, the results from this study showed large variation in the Bt protein concentration over the different cultivars despite the fact that all treatments express the same gene. This may be due to different genetic background and supports the findings of Trtivoka *et al.* (2015).

It cannot be concluded that glyphosate affects the Bt expression levels in the maize leaves. Trtivoka *et al.* (2015) showed in their study that different maize varieties containing the same transgene can react differently to environmental factors i.t.o. the Bt protein expression level. Therefore, further studies need to be done to investigate the effects of glyphosate on Bt/GR maize i.t.o. the physiology of the maize plant, transgene expression, and the relationship between the Bt and GR gene with regard to the Bt expression in the maize plant and the physiology of the plant.

3.7 References

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Chapter 4 - Conclusions

The control of weeds in maize cropping systems and of insect pests on maize is important since these biotic factors directly influence crop yield. During 2014 nearly 50% of all genetically modified (GM) maize that were planted globally contained the Bt/GR (glyphosate resistance) stacked genes (James, 2014). The use of the broad spectrum herbicide, glyphosate subsequently increased with the adoption of GR and stacked gene maize varieties (James, 2013). These GM maize varieties provide many benefits to farmers and it is therefore important that this technology be protected. The misuse of glyphosate in the past contributed to resistance development in weeds. Poor stewardship and lack of compliance to refuge requirements resulted in pest resistance evolution to Bt maize.

It is important to identify potential problems that threaten the sustainable use of GM crops as soon as possible in order to ensure continued success of GM technology. For example, it is important to monitor the status of resistance of pests to Bt crops and to investigate the efficacy of Bt maize in single and stacked maize varieties in order to put measures in place to delay evolution of resistance in key pests.

Numerous factors together with environmental conditions may influence the efficacy of insecticidal proteins in crops. One of the main reasons for product failure efficacy of Bt cotton against target insect pests was ascribed to reduced insecticidal protein expression levels (Benedict *et al*., 1996). Before the commercialization of Bt crops, there were however concerns with regard to the development of resistance to these insecticidal proteins (Gould, 1998; Tabashnik *et al*., 2003; Zhao *et al*., 2003).

Currently the high dose/refuge strategy is the only Insect Resistant Management (IRM) strategy used in South Africa (Kruger *et al*., 2011). Despite the use of the high-dose/refuge strategy in order to delay resistance development (Tabashnik *et al*., 2009), van Rensburg (2007) reported a *Busseola fusca* (Lepidoptera: Noctuidae) population in South Africa that was resistant to MON810 Bt maize, expressing Cry1Ab protein.

There are several reasons for the development of resistance in target pestS. For example, poor refuge compliance to high dose/refuge strategy was listed as the major reason that contributed to the high selection pressure for resistance development to Bt crops. Another reason is that target species are exposed to sub-lethal dosages of the Cry protein. Farmers assume that Bt technology controls the pests effectively and they therefore neglected to scout and monitor fields for pests (Kruger *et al*., 2009).

Previous studies confirmed that the expression levels of the Cry toxin in MON810 maize differ between young and older maize plants, and that environmental conditions influence Bt

expression levels in transgenic maize (Dutton *et al*., 2009).This was already confirmed by Van Rensburg (2001) by means of larval development studies. Bt concentration in plant tissue may vary during the growing season since leaves of young plants contain a higher Bt concentration compared to leaves of older plants (Greenplate, 1999; Coviella *et al*., 2000; Olsen and Daly, 2000; Dutton *et al*., 2004).

To ensure the success of IRM strategies for Bt maize pests, the expression levels of the Cry-toxin (Cry1Ab) in the stacked event needs to be assessed in further in depth studies. This will determine whether the Bt expression levels in the stacked event and the efficacy thereof is influenced by glyphosate spray applications.

The possible effect that stacking of insecticidal and herbicide tolerant traits could have on stem borer resistance evolution has never been investigated. No information exits on the effect of herbicide application on expression and efficacy of Cry protein and subsequent stem borer larval mortalities on Bt maize when treated with glyphosate. The relationship between the stacked traits and glyphosate application has also not been investigated

Bt expression levels in plants are influenced by abiotic factors and the expression levels may decrease with the maturity of the plant (Greenplate, 1999; Coviella *et al*., 2000; Coviella *et al*., 2002; Dutton *et al*., 2004; Chen *et al*., 2005; Jiang *et al*., 2006; Dutton *et al*., 2009). Studies and farmer reports also indicated that GR crops may be affected by glyphosate applications (Arregui *et al*., 2003; Duke *et al*., 2003a; Zablotowicz and Reddy, 2007; Zobiole *et al*., 2010a; Zobiole *et al*., 2010b).

If plant stress factors such as those described above and in chapter 3, influence the Bt protein expression in the plant, the possibility exists that application of agro-chemicals, could, under certain conditions also stress plant growth. In such cases the possibility exists that such applications may then influence Bt protein expression in plants with stacked traits such as Bt maize with GR traits. The idea that the herbicide tolerant gene may influence plant development and physiology, prompts the idea that it could also affect expression of Cry proteins inside plants of stacked events (Bt/GR).

This could imply that there may exist an interaction between the Bt and GR traits when stacked. Therefore the aim of the study was to determine if the combination of Bt and GR resistant traits, as well as the application of glyphosate have an effect on the efficacy of Bt maize against stem borers.

In the current study larval survival and mass was monitored as indicators to determine if glyphosate had an effect on the expression of the Bt toxin in a stacked gene maize variety.

Although there were no significant differences it is concluded that the GR trait or glyphosate application does not have an adverse effect on larval development as the survival results for *B. fusca* and *Chilo partellus* (Lepidoptera: Crambidae) tended to have the highest survival in most of the trials.

Chilo partellus has previously been reported to be more susceptible to Bt maize than *B. fusca*. The MON810 Bt maize event was also reported to be a low dose event against *B. fusca* (Tabashnik *et al*., 2009). The *B. fusca* population in this study showed some levels of resistance to MON810 maize which complicated the interpretation of the results. For that reason, a similar study to the one described above may yield different results if a Cry1Ab susceptible population of *B. fusca* is used.

The results further showed that the Bt concentration in the leaves decreased with the age of the maize plant. Although not significantly different, Bt concentration in the leaf samples decreased directly after glyphosate was applied. It can be concluded from this study that stacking of traits does not affect the Bt protein expression in maize leaves, but from the three cultivars observed, 8 out of the 15 sample intervals the Bt protein concentration tended to be higher in the Bt/GR maize leaves than in the single Bt gene maize leaves. When glyphosate is applied to the stacked gene maize varieties, the maize leaves tended to express lower levels of the Bt toxin. This only occurred in the Monsanto and Pannar cultivars.

Since large variation in the Bt concentration occur between different plant individuals (Nguyen and Jehle, 2007), between different parts in the leaf (Abel and Adamczyk, 2004), and different plant tissues (Greenplate, 1999) the question can be asked, why different parts of the plants produce different Bt concentration levels. We now know that the stability of the Bt concentration (Cry1Ab) is influenced by environmental factors, as this may be linked to the reason for the variation in Cry protein levels during the season (Nguyen, 2004) and different localities (Greenplate, 1999).

Large variation in the Bt protein concentration over the different cultivars and treatments were observed between plants grown under the same conditions. This may be due to different genetic backgrounds of the different cultivars and therefore supports the findings of Trtivoka *et al*. (2015) who reported large variation in the expression of the transgenes and Bt content. Since the expression of the Bt gene seems to be affected by the genetic background in which it is presented, experiential hybrids should be evaluated before commercialization. Due to high variation in the Bt concentration levels between plants grown under the same environmental conditions (Nguyen and Jehle, 2007), more studies with more replicates should be conducted with different plant tissues of the same plant over time for more accurate results.

In conclusion, glyphosate application does not affect the Bt expression levels in maize leaves directly. However results of previous studies showed, glyphosate does affect nutrient availability (Zobiole *et al*., 2010b), immobilize nutrients which is compulsory for chlorophyll production and function (Mg and Mn) (Taiz and Zeiger, 1998) and may also have long-term physiological impacts in soybean plants (Zobiole *et al*., 2010b).

Future studies are needed to investigate the effects of glyphosate on GR maize varieties under different conditions. These studies should include the investigation of physiology of the maize plant, transgene expression, and the relationship between the Bt and GR gene in regard to the Bt expression in the maize plant. Attention to the question as to why different parts of the plant express different Bt concentrations levels is required (Nguyen, 2004). This phenomenon should be assessed on different aspects as the Bt expression levels are highly sensitive to different abiotic and biotic factors.

The above mentioned effects of glyphosate may result in plant stress which might have an effect on expression levels of the Bt Cry concentration in the plant. When a plant experiences stress, it can be witnessed through chlorophyll a florescence (Strasser *et al*., 2004) as well as the chlorophyll content. It was also shown in a previous study that glyphosate may cause damage to chlorophyll (Reddy *et al*., 2000). Since chlorophyll a fluorescence provides valuable information regarding the function and structure of the photosynthetic system (Strasser *et al*., 2004), changes induced in the photosynthetic system can be detected and quantified (Mehta *et al*., 2010).

No data are available on the concentration of protein needed to fulfil the criterion of the high dose refuge strategy. Therefore reliable guideline data are needed regarding the minimum lethal Cry protein dosage to kill a target pest, this may contribute to the current problems experienced with the high/dose refuge strategy. Continued research, including the use of ELISA's to determine the effect of glyphosate on Cry protein expression levels is needed. It is important for future reference that the transgene expression be measured when Bt plants are assessed (Trtikova *et al*., 2015).

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