Bt expression in maize plant tissues and the impact of gene flow

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Bt expression in maize plant tissues and the impact of gene flow

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Declaration

I certify that the dissertation hereby submitted by me for the M.Med.Sc. (Molecular Biology) degree at the University of the Free State is my independent effort and not previously been submitted for a degree at another university/faculty. I furthermore waive copyright of the dissertation in favour of the University of the Free State.

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Contents

Abbreviations and acronyms

ANOVA	Analysis of variance
Bt	*Bacillus thuringiensis*
CI	Confidence interval
Cry	Crystal protein
cm	Centimetre
DAFF	Department of Agriculture, Forestry and Fisheries
ELISA	Enzyme-linked immunosorbent assay
et al.	*et alia* (and others)
F1	First filial generation
Fig.	Figure
g	Gram
GM	Genetically modified
GMO	Genetically modified organism
HCl	Hydrochloric acid
HT	Herbicide tolerant
IR	Insect resistant
LD_{50}	The median lethal dose of a substance, or the amount required to kill 50% of a given test population
M	Molar
m	Meter
ml	Millilitre
mg	Milligram
mm	Millimeter

ng	Nanogram
nm	Nanometer
PBS	Phosphate buffered saline
pH	Percentage hydrogen
R stage	Reproductive stage
rpm	Revolutions per minute
US EPA	United States Environmental Protection Agency
V stage	Vegetative stage
%	Percentage
µg	Microgram
µl	Microlitre
^{o}C	Degree Celsius

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Preface

The development of insect resistance to insect resistant (IR) crops is of concern as it negates the benefits of this technology. There are a few verified cases of evolved field resistance to IR crops including India, Puerto Rico and South Africa. In all three these cases, the development of insect resistance occurred rapidly when the requirement for the high dose/refugia strategy was not met. In the case of insect resistance to IR maize in South Africa, it has been reported that the initial lack of compliance to planting non-IR refugia resulted in selective pressure for resistance alleles. It has also been suggested that the required high dose of Cry1Ab was not always met in different plant tissue. For example, it has been observed that the feeding behaviour of African stem borer larvae (*Busseola fusca*, Fuller) on different plant tissues had an effect on mortality rate. However, prominent insect larvae feeding tissues, such as cob sheath, has not been included in studies to determine levels of Cry1Ab in different maize tissue. As a result, this question cannot be answered with current data.

A further consideration for insect resistance management is the introduction of IR technology in Africa. Subsistence and communal farming practice in Africa includes saving and exchanging seed. Under these conditions, gene flow is likely to occur between IR maize and traditional non-IR maize varieties. It is unknown what effect this will have on the requirements of the high dose/refugia strategy in terms of managing insect resistance development.

The aim of this study was to determine the levels of Cry1Ab within and between different tissue types, such as cob sheath which were not previously included in similar studies, over the growing season in a commercial MON810 maize hybrid under typical commercial dry land farming conditions in South Africa. In addition, the effect of gene flow from MON810 maize to non-IR maize, on the levels of Cry1Ab was also evaluated in different tissues of an F1 generation over the growing season.

This dissertation contains three chapters, including a literature review and two research chapters. The literature review presents a background to IR maize, and focuses on reported levels of Cry1Ab in different maize tissue as well as factors that may influence Cry1Ab expression. Chapter two was carried out over the 2008/2009 and 2009/2010 growing seasons using a white MON810 converted commercial maize variety. Chapter three comprised investigating the levels of Cry1Ab in an F1 generation, the result of gene flow between a MON810 converted yellow commercial maize variety and a white non-IR commercial maize variety over the 2009/2010 growing season. While I do not dispute the role of non-compliance to refugia in the evolution of insect resistance, it is hoped that this study may shed some light on the roles that levels of Cry1Ab may also play in this regard. I suggest that determining the levels of Cry1Ab in different plant tissue may be of especial importance in events with stacked *cry* genes and in the application of this technology in a typical African farming environment.

Chapter 1

A review of Cry1Ab in maize

1.1. Introduction to genetically modified organisms (GMOs)

1.1.1. The benefits of GM crops

Genetically modified (GM) crops have been deliberately altered through the use of recombinant DNA technology to develop varieties with improved traits (Uzogara, 2000). Genetic engineering can be used to introduce traits in plants such as insect resistance, herbicide tolerance, plant disease resistance, drought tolerance or enhanced nutrition (Uzogara, 2000). GM technology has the potential to benefit consumers, farmers and the environment by providing healthier, cheaper and environmentally friendlier foods compared to conventional crops (Uzogara, 2000). For example, agronomic traits, such as insect resistance and herbicide tolerance have been developed to improve crop management practices (Ando and Khanna, 2000; Hails, 2000; Phipps and Park, 2002; Sikorski and Gruys, 1997). The improvement in agricultural practice is that insect resistant (IR) plants do not require insecticidal applications to control targeted insect pests and herbicide tolerant (HT) crops can be sprayed with herbicide to control weeds. Thus, GM technology has the potential to improve food production (Uzogara, 2000).

1.1.2. The status of GMO production

1.1.2.1. GMO production in the world

There are currently four major GM crops being commercially produced in the world, including canola, cotton, maize and soybean. There are also many other minor GM crops such as alfalfa, papaya, potato, squash, sugar beet and sweet pepper (James, 2011). In 2011, a total of 160 million hectares of biotech crop was planted, which is estimated to contribute approximately 10% of global crop production (James, 2011). Approximately 26% of canola, 82% of cotton, 32% of maize and 75% of soybean produced worldwide is a result of GM technology (James, 2011). The major producers of GM crops (1 million hectares or more) include Argentina, Brazil, Canada, China, India, Pakistan, Paraguay, South Africa, United States and Uruguay.

1.1.2.2. GMO production in South Africa

In South Africa, GM cotton, soybean and maize have been approved for commercial production (James, 2010). In 2010, approximately 2.2 million hectares of GM crop was cultivated in South Africa (James, 2010). It is estimated that 100% (15,000 hectares) of cotton, 85.0% (331,500 hectares) of soybean and 76.9% (1.9 million hectares) of maize grown in South Africa is GM. In 2010, approximately 95% (14,250 hectares) of the GM cotton that was planted in South Africa was stacked HT/IR and 5% (750 hectares) was HT, all (331,500 hectares) GM soybean was HT, whereas 45.6% (865,589 hectares) of the GM maize grown was IR, 13.4% (254,211 hectares) was HT and 41.0% (777,820 hectares) was stacked IR/HT. It is estimated that 20,000 hectares of

GM maize (HT and/or IR) was planted by small scale or subsistence farmers in South Africa in 2010 (James, 2010). The GM events that have been released in South Africa for cotton include MON15985 (IR) (Cry1Ac and Cry2Ab2), MON531/757/1076 (IR) (Cry1Ac), MON1445/1698 (HT) and MON88913 (HT). Only one GM event has been released for soybean, namely MON 40-3-2 (HT). The GM maize events that have been released are MON810 and Bt11 (IR) (Cry1Ab), NK603 and GA21 (HT), and stacked event MON89034 (IR) (Cry1A.105 and Cry2Ab) (DAFF, 2011). Currently, the majority of insect resistant maize grown in South Africa is MON810, which was introduced into local maize varieties through conventional breeding (Kruger *et al*., 2011).

1.2. Introduction to IR crops

IR crops have been transformed to express different *cry* genes, that originate from the soil bacterium *Bacillus thuringiensis* (Bt). The Cry protein is an endotoxin to different insect groups, including those of agricultural importance (Crickmore *et al*., 1998). Although different *cry* genes have been used to develop IR plants, the most common *cry* gene currently used in commercial crop production is Cry1Ab. The first maize event containing Cry1Ab was MON810, initially developed in the United States to control the European stem borer (*Ostrinia nubilalis*, Hübner) and the South-Western corn borer (*Diatraea grandiosella,* Dyar*)* (Archer *et al*., 2001). MON810 was introduced into South Africa in 1997 to control the African stem borer (*Busseola fusca*, Fuller) and spotted stem borer (*Chilo partellus,* Swinhoe) (Van Rensburg, 1999). When

MON810 was introduced into South Africa, it was found to be 100% effective against *B. fusca* and *C. partellus* (Van Rensburg, 1999).

1.3. Considerations for the cultivation of IR maize

1.3.1. Benefits of IR maize

IR maize has several agricultural and environmental benefits. Farmers benefit by planting IR maize as a result of yield protection from stem borer infestation (Park *et al*., 2011). Furthermore, IR maize is more profitable than conventional maize due to reduced costs from not having to apply insecticides, and the reduction in insecticide benefits the environment (Gouse *et al*., 2005; Huesing and English, 2004; Phipps and Park, 2002; Qaim and Zilberman, 2003; Qaim, 2009; Raney, 2006; Yorobe and Quicoy, 2006). In South Africa, it is estimated that *B. fusca* and *C. partellus* can result in crop losses up to 10%, which can have a substantial financial impact on crop production (Gouse *et al*., 2005). Thus, despite the extra cost of IR maize seed, due to the royalty costs on GM technology, commercial farmers consider it economically beneficial due to the benefit of yield protection as well as the reduced cost of insecticides (Gouse *et al*., 2005; Kruger *et al*., 2009; Qaim, 2009).

1.3.2. Considerations associated with the introduction of IR maize

With the first environmental release of IR maize in the United States in 1996, there was a consideration that insect pests could develop resistance to the Cry endotoxin, which would threaten its long term use (Gould, 1998). Resistance to Cry endotoxin in the target insect can be defined as a failure or decreased efficacy to control the target insect (Huang *et al*., 2011). It was suggested that widespread planting of IR maize could result in strong selective pressure on target insect populations to develop resistance to the Cry endotoxin (Bates *et al*., 2005; Gould, 1998). The development of resistance to endotoxin in insect pests is a concern as it negates the benefits of IR technology and would result in farmers having to spray insecticides to control stem borers, in addition of the cost of IR seed (Kruger *et al*., 2012). Furthermore, the application of insecticides would negate the environmental benefit of planting IR maize. In order to ensure sustainable use of IR technology, management strategies were incorporated when IR crops were introduced to ensure its successful use (Gould, 1998).

1.4. Management of IR crops

1.4.1. High dose/refugia strategy

In order to minimize the development of resistance to Cry endotoxin in IR maize, a combination of high dose endotoxin and non-IR maize refugia is used, known as the high dose/refugia strategy (Gould, 1998; McGaughey *et*

al., 1998). A requirement of the high dose strategy is that IR maize must express sufficiently high levels of endotoxin to kill between 95 and 100% of the target insects (Gould, 1998; Meihls *et al*., 2008; Tabashnik, 2008; US EPA, 2001). MON810 was developed to theoretically produce 25 times the lethal dose (LD_{50}) required to kill 50% of *O. nubilalis* (US EPA, 2001). However, LD_{50} values differ for different insects (Huang *et al*., 2011) (Table 1.1.). For example, Van Rensburg (1999) found that *B. fusca* was less susceptible to Cry1Ab than *C. partellus*, suggesting that the former had a higher LD_{50} value. Despite the differences in LD_{50} values of target insects, it was assumed that levels of Cry1Ab in MON810 maize are produced in excess of the required LD_{50} dose (US EPA, 2001). Huang *et al*. (2007) and Singh *et al*. (2005) found that there was a 'functional mortality' dose-response for the sugarcane borer (*Diatraea saccharalis,* Fabricius) and *C. partellus*, where an increase in Cry1Ab concentration had a negative effect on larval development, resulting in stunted growth and consequent mortality. Thus, it appears that the effect of Cry1Ab on a given target insect is dose dependent.

Table 1.1.: ***Summary of studies that determined the LD_{50} values of Cry1Ab for different insect pests.***

Author	Species	LD_{50}
Ayra-Pardo *et al*. (2006)	*Spodoptera frugiperda*	>3000 ng cm^{-2}
He *et al*. (2005)	*Ostrinia furnacalis*	0.10 - 0.81 µg g^{-1}
Huang *et al*. (2007)	*Diatraea saccharalis*	0.11 µg $g^{-1\ S}$ 11.17 µg $g^{-1\ R}$
Jalali *et al*. (2010)	*Chilo partellus*	0.01 - 0.07 µg ml^{-1}
	Helicoverpa armigera	0.12 - 1.99 µg ml^{-1}
	Sesamia inferens	0.45 - 0.56 µg ml^{-1}
Singh *et al*. (2005)	*Chilo partellus*	0.12 ng ml^{-1}

^{S} – LD_{50} value for susceptible insects

^{R} – LD_{50} value for resistant insects

In addition to the high dose strategy, Gould (1998) suggested that the use of non-IR refugia would delay the development of resistance to IR crops in the target insect. It was hypothesized that planting of non-IR refugia together with the IR crop would maintain a susceptible insect population (Gould, 1998; US EPA, 2001). This approach was based on the premise that homozygous resistant insects emerging from the IR field would mate with the susceptible insects from the refugia and help maintain a low frequency of resistant alleles where heterozygous insects, that were low to moderately resistant, would be killed when feeding on the IR maize (Gould, 1998). Thus similar to the introduction of IR maize in other countries, it is a regulatory requirement in

South Africa to plant non-IR refugia. Current requirements for planting refugia in South Africa include 20% refugia that may be sprayed with insecticide or 5% refugia that may not be sprayed with insecticide (Monsanto, 2011). In addition to a regulatory requirement to plant refugia, stewardship programmes, managed by the biotech seed companies, were also implemented in South Africa. The stewardship programmes involve farmer education and a legal requirement by farmers to comply with planting refugia (Kruger *et al*., 2009). When IR crops were first commercialized, it was thought that the development of resistance would be highly delayed if the high dose/refugia strategy was implemented correctly (Bates *et al*., 2005; Gould, 1998; Tabashnik *et al*., 2008).

1.4.2. Development of resistance to IR maize in South Africa

Despite the implementation of strategies to delay the development of resistance to Cry endotoxin in the target insect, Van Rensburg (2007) reported the discovery of a resistant population of *B. fusca* to Cry1Ab maize in the Christiana area, South Africa. Van Rensburg (2007) found that a population of *B. fusca* was able to survive on MON810 maize, although there was a marked reduction in larval growth rate compared to larvae feeding on non-Bt plant tissue. A follow up study by Kruger *et al*. (2011) confirmed the presence of resistance, as well as its spread from the point of discovery. As a result, farmers, especially when planting maize under irrigation, were forced to apply insecticides to IR maize at additional cost (Kruger *et al*. 2012). The

development and spread of resistance to Cry1Ab in the target pest was and remains a concern in South Africa as it negates the benefits of IR technology.

1.4.3. Factors contributing towards insect resistance development in South Africa

In an attempt to determine the reasons for resistance development to Cry1Ab in South Africa, Kruger *et al*. (2009) conducted a survey to evaluate farmer compliance to refugia. It was found that compliance to planting refugia with maize under irrigation by farmers in the region where resistance to Cry1Ab was first reported, was poor in the first five to seven years. The lack of compliance was attributed to small farming units and the impracticality of planting refugia in plots under pivot irrigation (Kruger *et al*., 2009). Predictably, compliance to planting refugia increased to almost 100% after resistance to IR maize was reported. It is thought that non-compliance to planting refugia resulted in high selective pressure for resistance alleles to Cry1Ab in *B. fusca* (Bates *et al*., 2005, Kruger *et al*., 2009, 2012; Van Rensburg, 2007).

Although there has been a dramatic increase in compliance under commercial farmers, it is not certain whether compliance to planting refugia is similar for subsistence and communal farmers. South Africa has approximately 20,000 hectares of IR maize planted by subsistence and communal farmers. Many subsistence and communal farmers tend to practice seed saving and will also exchange seed amongst themselves (Gouse *et al*., 2005). Another

consideration is that planting refugia would not be considered practical for subsistence and communal farmers since the farms are too small, where communal or subsistence farm plots range from 100 m^2 to 4,550 m^2 (Aliber and Hart, 2009). Furthermore, stewardship programmes will not be effective since seed companies would have to deal with thousands of small-scale farmers (Gouse *et al*., 2005). It is unknown what effect communal and subsistence farming will have on the development of resistance due to the practice of saving and exchanging seed.

Van Rensburg (2007) suggested that resistance to Cry1Ab in the target insect could have developed as result of continuous exposure of larvae from the second moth flight to sub-lethal levels of endotoxin at the end of the growing season. Van Rensburg (2001) found that the feeding behaviour of *B. fusca* larvae on different tissue types had an influence on insect mortality. He found that larvae had a higher survival rate when feeding on silk and cob sheath tissue compared to leaf and stem tissue. Furthermore, larvae that had first fed on silk tissue had a greater chance of survival if they migrated to kernels or soft cob tissue. Van Rensburg (2001) suggested that this was an indirect indication of lower production of Cry1Ab in these tissues when compared to other tissues.

1.4.4. Factors that contribute to variation in expression of Cry1Ab

Several studies have shown that environmental factors, such as fertilization practice, soil salinity and water logging, can affect the expression of Cry1Ab in

maize and other crop types (Bruns and Abel, 2003; Coll *et al*., 2010; Luo *et al*., 2008). Bruns and Abel (2003) found that an increase in soil nitrogen positively affected Cry1Ab production in leaf tissue. Compared to this, salinity and water logging appears to have a negative effect on endotoxin expression. An interesting study by Abel and Adamczyk (2004) and Székács *et al*. (2010b) demonstrated that production of endotoxin was related to photosynthetic activity. Coll *et al*. (2010) suggested that Cry1Ab expression is determined by cellular metabolism. Thus, factors affecting cellular metabolism, such as photosynthesis, also appear to affect the expression of endotoxin. This suggests that environmental factors affecting cell metabolism also have an effect on production of endotoxin.

A further consideration that could influence the expression of Cry1Ab is the genetic background of the maize variety. Some studies have investigated the effect of genetic background on Cry1Ab production (Coll *et al*., 2008; Levandi *et al*., 2008; Piccioni *et al*., 2009). Abel and Adamczyk (2004) found significant differences in levels of Cry1Ab in leaf tissue from different MON810 varieties, suggesting that cellular metabolism is also affected by genetic variation. In cotton it has been found that plant maturation results in a decrease in Cry1Ac mRNA (Dong and Li, 2007) and it has been suggested that methylation of the 35S promoter results in lower levels of Cry1Ac. From these studies it is evident that endotoxin expression is affected by genetic as well as epigenetic factors.

In addition to genetic background, gene flow is also a consideration for affecting Cry1Ab expression. GM gene flow describes the movement of genes from a GM variety to a non-GM variety. Chilcutt and Tabashnik (2004) proposed that gene flow from IR to non-IR maize refugia has the potential accelerate the development of resistance in pest insects by either killing susceptible larvae in the refugia, or by selecting for heterozygotes. Additionally, Krupke *et al*. (2009) found that volunteer MON88017 maize had similar insect damage when compared to non-IR volunteer maize in the same field. Aheto *et al*. (2011) suggested that based on modelling data, there would be an increase in gene flow due the combination of small plots, typical of communal and subsistence farming, and heterogeneity of seed sources. Thus, it appears that gene flow may also impact on the development of resistance to IR maize in insect pests, which could be exacerbated in a subsistence farming environment.

1.4.5. Expression levels of Cry1Ab in MON810

When IR maize was first commercialized, there was limited information available on the expression of Cry1Ab in maize tissue (Nguyen and Jehle, 2007). It was initially thought that Cry1Ab expression in leaf tissue was stable and similar to other tissues, with the exception of kernels (US EPA, 2001). Several post-release studies have investigated the levels of Cry1Ab in different tissue, and there appears to be a range in endotoxin production which changes over the growing season (Habuštová *et al*., 2012; Nguyen and Jehle, 2007; Kamath *et al*., 2010; Szèkács *et al*., 2010a) (Table 1.2.). The

highest levels of Cry1Ab were determined in the leaves (7.9 - 10.3 µg g^{-1}, 0.7 - 2.4 µg g^{-1}, 0.1 - 11.1 µg g^{-1}, 8.1 - 17.2 µg g^{-1}, 9.7 - 50.1 µg g^{-1} and 0.7 – 1.4 µg g^{-1}) (US EPA, 2001; Abel and Adamczyk, 2004; Nguyen and Jehle, 2007; Szèkács *et al*., 2010a; Kamath *et al*., 2010; Habuštová *et al*., 2012), followed by anthers or tassel (0.3 – 6.7 µg g^{-1},5.0 µg g^{-1} and 0.2 µg g^{-1}) (Nguyen and Jehle, 2007; Szèkács *et al*., 2010a; Habuštová *et al*., 2012), root (0.3 – 4.2 µg g^{-1},2.3 – 5.0 µg g^{-1} and 0.2 – 0.4 µg g^{-1}) (Nguyen and Jehle, 2007; Szèkács *et al*., 2010a; Habuštová *et al*., 2012), stem (0.1 – 2.4 µg g^{-1},1.4 µg g^{-1} and 0.1 – 0.3 µg g^{-1}) (Nguyen and Jehle, 2007; Szèkács *et al*., 2010a; Habuštová *et al*., 2012), kernels (0.2 – 0.9 µg g^{-1}, 0.01 – 0.5 µg g^{-1}, 1.0 µg g^{-1} and 0.1 µg g^{-1}) (US EPA, 2001; Nguyen and Jehle, 2007; Szèkács *et al*., 2010a; Habuštová *et al*., 2012) and silk or bloom (0.01 µg g^{-1}) (Habuštová *et al*., 2012). In addition, Abel and Adamczyk (2004) found Cry1Ab expression also varied within different sections of the same leaf. Furthermore, Kamath *et al*. (2010), Nguyen and Jehle (2007) and Szèkács *et al*. (2010a) have that found that Cry1Ab expression increases until flowering, after which levels of endotoxin decrease up to maturity. A recent study by Kamath *et al*. (2010) found that levels of Cry1Ab in leaf tissue differed between wet and dry seasons. Unfortunately, no studies have investigated the levels of Cry1Ab in cob sheath, an important feeding tissue for *B. fusca*.

Table 1.2.: ***Summary of available data on the levels of Cry1Ab in various tissue types for MON810 maize.*** *The Cry1Ab data is presented as the mean, range and growth stage.*

Plant tissue		Levels of Cry1Ab[5, 6]																			
		US EPA (2001)[1]	Abel and Adamczyk (2004)[1, 5]	Nguyen and Jehle (2007)[1, 5, 6]				Székács *et al*. (2010a)[1, 5, 6]					Kamath *et al*. (2010)[5, 6]			Habuštová *et al*., (2012)[1, 5, 6]				
Roots	Mean Range Growth stage[5,6]	ND	ND	1.4, 1.6 0.3 - 3.9 V4	1.4, 1.7 0.3 - 4.2 ~V7	1.4, 1.6 0.6 - 2.7 R1	1.4, 1.6 0.3 - 2.8 R6	5.3 ND V1	~2.3 ND V3 - R5				ND			0.2 ND V4-6	0.4 ND ~V15	0.2 ND R1	0.2 ND R4	0.2 ND R6
Stems	Mean Range Growth stage[5,6]	ND	ND	0.4, 0.5 0.1 - 1.1 V4	0.3, 0.4 0.1 - 0.9 ~V7	1.0 0.4 - 2.4 R1	1.1, 1.2 0.4 - 2.6 R6	ND	ND	~1.4 ND R1 - R5			9.3[2], 14.3[3] ND V3 - V4	ND	3.5[2], 4.7[3] ND R1	0.3 ND V4-6	0.1 ND ~V15	0.2 ND R1	0.1 ND R4	0.1 ND R6
Leaves	Mean Range Growth stage[5,6]	8.6, 9.0, 9.4, 12.2 5.2 - 15.1 ND	0.7 - 2.4[4] ND V7	2.5, 3.3 0.3 - 4.7 V4	2.9, 3.2, 4.4, 4.6 0.7 - 7.8 ~V7	2.5, 2.7, 4.2, 5.1 2.0 - 8.6 R1	4.0, 5.5, 5.8, 6.4 1.4 - 11.1 R6	8.1 ND V1	17.2 ND V5	ND	9.6 ND R4	13.5 ND R5	50.1[2], 19.3[3] ND V3 - V4	38.1[2], 9.7[3] ND V9	21.0[2], 11.1[3] ND R1	0.9 ND V4-6	1.0 ND ~V15	1.4 ND R1	0.7 ND R4	0.7 ND R6
Anthers or tassel	Mean Range Growth stage[5,6]	ND	ND	ND	ND	2.1, 2.8 0.3 - 6.7 R1	ND	ND	ND	5.0 ND R1	ND	ND	ND			ND	0.2 ND ~V15	0.2 ND R1	ND		
Silk or bloom	Mean Range Growth stage[5,6]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND			ND		0.01 ND R1	ND		
Kernel	Mean Range Growth stage[5,6]	0.3, 0.4, 0.5, 0.6 0.2 - 0.9 ND	ND	ND	ND	ND	0.2, 0.3 0.01 - 0.5 R6	ND	ND	ND	ND	1.0 ND R5	ND			ND			0.1 ND R4	0.1 ND R6

ND - Not determined

[1] Levels of Cry1Ab in µg g^{-1}fresh weight

[2] Dry season (µg g^{-1}dry weight)

[3] Wet season (µg g^{-1}dry weight)

[4] Range in mean values for multiple data sets

[5] V - Vegetative stage

[6] R - Reproductive stage

1.5. Quantification of Cry1Ab in plant tissue using ELISA

The most commonly used method to quantify levels of Cry1Ab in plant tissue is ELISA (enzyme-linked immunosorbant assay) (Ahmed, 2002; Anklam *et al*., 2002). This method is based on immunological detection, is highly specific and allows the recognition of an antigen in the presence of other compounds (Anklam *et al*., 2002). Commercially available monoclonal ELISA kits can be used to screen or quantify a specific GM protein in raw or processed products, as long as the expressed protein is not degraded and the epitope can still be detected reliably (Asensio *et al*., 2008; Grothaus *et al*., 2006). The sandwich ELISA format is based on the antigen binding between a primary (capture) antibody and a secondary (detection) antibody (Ahmed, 2002; Grothaus *et al*., 2006). A 96 well microtiter plate is coated with a primary antibody, and the antibody binds to a specific antigen in a sample. A secondary antibody is added to the reaction, and binds to the antigen/primary antibody complex, resulting in the sandwich of the antigen between the primary and secondary antibodies (Grothaus *et al*., 2006). A horseradish peroxidise is coupled to the secondary antibody that induces a chromogenic signal in the presence of an enzyme substrate (Asensio *et al*., 2008). The chromogenic signal is detectable at a wavelength of 450 nm and is measured using an optical plate reader. Consequently, the levels of Cry1Ab can be determined against a standard curve. There are various commercial ELISA kits available to screen or quantify Cry1Ab endotoxin in maize tissue. This technique takes approximately four hours to complete and is suitable for bulk-volume analysis to quantify endotoxin in MON810 maize (Ahmed, 2002).

A consideration in the quantification of Cry1Ab in maize is the use of bacterially produced or plant derived endotoxin standards (Grothaus *et al*., 2006; US EPA, 2001). Grothaus *et al*. (2006) suggested that bacterially produced Cry standards can be used if there is similarity in sensitivity and specificity in antibody binding compared to plant derived endotoxin. Likewise, the US EPA (2001) recommended the substitution of microbial produced Cry1Ab protein for the plant source based on protein sequence, structural and toxicological similarities. An advantage of using microbial produced Cry1Ab is that large amounts of protein can be manufactured in a short period of time, as appose to plant-derived Cry1Ab (Mendelsohn *et al*., 2003; US EPA, 2001).

1.6. Conclusion

In South Africa, approximately 1.9 million hectares of maize grown is GM (HT or IR), of which majority of the GM maize is IR (James, 2010). There are several financial and environmental benefits to planting IR maize, due to reduced insecticidal application (Phipps and Park, 2002). Regardless of the financial and environmental benefits, there is a concern that insect pests could develop resistance to IR maize, which would negate IR technology.

When IR maize was commercialized in 1995, it was suggested that the high dose/refugia strategy be used to delay the development of resistance to Cry1Ab endotoxin in the target insect (Gould, 1998). Despite South Africa adopting the high dose/ refugia strategy to delay the development of resistance to Cry1Ab in insect pests, in 2007, Van Rensburg (2007) described

a population of *B. fusca* that was able to survive on MON810 maize. A recent study by Kruger *et al*. (2011) confirmed the presence of resistance to Cry1Ab, and its spread from the point of discovery. Studies have suggested that the lack of compliance to planting refugia resulted in a high selective pressure for resistance to develop in *B. fusca* (Kruger *et al*., 2009; Van Rensburg, 2007). In addition to non-compliance to planting refugia, Van Rensburg (2007) also suggested that sub-lethal production of Cry1Ab endotoxin in maize could have contributed to the development of resistance in *B. fusca*.

Although there were initially few studies on Cry1Ab expression in IR maize, current data shows that levels of Cry1Ab can vary within a tissue type, as well as between different tissue types, over the growing season (Habuštová *et al*., 2012; Kamath *et al*., 2010; Nguyen and Jehle, 2007; Szèkács *et al*., 2010a). It appears that environmental factors, such as soil fertilization, soil salinity and water logging, affect Cry1Ab expression (Coll *et al*., 2010; Levandi *et al*., 2008; Piccioni *et al*., 2009). In addition, the genetic background of the maize also affects Cry1Ab expression (Coll *et al*., 2008; Levandi *et al*., 2008; Piccioni *et al*., 2009). This raises the question as to what effect changes in genetic background as a result of gene flow could have on the levels of endotoxin.

Although several studies have investigated the levels of Cry1Ab in specific tissues, as such root, stem, leaves, silk, tassel and cob, there is still no data on Cry1Ab expression for cob sheath, which is a primary plant tissue that *B. fusca* larvae feed on. In order to counter the development of resistance to IR maize in the target insect, new varieties of IR stacked events are currently

being released in South Africa. However, without a comprehensive understanding of the factors that contribute to the development of resistance to IR maize in the target insect, there is a concern that the introduction of IR stacked events may not provide durable control of insect pests, thus denying farmers the benefit of this technology.

Chapter 2

Levels of Cry1Ab endotoxin in MON810 maize plant tissue

2.1. Introduction

Currently, the biggest threat to the successful use of IR crops is the development of resistance in the target insect (Bates *et al*., 2005; Shelton *et al*., 2000). As a result, management strategies, to delay or prevent the evolution of resistance in pest populations, have been implemented since the commercialization of insect resistant crops. Management approaches usually include the planting of non-Bt refugia to dilute insect resistance alleles. In conjunction with the use of refugia is the assumption that endotoxin, if produced in a sufficiently 'high dose' will kill almost all insects with resistant alleles (Bates *et al*., 2005; Gould, 1998, 2000; Huang *et al*., 2011; Tabashnik *et al*., 2009). Bates *et al*. (2005) suggested that the high dose strategy may be compromised by a number of practical considerations, including the contamination of IR seed with non-expressing 'off-types' or variable expression of Cry1Ab as a result of environmental influences (Gianessi and Carpenter, 1999). Meihls *et al*. (2008) demonstrated that under laboratory conditions, the exposure of rootworm to a low to moderate dose of endotoxin results in the evolution of resistance in as few as three generations (Huang *et al*., 2011).

The development of insect resistance to IR crops in the field has been limited with only a few confirmed reports, including Bt cotton in India and IR maize in Puerto Rico and South Africa (Karihaloo and Kumar, 2009; Matten *et al*., 2008; Van Rensburg, 2007). In all three cases, field resistance to IR crops occurred rapidly when requirements for the high dose/refugia strategy were not met (Huang *et al*., 2011). In the case of insect resistance to IR maize in South Africa, it was determined that compliance to refugia was initially low (Kruger *et al*., 2009). It is suggested that the lack of compliance to planting refugia resulted in a high selective pressure for resistance alleles to evolve. In addition to this, Van Rensburg (2007) suggested that levels of Cry1Ab were not at a sufficiently high dose during late growth stages of Bt maize. Van Rensburg (2001) also noted that the feeding behaviour of larvae of *B. fusca* had an effect on mortality. For example, it was observed that larvae had a higher survival rate when feeding on silk and cob sheath compared to leaf and stem tissue. In a study on IR cotton, it has been found that cotton bollworm (*Helicoverpa arnigera*) exhibited a higher rate of survival when feeding on reproductive compared to vegetative tissue (Kranthi *et al.,* 2005). This suggests that the expression of Bt in different tissue of a maize plant may not always meet the high dose requirement.

Several studies have investigated the levels of Cry1Ab in different maize tissue (Table 1.2. Chapter 1) (Abel and Adamczyk, 2004; Habuštová *et al*., 2012; Nguyen and Jehle, 2007; Kamath *et al*., 2010; Szèkács *et al*., 2010a; US EPA, 2001). While all studies have found differences between levels of Cry1Ab in different tissue, some results are incongruent. For example,

Nguyen and Jehle (2007) found that with the exception of roots, there were significant differences in Cry1Ab at different reproductive stages. Compared to this, it has been reported that levels of Cry1Ab does not differ significantly over the reproductive stages (Szèkács *et al*., 2010a). The highest concentration of Cry1Ab has been detected in leaf tissue (Nguyen and Jehle, 2007; Habuštová *et al*., 2012; Kamath *et al*., 2010; Szèkács *et al*., 2010a). The second highest levels of Cry1Ab have been found in anther (Nguyen and Jehle, 2007; Szèkács *et al*., 2010a), followed by roots (Nguyen and Jehle, 2007; Kamath *et al*., 2010; Szèkács *et al*., 2010a), stem (Nguyen and Jehle, 2007; Kamath *et al*., 2010; Szèkács *et al*., 2010a), kernel (Nguyen and Jehle, 2007; Szèkács *et al*., 2010a) and then silk (Habuštová *et al*., 2012). Nguyen and Jehle (2007) found significant differences in the expression of Cry1Ab between growing seasons, compared to Kamath *et al*. (2010) who found differences in leaves but not stems. From all these studies, it appears that while plant tissue and development are the main factors affecting Cry1Ab content, there is no clear indication of the trend that could inform the discussion on resistance development in terms of high dose strategy in South Africa. In addition, important target insect larvae feeding tissues, such as cob sheath, has not been studied in terms of Cry1Ab expression. Thus, the aim of the study was to determine the levels of Cry1Ab in different tissue in MON810 maize, including those important in larvae feeding, at different growth stages over the growing season.

2.1. Materials and methods

2.2.1. Field trial layout

A white MON810 converted commercial maize variety (PAN6Q-321 B) was grown at the University of the Free State experimental farm, outside Bloemfontein in the Free State, over two consecutive growing seasons (2008/2009 and 2009/2010). The trial comprised of a four hectare plot cultivated under conventional farming practice for the region, without the application of insecticides. A three week temporal isolation to other maize planted in adjacent plots in a three km radius was employed to prevent cross pollination.

2.2.2. Collection and storage of plant material

Maize plants were collected at one vegetate (V) and three reproductive (R) growth stages, namely, pre-flowering (V20 stage), flowering (R1 stage), green cob or dough stage (R4 stage) and cob maturity (R6 stage). Roots (carefully removed from the soil and washed with distilled water to remove excess soil), stem (45 cm section of the mid stem), leaves (the 5^{th}, 7^{th} and 9^{th} leaves were collected to represent a composite leaf sample for each plant), silk, tassel, cob sheath and whole cob was sampled from 55 randomly selected plants per growth stage (as applicable to the growth stage). Approximately 40 g of plant tissue was collected per tissue type, cut into smaller pieces and placed in a sealable bag on ice at the trial site. Samples were transported to the laboratory on ice and stored at -20^{o}C. Plant tissue was stored at -80^{o}C for 24 hours prior to lyophilisation using the VirTis Benchtop K. Lyophilized plant

material was homogenized using a Waring blender at 4^{o}C in a cold room and the homogenized plant material stored at -20^{o}C.

2.2.3. Quantification of Cry1Ab using ELISA

Levels of Cry1Ab were quantified using the commercial Envirologix Cry1Ab/Cry1Ac QuantiPlate® 96-well microplate ELISA kit according to a modification of the manufacturer's instructions and taking the methods of Abel and Adamczyk (2004), Kamath *et al*. (2010); Nguyen and Jehle (2007) and Szèkács *et al*. (2010a) into consideration. All reactions were performed in duplicate, and each assay included 1, 2, 6 and 10 ng Cry1Ab standards (Biosence, Norway) and a blank control. Phosphate buffered saline (PBS) (pH 7.2) (Diagnostic and Technical Services) (1 ml) containing 0.55% Tween20, was added to 100 mg plant tissue followed by vortexing and centrifugation at 10,000 rpm for five minutes. The supernatant (100 µl) of the sample extract was retained and diluted with extraction buffer (PBS (pH 7.2) containing 0.55% Tween20) to fall within a final concentration of between 1 to 10 ng Cry1Ab. Conjugate solution (50 µl) (provided with the kit) was added to the plate, followed by the addition of 50 µl of the diluted sample extract. The plate was incubated on a rotary shaker at 100 rpm at room temperature for one hour after which each microwell was washed three times with 100 µl PBS (pH 7.2) containing 0.05% Tween20. Substrate solution (100 µl) (provided with the kit) was then added to the plate and incubated on a rotary shaker at 100 rpm at room temperature for 15 minutes after which 100 µl of 1 M HCl stop solution was added to the plate. The absorbance was read on the

BioTek Synergy HT Plate Reader at 450 nm within 30 minutes after adding the stop solution. Compensation for background noise was done by subtracting the mean optical density of the blank control from each sample reading. Samples with a mean reading outside of the range of the standard curve were diluted as necessary and the assay repeated.

2.2.4. Weather data

General weather data for the area was obtained from the South African Weather Service for Bloemfontein. The weather data included daily recording of average temperature (^{o}C), humidity (%) and precipitation (mm) over the growing season. ANOVA was used to compare average temperature, humidity and precipitation between the 2008/2009 and 2009/2010 growing seasons.

2.2.5. Data analysis

The mean, standard deviation and range for each tissue type was determined using Excel 2003 (Windows XP). The Bonferroni-Holm test (Daniel's XL Toolbox, version 2.60) was used as a post hoc test to compare the statistical difference in levels of Cry1Ab between tissue type, growth stage and growing season. The distribution and skewness of each data set was determined using Easyfit 5.5 Professional to evaluate the variability of Cry1Ab expression in each tissue type over the growing season. A calculation of the 95%

confidence interval (CI) was performed to exclude all outlying data points. Statistical significance was set at $p<0.01$ for all tests performed.

2.3. Results and discussion

While several studies have investigated the levels of Cry1Ab in MON810 maize, there does not appear to be a clear trend other than that differences within and between tissue types have been reported (Abel and Adamczyk, 2004; Habuštová *et al*.. 2012; Kamath *et al*., 2010; Nguyen and Jehle, 2007; Szèkács *et al*., 2010a; US EPA, 2001). In the current study, levels of Cry1Ab were monitored in different tissue over the growing season and between two growing seasons (Table 2.1. and 2.2.; Fig. 2.1. and 2.2.). In addition, levels of Cry1Ab were determined for cob sheath, not previously reported in the literature but that is important in target insect feeding. Variations in Cry1Ab between maize tissue is similar to findings of Cry1Ac in Bt cotton (Adamczyk *et al*., 2001; Adamczyk and Summerford, 2001; Greenplate *et al*.,1999; Kranthi et al., 2005).

The levels of Cry1Ab within the same tissue in different plants at different growth stages were shown to have a considerable range. A calculation of the 95% CI for each data set, excluded the majority of data points indicating the high extent of variation for levels of Cry1Ab (Table 2.3. and 2.4.). Furthermore, the data for the majority of the sampling points was moderately to highly skewed (Table 2.5. and 2.6.). The data distribution over the two growing seasons did not follow a distinct trend for roots, stems, leaves, silk

and tassel. The data for cob sheath and cob tended to bias towards lower values of Cry1Ab. Based on these data, it appears that the levels of Cry1Ab in MON810 maize are highly variable.

Table 2.1.: Mean levels of Cry1Ab (µg g^{-1}dry weight) in different tissues over the 2008/2009 growing season for MON810 maize. *The Cry1Ab data is presented as the mean with standard deviation, range and number of samples.*

Plant tissue		Cry1Ab (µg/g dry weight)			
		V20 (Pre-flowering)	R1 (Flowering)	R4 (Green cob)	R6 (Seed maturity)
Roots	Mean ±SD	24.2 ± 8.0	46.8 ± 17.1	21.1 ± 7.4	6.8 ± 6.7
	Range	11.4 - 49.0	18.5 - 91.8	8.0 - 38.9	0.8 - 29.0
	n	55	55	55	55
Stems	Mean ±SD	7.1 ± 4.9	18.6 ± 4.6	17.4 ± 3.9	9.9 ± 4.6
	Range	1.4 - 23.1	6.0 - 30.0	9.4 - 24.6	3.0 - 22.3
	n	55	55	55	55
Leaves	Mean ±SD	57.0 ± 18.1	65.4 ± 15.4	36.8 ± 9.7	9.5 ± 4.3
	Range	27.3 - 102.2	36.4 - 100.0	19.9 - 70.8	1.8 - 21.2
	n	55	55	55	55
Silk	Mean ±SD	NA	31.5 ± 13.0	NA	NA
	Range		11.0 - 69.8		
	n		55		
Tassel	Mean ±SD	NA	31.5 ± 7.3	NA	NA
	Range		10.7 - 51.0		
	n		55		
Cob sheath	Mean ±SD	NA	NA	8.6 ± 3.4	1.7 ± 1.0
	Range			4.2 - 20.9	0.6 - 5.9
	n			55	55
Cob	Mean ±SD	NA	NA	2.8 ± 1.4	1.1 ± 0.3
	Range			0.5 - 7.7	0.3 - 1.7
	n			55	55

NA – Not applicable

Table 2.2.: Mean levels of Cry1Ab (µg g^{-1} dry weight) in different tissues over the 2009/2010 growing season for MON810 maize. *The Cry1Ab data is presented as the mean with standard deviation, range and number of samples.*

Plant tissue		Cry1Ab (µg/g dry weight)			
		V20 (Pre-flowering)	R1 (Flowering)	R4 (Green cob)	R6 (Seed maturity)
Roots	Mean ±SD	37.6 ± 13.0	22.5 ± 7.8	16.4 ± 7.1	18.5 ± 8.5
	Range	14.7 - 70.0	6.0 - 37.1	4.1 - 31.7	2.4 - 35.5
	n	55	55	45	55
Stems	Mean ±SD	17.5 ± 5.3	17.1 ± 4.5	14.9 ± 3.9	13.7 ± 4.1
	Range	5.1 - 28.8	7.7 - 28.3	8.3 - 25.4	2.0 - 25.7
	n	55	55	45	55
Leaves	Mean ±SD	55.3 ± 10.1	64.3 ± 12.6	36.7 ± 9.8	12.5 ± 5.7
	Range	38.06 - 80.83	42.6 - 94	18.6 - 64.9	3.3 - 29.0
	n	55	55	45	55
Silk	Mean ±SD	NA	29.5 ± 11.3	NA	NA
	Range		8.6 - 57.8		
	n		55		
Tassel	Mean ±SD	NA	28.2 ± 9.8	NA	NA
	Range		12.2 - 65.1		
	n		55		
Cob sheath	Mean ±SD	NA	NA	10.5 ± 2.3	1.3 ± 0.9
	Range			6.5 - 18.7	0.3 - 4.0
	n			45	55
Cob	Mean ±SD	NA	NA	2.2 ± 1.2	0.7 ± 0.3
	Range			0.5 - 6.2	0.3 - 1.5
	n			45	55

NA – Not applicable

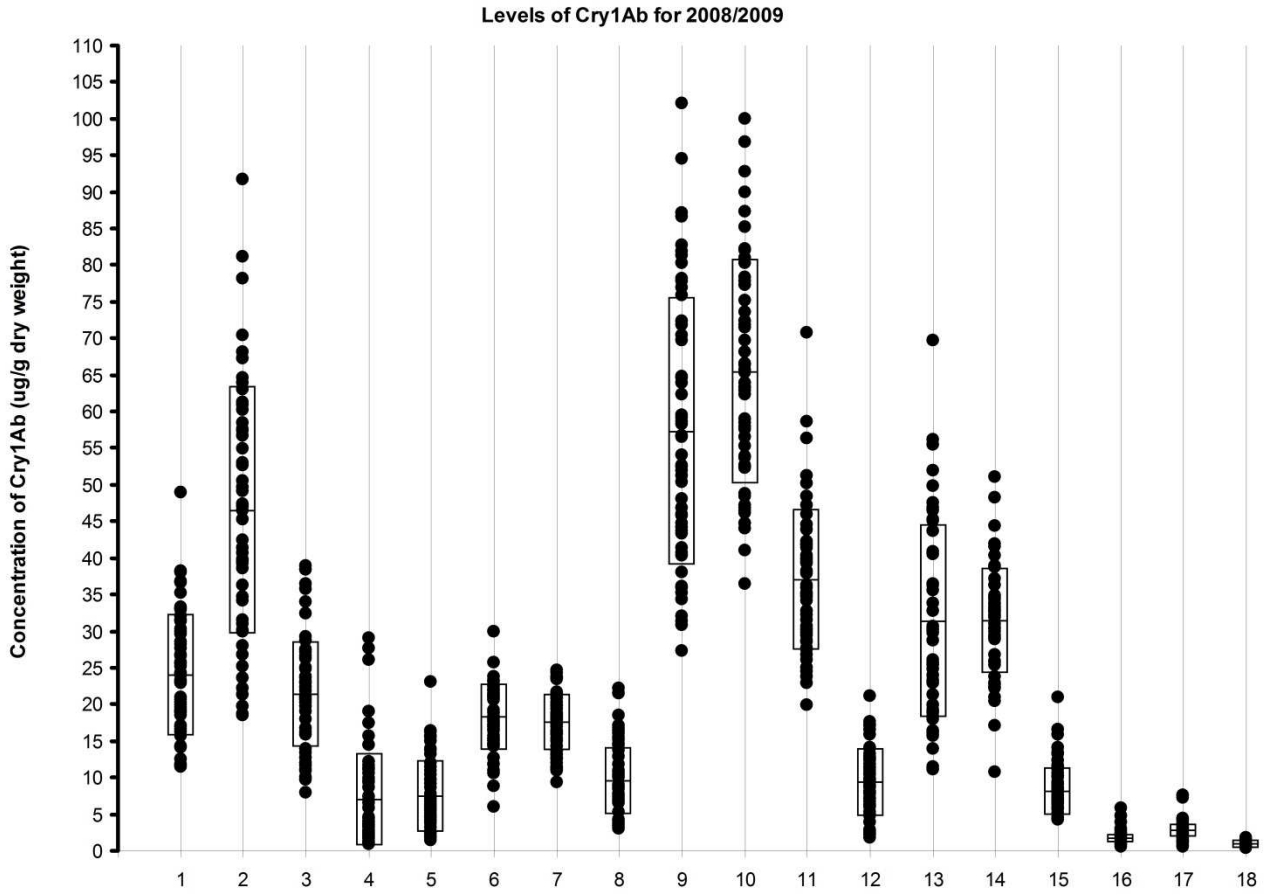

Figure 2.1.: ***Scatter plot of the levels of Cry1Ab (µg g^{-1} dry weight) in different tissues at V20, R1, R4 and R6 growth stages over the 2008/2009 growing season.*** *The standard deviation and mean are indicated by the box and horizontal line. 1 – roots at V20, 2 – roots at R1, 3 – roots at R4, 4 – roots at R6, 5 – stems at V20, 6 – stems at R1, 7 – stems at R4, 8 – stems at R6, 9 – leaves at V20, 10 – leaves at R1, 11 – leaves at R4, 12 – leaves at R6, 13 – silk at R1, 14 – tassel at R1, 15 – cob sheath at R4, 16 – cob sheath at R6, 17 – cob at R4 and 18 – cob at R6.*

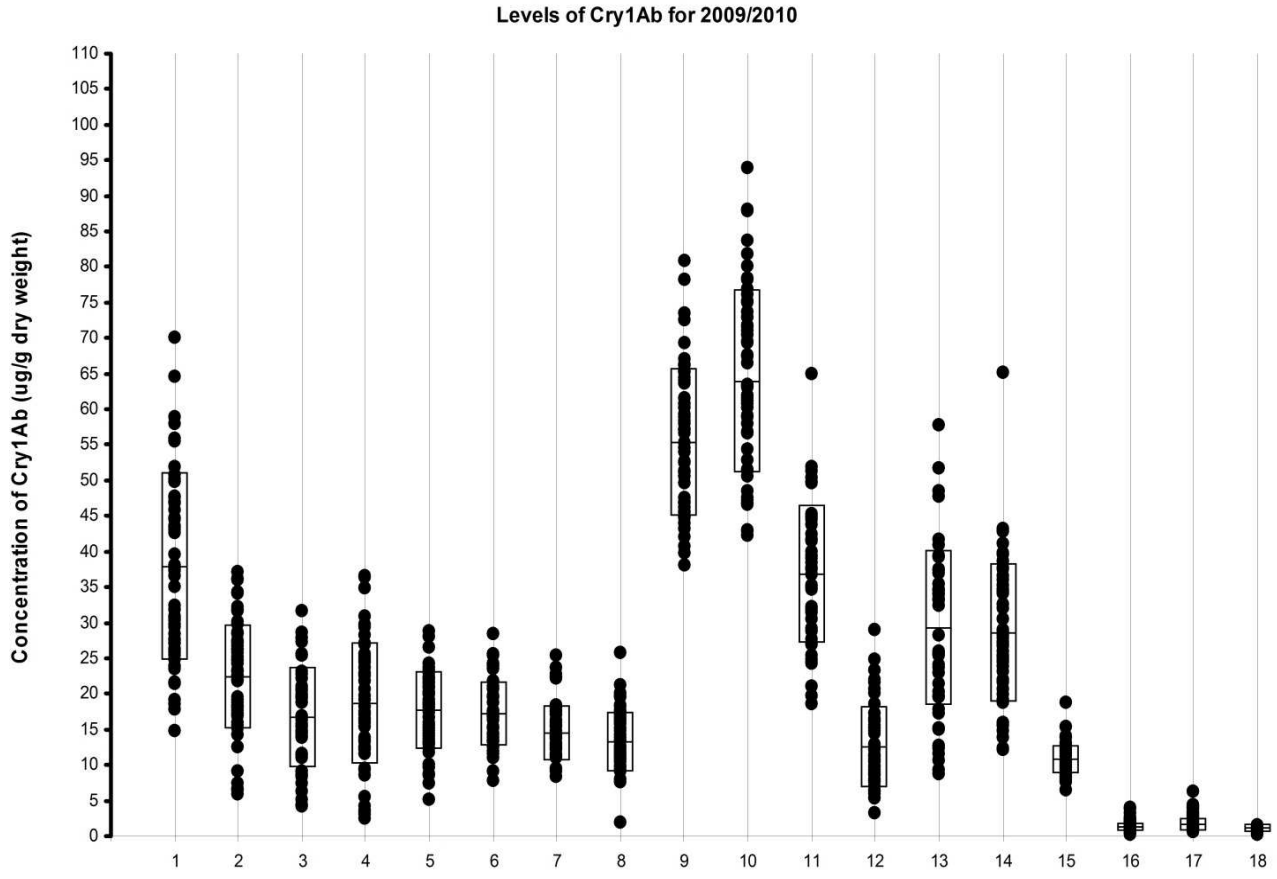

Figure 2.2.: ***Scatter plot of the levels of Cry1Ab (µg g^{-1} dry weight) in different tissues at V20, R1, R4 and R6 growth stages over the 2009/2010 growing season.*** *The standard deviation and mean are indicated by the box and horizontal line. 1 – roots at V20, 2 – roots at R1, 3 – roots at R4, 4 – roots at R6, 5 – stems at V20, 6 – stems at R1, 7 – stems at R4, 8 – stems at R6, 9 – leaves at V20, 10 – leaves at R1, 11 – leaves at R4, 12 – leaves at R6, 13 – silk at R1, 14 – tassel at R1, 15 – cob sheath at R4, 16 – cob sheath at R6, 17 – cob at R4 and 18 – cob at R6.*

Similar to observations by Nguyen and Jehle (2007) significant differences were observed in levels of Cry1Ab over the 2008/2009 and 2009/2010 growing seasons (Table 2.7. amd 2.8.). In the 2008/2009 growing season there was a lower than average rainfall compared to 2009/2010 with a higher than average rainfall (Fig. 2.3.). Thus 2008/2009 can be considered a “dry” season and 2009/2010 a “wet” season. The average temperature and humidity was similar over both growing seasons (Fig. 2.4. and 2.5.). Kamath et al. (2010) reported that in a “wet” season, levels of Cry1Ab were higher in leaves but not in stems. Compared to this, Habuštová *et al*. (2012) found that increased rainfall had an adverse effect on toxin concentration in Bt maize. These data suggest that although increases in rainfall may result in increased toxin production, water logging decreases toxin production (Luo *et al*., 2008). Based on these data, we suggest that the significant increase in Cry1Ab may be attributed to increased rainfall (Table 2.9.).

In the 2008/2009 growing season there was an increase in levels of Cry1Ab up to flowering (R1 stage) followed by a decline up to seed maturity (R6), in roots, stems and leaves. However, this observation was only found in leaves in the 2009/2010 growing season. Compared to this, the highest level of Cry1Ab in cob sheath and cob was observed at green cob stage (R4 stage) in both seasons (Table 2.3. and 2.4.; Fig. 2.1. and 2.2.). Levels of Cry1Ab were similar in silk and tassel over both seasons (Table 2.3. and 2.4.; Fig. 2.1. and 2.2.).

Table 2.3.: Mean levels of Cry1Ab (µg g^{-1} dry weight) in different tissues over the 2008/2009 growing season of MON810 maize, with a 95% CI. *The Cry1Ab data is presented as the mean with standard deviation, range and number of samples.*

Plant tissue		Cry1Ab (µg/g dry weight)			
		V20 (Pre-flowering)	R1 (Flowering)	R4 (Green cob)	R6 (Seed maturity)
Roots	Mean ±SD	24.0 ± 1.0	47.2 ± 2.3	21.3 ± 0.9	6.6 ± 0.7
	Range	22.9 - 25.7	42.5 - 50.6	20.2 - 23.0	5.7 - 7.4
	n	12	10	14	7
Stems	Mean ±SD	6.7 ± 0.6	18.0 ± 0.5	17.9 ± 0.5	9.9 ± 1.0
	Range	5.8 - 7.7	17.7 - 19.2	17.0 - 18.5	8.6 - 11.8
	n	10	9	12	21
Leaves	Mean ±SD	56.0 ± 2.9	64.7 ± 1.9	36.3 ± 1.9	9.6 ± 0.7
	Range	52.4 - 59.4	62.4 - 68.1	34.2 - 39.3	8.6 - 10.6
	n	10	10	12	16
Silk	Mean ±SD	NA	30.5 ± 1.6	NA	NA
	Range		28.7 - 33.7		
	n		10		
Tassel	Mean ±SD	NA	31.9 ± 1.2	NA	NA
	Range		29.9 - 33.3		
	n		17		
Cob sheath	Mean ±SD	NA	NA	8.4 ± 0.5	1.6 ± 0.2
	Range			7.9 - 9.3	1.5 - 1.9
	n			11	10
Cob	Mean ±SD	NA	NA	2.7 ± 0.2	1.1 ± 0.1
	Range			2.4 - 3.0	1.0 - 1.1
	n			12	11

NA – Not applicable

Table 2.4.: Mean levels of Cry1Ab (µg g^{-1} dry weight) in different tissues over the 2009/2010 growing season of MON810 maize, with a 95% CI. *The Cry1Ab data is presented as the mean with standard deviation, range and number of samples.*

Plant tissue		Cry1Ab (µg/g dry weight)			
		V20 (Pre-flowering)	R1 (Flowering)	R4 (Green cob)	R6 (Seed maturity)
Roots	Mean ±SD	37.3 ± 1.5	23.2 ± 1.2	15.5 ± 1.4	17.7 ± 1.5
	Range	35.0 - 39.5	21.8 - 25.2	14.1 - 18.7	16.2 - 20.7
	n	6	13	11	13
Stems	Mean ±SD	17.6 ± 1.1	16.8 ± 0.8	14.7 ± 0.9	13.2 ± 0.8
	Range	15.5 - 19.1	15.3 - 18.7	13.6 - 16.0	12.3 - 14.7
	n	12	15	14	11
Leaves	Mean ±SD	55.8 ± 2.2	63.0 ± 2.6	37.4 ± 1.9	11.9 ± 1.2
	Range	52.4 - 58.4	60.2 - 67.5	34.6 - 39.9	10.5 - 14.3
	n	11	13	13	13
Silk	Mean ±SD	NA	29.3 ± 3.7	NA	NA
	Range		25.6 - 33.4		
	n		9		
Tassel	Mean ±SD	NA	26.9 ± 1.7	NA	NA
	Range		25.0 - 30.4		
	n		13		
Cob sheath	Mean ±SD	NA	NA	10.2 ± 0.5	1.3 ± 0.2
	Range			9.3 - 11.2	1.1 - 1.6
	n			16	12
Cob	Mean ±SD	NA	NA	2.2 ± 0.3	0.6 ± 0.1
	Range			1.8 - 2.5	0.6 - 0.7
	n			8	12

NA – Not applicable

Table 2.5.: The skewness of data in levels of Cry1Ab for each tissue type over the 2008/2009 growing season.

Tissue type	Growth stage V20 to R6	Comment[1]
Roots	0.3 to 1.9	Normal to moderate to highly skewed
Stems	-0.1 to 1.1	Normal to moderate to highly skewed
Leaves	0.3 to 1.0	Normal to moderate to highly skewed
Silk	0.6	Moderately skewed
Tassel	0.02	Normal
Cob sheath	1.5 to 2.2	Highly skewed
Cob	-0.3 to 1.3	Normal to highly skewed

[1] Skewness between -1 and 1 is considered moderately skewed. Skewness <-1 and >1 is considered is highly skewed.

Table 2.6.: The skewness of data in levels of Cry1Ab for each tissue type over the 2009/2010 growing season.

Tissue type	Growth stage V20 to R6	Comment[1]
Roots	-0.3 to 0.4	Normal to moderately skewed
Stems	-0.2 to 0.9	Normal to moderately skewed
Leaves	0.2 to 0.9	Normal to moderate skewed
Silk	0.02	Normal
Tassel	0.9	Moderately skewed
Cob sheath	1.2	Highly skewed
Cob	1.1 to 1.2	Highly skewed

[1] Skewness between -1 and 1 is considered moderately skewed. Skewness <-1 and >1 is considered is highly skewed.

Table 2.7.: Statistical differences (p-value) using Bonferroni-Holm test in levels of Cry1Ab, within and between different tissue at different growth stages for the 2008/2009 growing season (p<0.01).

Plant tissue		Roots				Stems				Leaves				Silk	Tassel	Cob sheath		Cob	
		V20	R1	R4	R6	V20	R1	R4	R6	V20	R1	R4	R6	R1	R1	R4	R6	R4	R6
Roots	V20		1.6E-14*	0.03*	1.6E-22*	4.9E-25*	1.6E-5*	1.2E-7*	1.4E-20*	3.3E-22*	1.9E-33*	2.4E-11*	9.2E-22*	0.0006*	2.8E-6*	1.1E-24*	1.7E-39*	2.0E-37*	9.0E-41*
	R1	1.6E-14*		1.3E-17*	1.3E-30*	1.9E-31*	3.6E-21*	1.5E-22*	3.4E-29*	0.003*	2.7E-8*	0.0003*	1.1E-29*	7.0E-7*	1.6E-8*	7.6E-31*	2.8E-37*	2.4E-36*	7.8E-38*
	R4	0.03	1.3E-17*		1.8E-18*	7.4E-21*	0.04	0.002*	5.3E-16*	3.6E-25*	1.3E-36*	4.0E-16*	3.3E-17*	1.0E-7*	3.6E-11*	3.2E-20*	1.2E-36*	2.5E-34*	4.2E-38*
	R6	1.6E-22*	1.3E-30*	1.8E-18*		0.8	7.5E-19*	1.4E-17*	0.006*	8.8E-37*	4.6E-48*	3.5E-36*	0.01	7.2E-23*	4.2E-35*	0.08	1.6E-7*	2.8E-5*	5.4E-9*
Stems	V20	4.9E-25*	1.9E-31*	7.4E-21*	0.8		4.3E-23*	3.0E-22*	0.002*	1.3E-37*	2.2E-49*	9.0E-39*	0.008*	5.7E-24*	6.5E-39*	0.07	1.8E-12*	7.7E-9*	7.1E-15*
	R1	1.6E-5*	3.6E-21*	0.04	7.5E-19*	4.3E-23*		0.1	6.2E-17*	1.2E-28*	6.7E-41*	5.4E-23*	8.6E-19*	2.8E-10*	2.8E-19*	7.8E-24*	3.9E-49*	1.0E-45*	1.9E-51*
	R4	1.2E-7*	1.5E-22*	0.002*	1.4E-17*	3.0E-22*	0.1		1.4E-15*	6.1E-30*	2.5E-42*	1.2E-25*	1.2E-17*	6.4E-12*	9.2E-23*	2.0E-23*	3.3E-53*	3.4E-49*	4.9E-56*
	R6	1.4E-20*	3.4E-29*	5.3E-16*	0.006*	0.002*	6.2E-17*	1.4E-15*		1.1E-35*	1.2E-47*	1.2E-35*	0.6	7.5E-21*	3.4E-35*	0.1	1.7E-23*	3.4E-19*	3.1E-26*
Leaves	V20	3.3E-22*	0.003*	3.6E-25*	8.8E-37*	1.3E-37*	1.2E-28*	6.1E-30*	1.1E-35*		0.01	5.5E-11*	3.9E-36*	1.3E-13*	2.5E-16*	3.6E-37*	8.8E-43*	5.8E-42*	2.9E-43*
	R1	1.9E-33*	2.7E-8*	1.3E-36*	4.6E-48*	2.2E-49*	6.7E-41*	2.5E-42*	1.2E-47*	0.01		8.8E-21*	3.7E-48*	1.3E-22*	1.5E-27*	2.3E-49*	5.5E-55*	3.4E-54*	1.8E-55*
	R4	2.4E-11*	0.0003*	4.0E-16*	3.5E-36*	9.0E-39*	5.4E-23*	1.2E-25*	1.2E-35*	5.5E-11*	8.8E-21*		1.2E-36*	0.02	0.001*	5.2E-39*	1.3E-49*	3.6E-48*	1.6E-50*
	R6	9.2E-22*	1.1E-29*	3.3E-17*	0.01	0.008*	8.6E-19*	1.2E-17*	0.6	3.9E-36*	3.7E-48*	1.2E-36*		1.5E-21*	1.5E-36*	0.2	4.6E-24*	2.0E-19*	4.6E-27*
Silk	R1	0.0006*	7.0E-7*	1.0E-7*	7.2E-23*	5.7E-24*	2.8E-10*	6.4E-12*	7.5E-21*	1.3E-13*	1.3E-22*	0.02	1.5E-21*		0.98	3.7E-23*	2.6E-32*	5.6E-31*	4.1E-33*
Tassel	R1	2.8E-6*	1.6E-8*	3.6E-11*	4.2E-35*	6.5E-39*	2.8E-19*	9.2E-23*	3.4E-35*	2.5E-16*	1.5E-27*	0.001*	1.5E-36*	0.98		5.4E-52*	7.4E-54*	3.4E-54*	4.7E-55*
Cob sheath	R4	1.1E-24*	7.6E-31*	3.2E-20*	0.08	0.07	7.8E-24*	2.0E-23*	0.1	3.6E-37*	2.3E-49*	5.2E-39*	0.2	3.7E-23*	5.4E-52*		4.3E-27*	3.5E-21*	4.5E-31*
	R6	1.7E-39*	2.8E-37*	1.2E-36*	1.6E-7*	1.8E-12*	3.9E-49*	3.3E-53*	1.7E-23*	8.8E-43*	5.5E-55*	1.3E-49*	4.6E-24*	2.6E-32*	7.4E-54*	4.3E-27*		6.1E-6*	3.1E-5*
Cob	R4	2.0E-37*	2.4E-36*	2.5E-34*	2.8E-5*	7.7E-9*	1.0E-45*	3.4E-49*	3.4E-19*	5.8E-42*	3.4E-54*	3.6E-48*	2.0E-19*	5.6E-31*	3.4E-54*	3.5E-21*	6.1E-6*		8.1E-15*
	R6	9.0E-41*	7.8E-38*	4.2E-38*	5.4E-9*	7.1E-15*	1.9E-51*	4.9E-56*	3.1E-26*	2.9E-43*	1.8E-55*	1.6E-50*	4.6E-27*	4.1E-33*	4.7E-55*	4.5E-31*	3.1E-5*	8.1E-15*	

* Statistically significantly different at p<0.01

Table 2.8.: Statistical differences (p-value) using Bonferroni-Holm test in levels of Cry1Ab, within and between different tissue at different growth stages for the 2009/2010 growing season (p<0.01).

Plant tissue		Roots				Stems				Leaves				Silk	Tassel	Cob sheath		Cob	
		V20	R1	R4	R6	V20	R1	R4	R6	V20	R1	R4	R6	R1	R1	R4	R6	R4	R6
Roots	V20		3.3E-11*	3.5E-16*	4.3E-15*	1.7E-18*	1.9E-19*	1.8E-19*	6.3E-24*	1.6E-12*	2.4E-19*	0.7	3.6E-24*	0.0008*	4.1E-5*	8.6E-25*	2.4E-39*	2.9E-33*	3.9E-40*
	R1	3.3E-11*		0.0001*	0.01	0.0001*	2.4E-5*	4.3E-8*	2.6E-11*	2.5E-36*	6.3E-40*	1.8E-12*	6.6E-12*	0.0003*	8.9E-12*	1.3E-16*	4.3E-38*	1.5E-31*	1.7E-39*
	R4	3.5E-16*	0.0001*		0.2	0.4	0.5	0.2	0.02	2.9E-39*	6.7E-41*	1.1E-18*	0.003*	1.2-E09*	1.0E-9*	1.2E-9*	3.4E-28*	1.4E-22*	8.2E-30*
	R6	4.3E-15*	0.01	0.2		0.4	0.3	0.01	0.0002*	2.2E-39*	1.7E-42*	1.5E-16*	2.7E-5*	7.5E-8*	1.8E-7*	1.5E-8*	4.6E-28*	1.1E-22*	1.8E-29*
Stems	V20	1.7E-18*	0.0001*	0.4	0.4		0.7	0.003*	5.7E-5*	5.7E-46*	1.8E-47*	5.7E-22*	6.2E-6*	1.2E-10*	1.0E-10*	1.0E-12*	7.6E-42*	3.0E-34*	6.6E-44*
	R1	1.9E-19*	2.4E-5*	0.5	0.3	0.7		0.01	4.5E-5*	1.1E-47*	1.0E-48*	1.5E-23*	5.3E6*	1.6E-11*	9.0E-12*	1.2E-14*	6.9E-48*	2.2E-39*	2.8E-50*
	R4	1.8E-19*	4.3E-8*	0.2	0.01	0.003*	0.01		0.1	9.1E-45*	6.4E-45*	6.8E-24*	0.02	7.7E-13*	1.4E-13*	2.7E-9*	1.2E-44*	4.2E-36*	1.9E-47*
	R6	6.3E-24*	2.6E-11*	0.02	0.0002*	5.7E-5*	4.5E-5*	0.1		8.4E-52*	5.3E-52*	8.0E-29*	0.2	1.7E-16*	1.7E-17*	9.2E-6*	1.9E-41*	3.4E-33*	2.6E-44*
Leaves	V20	1.6E-12*	2.5E-36*	2.9E-39*	2.2E-39*	5.7E-46*	1.1E-47*	9.1E-45*	8.4E-52*		5.9E-5*	3.9E-15*	1.9E-50*	7.0E-23*	1.2E-26*	4.6E-50*	5.5E-66*	3.1E-57*	1.1E-66*
	R1	2.4E-19*	6.3E-40*	6.7E-41*	1.7E-42*	1.8E-47*	1.0E-48*	6.4E-45*	5.3E-52*	5.9E-5*		3.7E-21*	3.2E-51*	9.9E-29*	5.4E-32*	4.7E-49*	2.6E-63*	6.0E-55*	7.1E-64*
	R4	0.7	1.8E-12*	1.1E-18*	1.5E-16*	5.7E-22*	1.5E-23*	6.8E-24*	8.0E-29*	3.9E-15*	3.7E-21*		4.6E-28*	0.001*	4.0E-5*	1.9E-30*	8.1E-47*	1.0E-39*	1.1E-47*
	R6	3.6E-24*	6.6E-12*	0.003*	2.7E-5*	6.2E-6*	5.3E6*	0.02	0.2	1.9E-50*	3.2E-51*	4.6E-28*		5.5E-17*	7.9E-18*	0.03	6.7E-27*	7.0E-21*	4.0E-29*
Silk	R1	0.0008*	0.0003*	1.2-E09*	7.5E-8*	1.2E-10*	1.6E-11*	7.7E-13*	1.7E-16*	7.0E-23*	9.9E-29*	0.001*	5.5E-17*		0.5	5.7E-19*	5.3E-35*	3.1E-29*	5.8E-36*
Tassel	R1	4.1E-5*	8.9E-12*	1.0E-9*	1.8E-7*	1.0E-10*	9.0E-12*	1.4E-13*	1.7E-17*	1.2E-26*	5.4E-32*	4.0E-5*	7.9E-18*	0.5		9.0E-21*	1.1E-38*	2.1E-32*	8.8E-40*
Cob sheath	R4	8.6E-25*	1.3E-16*	1.2E-9*	1.5E-8*	1.0E-12*	1.2E-14*	2.7E-9*	9.2E-6*	4.6E-50*	4.7E-49*	1.9E-30*	0.03	5.7E-19*	9.0E-21*		2.4E-47*	1.4E-36*	9.5E-53*
	R6	2.4E-39*	4.3E-38*	3.4E-28*	4.6E-28*	7.6E-42*	6.9E-48*	1.2E-44*	1.9E-41*	5.5E-66*	2.6E-63*	8.1E-47*	6.7E-27*	5.3E-35*	1.1E-38*	2.4E-47*		0.0001*	1.1E-7*
Cob	R4	2.9E-33*	1.5E-31*	1.4E-22*	1.1E-22*	3.0E-34*	2.2E-39*	4.2E-36*	3.4E-33*	3.1E-57*	6.0E-55*	1.0E-39*	7.0E-21*	3.1E-29*	2.1E-32*	1.4E-36*	0.0001*		8.7E-15
	R6	3.9E-40*	1.7E-39*	8.2E-30*	1.8E-29*	6.6E-44*	2.8E-50*	1.9E-47*	2.6E-44*	1.1E-66*	7.1E-64*	1.1E-47*	4.0E-29*	5.8E-36*	8.8E-40*	9.5E-53*	1.1E-7*	8.7E-15*	

* Statistically significantly different at p<0.01

Table 2.9.: Statistical differences (p-value) in levels of Cry1Ab, at different growth stages between the 2008/2009 and 2009/2010 growing season.

Plant tissue	P-value			
	V20	**R1**	**R4**	**R6**
Roots	2.6E-09*	3.7E-16*	0.001*	1.1E-12*
Stems	1.9E-18*	0.09	0.001*	1.2E-05*
Leaves	0.5	0.6	0.9	0.002*
Silk	NA	0.3	NA	NA
Tassel	NA	0.04	NA	NA
Cob sheath	NA	NA	0.001*	0.06
Cob	NA	NA	0.02	3.3E-11*

NA – Not applicable

* Statistically significantly different at p<0.01

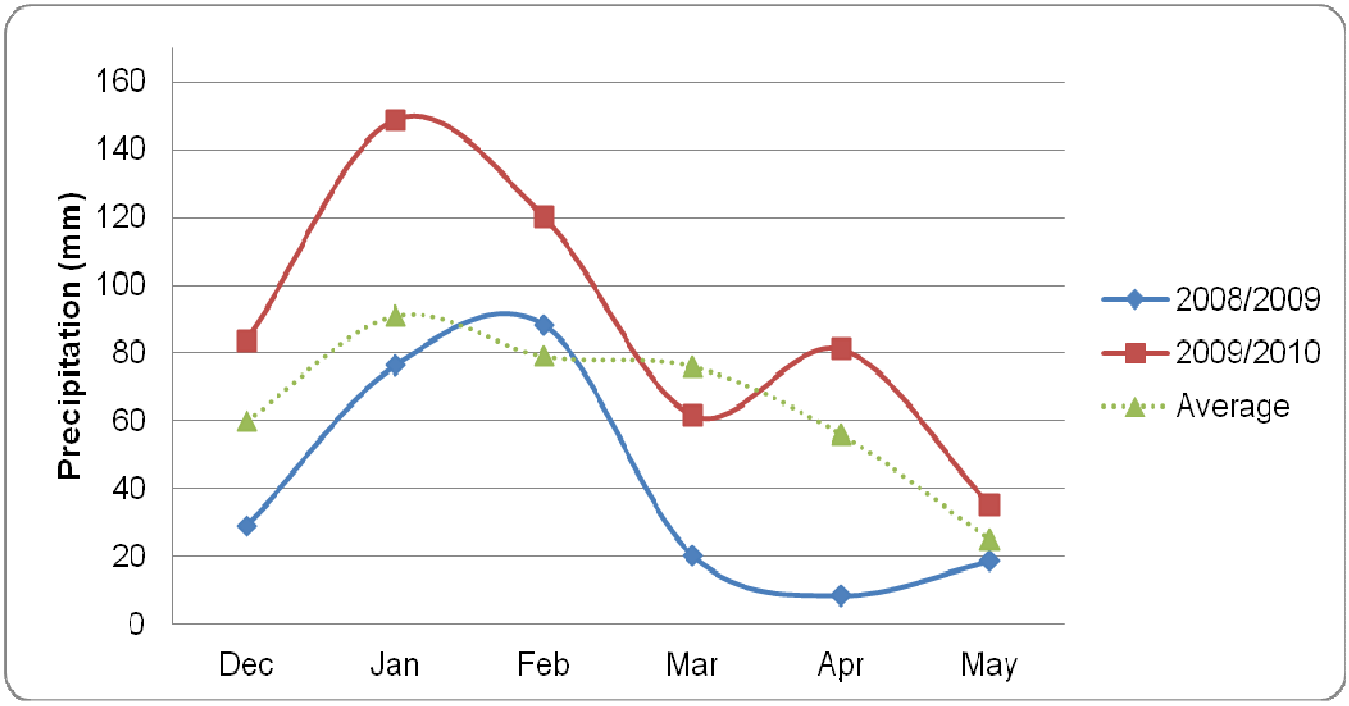

Figure 2.3.: Precipitation (mm) over the 2008/2009 (blue line) and 2009/2010 (red line) growing season for Bloemfontein. *The monthly average over 10 years indicated by the dotted line.*

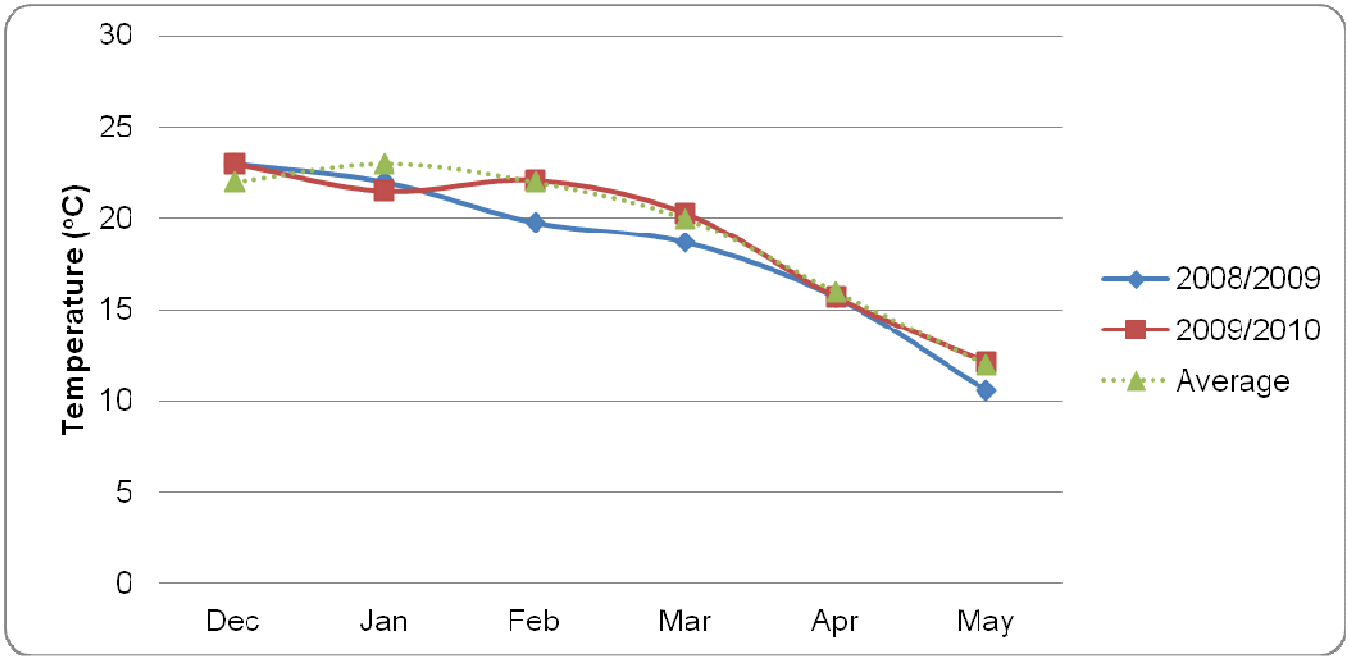

Figure 2.4.: Temperature (°C) over the 2008/2009 (blue line) and 2009/2010 (red line) growing season for Bloemfontein. *The monthly average over 10 years is indicated by the dotted line.*

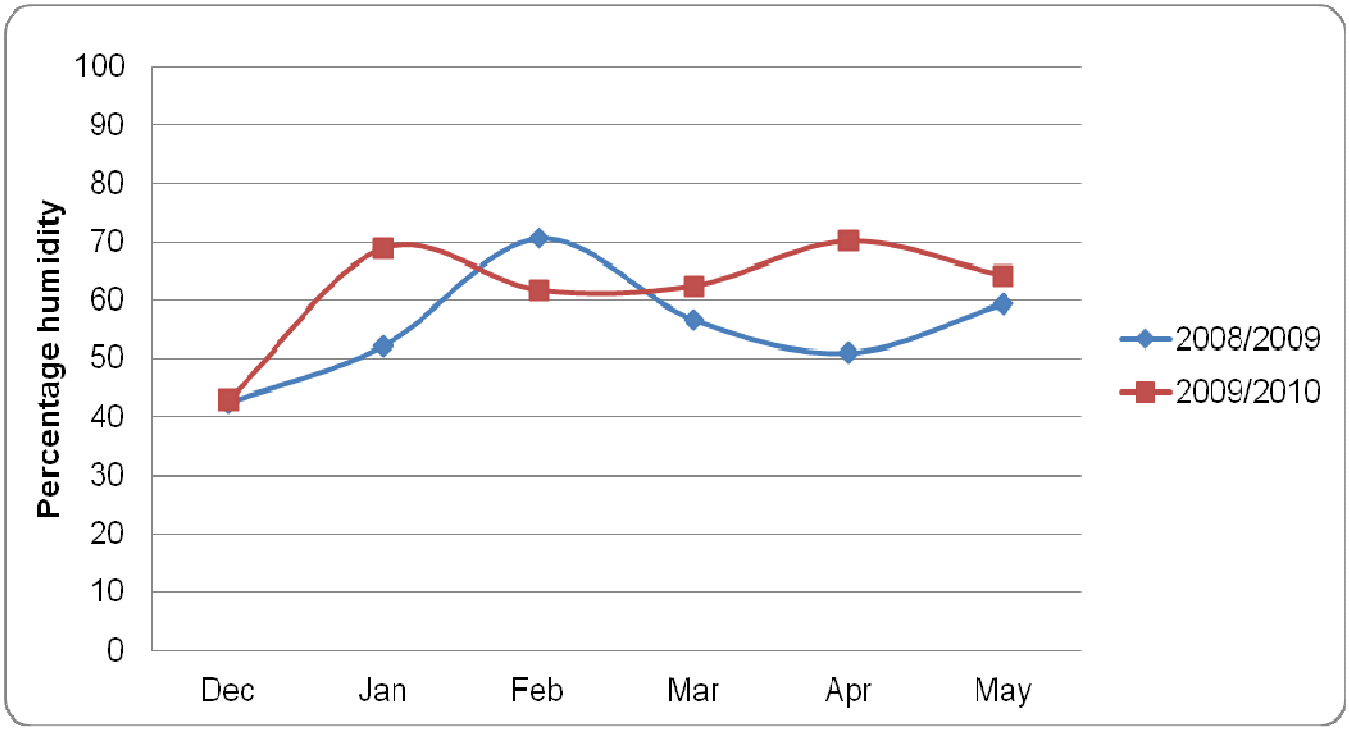

Figure 2.5.: Percentage humidity over the 2008/2009 (blue line) and 2009/2010 (red line) growing season for Bloemfontein.

Similar to the overall published trends, the highest levels of Cry1Ab were found in leaves, followed by silk and tassel, roots, stems, cob sheath and cob (Nguyen and Jehle, 2007; Kamath *et al*., 2010; Szèkács *et al*., 2010a). Studies of Cry1Ac in Bt cotton have shown a similar trend with the highest levels of endotoxin production in vegetative tissue compared to reproductive tissue (Adamczyk *et al*., 2001; Adamczyk and Summerford, 2001; Greenplate *et al*., 1999; Kranthi *et al*., 2005). These data provide evidence for the observation by Van Rensburg (2001) that target insect larvae had a higher rate of survival when feeding on silk and cob sheath compared to leaves and stems. A similar observation has been made for corn earworm (*Helicoverpa zea*) on Cry1Ab maize (Buntin *et al*., 2001; Sims *et al*., 1996). Based on these data we predict that the pattern of target insect larvae survival on MON810 maize follows the trend of levels of Cry1Ab. Meihls *et al*. (2008) found that the rate of survival increased when larvae first fed on non-Bt maize followed by IR maize. The observations by Van Rensburg (2001) suggest a similar trend when insect larvae feed on tissue with a lower level of Cry1Ab and then migrate to tissue with higher levels. This suggests levels of Cry1Ab in certain tissues, including silk, tassel, cob sheath and cob, also taking into consideration the variation in levels of Cry1Ab within a tissue, may be at sub-lethal doses as suggested by Van Rensburg (2001).

Van Rensburg (2007) also noted that during the late growth stages of MON810 maize, the mortality of *B. fusca* decreased. These data support this observation and a decline in levels of Cry1Ab was evident in stems, leaves and cob sheath towards the end of the growing season. Regarding the

decrease in Cry1Ab towards the end of the growing season, a similar observation has been made for Cry1Ac in IR cotton (Adamczyk *et al*., 2001; Adamczyk and Summerford, 2001; Greenplate *et al*., 1999; Kranthi *et al*., 2005).

The decline in levels of Cry1Ab may be explained by the observation of Abel and Adamczyk (2004) that low chlorophyll content appears to adversely affect Cry1Ab production. Thus, it appears that levels of Cry1Ab are determined by the metabolic activity of the maize plant, which is lower towards the end of the growing season.

Several papers have focussed on the possible reasons for the development of resistance in the target insect, *B. fusca*, to IR maize in South Africa (Huang *et al*., 2011; Kruger *et al*., 2009; Van Rensburg, 2007). These studies have determined that the major contributing factor was a lack of compliance by commercial farmers to plant refugia. Based on the current study, we suggest that potential sub-lethal levels of Cry1Ab may also be a contributing factor.

Tabashnik *et al*. (2009) suggested that a pyramid of *cry* genes is expected to delay the evolution of insect resistance to IR maize most effectively. However, it is uncertain how a pyramid of *cry* genes will affect levels of Cry1Ab in different maize tissue. It may be that the metabolic burden of multiple *cry* genes on the cell will result in sub-optimal gene expression and become a contributing factor to resistance development as was the case for a single gene.

2.4. Conclusion

In conclusion, the current study has determined that there is a wide range of levels of Cry1Ab within and between different maize tissues, with a general decline towards the end of the growing season. The production of Cry1Ab appears to be adversely influenced by higher than normal precipitation. Finally, the observations by Van Rensburg (2001) regarding the differential rates of insect larvae survival when feeding on different MON810 maize tissue are supported by the data from the current study and provide an important basis to understand the potential role of levels of Cry1Ab in the development of insect resistance to MON810 maize in South Africa.

Chapter 3

Impact of gene flow on Cry1Ab expression

3.1. Introduction

The introduction of IR maize in developing countries has the potential to improve crop production. Especially since the majority of farmers in Africa are resource poor and do not have access to insecticides to control target pests (Keetch *et al*., 2005; Mugo *et al*., 2005). The advantage of IR maize is that it can provide protection against stem borer damage in the absence of insecticides (Gouse *et al*., 2005; Park *et al*., 2011). Thus, IR technology can contribute to sustainable food production in developing countries (Raybould and Quemada, 2010).

Unlike commercial farming in developed countries, the majority of farmers in Africa are communal or subsistence farmers (Aliber and Hart, 2009). While South Africa has a large commercial farming sector, it is estimated that there are up to 3 million communal or subsistence farmers in South Africa alone. Communal or subsistence farm plots range from 100 m^2 to 4,550 m^2 (Aliber and Hart, 2009). These farmers practice seed saving, based on phenotypic selection, and exchange seed among themselves (Gouse *et al*., 2005). Furthermore, due to the small plot size and farming practices, these farmers generally do not comply with regulatory requirement to plant refugia when planting IR maize (Gouse *et al*., 2005).

Chilcutt and Tabashnik (2004) suggested that gene flow from IR to non-IR maize refugia could accelerate pest resistance development by either killing susceptible larvae in the refugia, or by selecting for heterozygotes. Furthermore, Krupte *et al*. (2009) suggested that gene flow could result in F1 IR plants with decreased fitness and that were less effective at controlling insect pests. Aheto *et al*. (2011) suggested that based on modelling data, the combination of small plots, typical of communal and subsistence farming, and heterogeneity of seed sources would result in increased gene flow. Thus, gene flow from IR to non-IR maize combined with the practice of seed saving is likely to increase selective pressure for resistance alleles in the target insect. The aim of this study was to evaluate the effects of gene flow from a commercial MON810 IR hybrid to a non-IR commercial hybrid on the levels of Cry1Ab in the F1 generation.

3.2. Materials and methods

3.2.1. Field trial layout

A MON810 converted yellow commercial maize variety (PAN6Q-321 B) and a white non-IR commercial maize variety (PAN6Q-321) was grown at the University of the Free State experimental farm, outside Bloemfontein in the Free State, during the 2008/2009 growing season. The trial comprised of a 4 hectare plot of yellow MON810 maize adjacent to a 4 hectare plot of white non-Bt maize, cultivated under conventional farming practice for the region,

without the application of insecticides (Fig. 3.1.). A three week temporal isolation to other maize planted in adjacent plots was employed to prevent cross pollination. Cobs from the first two rows of the non-IR maize adjacent to the MON810 plot were collected at the end of the 2008/2009 growing season based on the presence of the yellow phenotype. The yellow kernels were separated and approximately 800 F1 seed was planted at the University of the Free State experimental farm in the 2009/2010 growing season. Approximately 800 F1 plants were screened using the Envirologix Cry1Ab/Cry1Ac QuantiPlate® 96-well microplate ELISA kit (as described in paragraph 2.2.3. in Chapter 2), to confirm the presence of Cry1Ab.

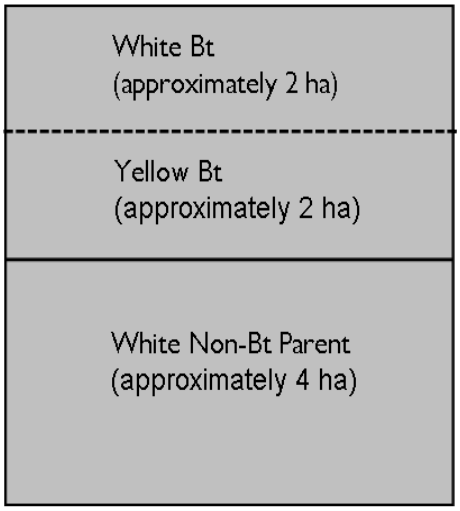

Figure 3.1.: Field trial layout of the gene flow experiment. *A 2 hectare plot of yellow MON810 maize was planted adjacent to a 4 hectare plot of white non-Bt maize. The yellow kernels that were collected from the cobs of first two rows of white non-IR maize adjacent to the yellow MON810 maize were planted next to the field trial.*

3.2.2. Collection and storage of plant material

The F1 maize plants were collected at one vegetative (V) and three reproductive (R) stages, namely pre-flowering (V20 stage) (28 plants), flowering (R1 stage) (30 plants), green cob or dough stage (R4 stage) (22 plants) and seed maturity (R6 stage) (23 plants), according to plant availability (as described in paragraph 2.2.2. in Chapter 2).

3.2.3. Quantification of Cry1Ab using ELISA

Levels of Cry1Ab were quantified using the commercial Envirologix Cry1Ab/Cry1Ac QuantiPlate® 96-well microplate ELISA kit (as described in paragraph 2.2.3. in Chapter 2).

3.2.4. Data analysis

The mean, standard deviation and range for each tissue type was determined using Excel 2007 (Windows XP). ANOVA and the Bonferroni-Holm test (Daniel's XL Toolbox, version 2.60) were used to compare levels of Cry1Ab between growth stages, tissue types and MON810 (white PAN6Q-321) maize grown in the same season as the F1 plants. The data distribution and skewness was determined using EasyFit 5.5 Professional. A 95% confidence interval (CI) to exclude all outlying data points was performed using Excel 2007 (Windows XP). Statistical significance was set at $p<0.01$ for all tests performed. The reduction in levels of Cry1Ab in F1 plants compared to the

MON810 hybrid was calculated by dividing the mean of the level of Cry1Ab in different tissue at each growth stage in the MON810 hybrid by the corresponding mean level of Cry1Ab in the F1 plants.

3.3. Results and discussion

As expected, the levels of Cry1Ab in F1 plants were significantly lower than the commercial MON810 hybrid (Table 3.1. and 3.2.; Fig 3.2.). The highest average reduction in Cry1Ab levels in F1 plants compared to the MON810 hybrid was -4.3x in silk, -4.2x in stems, -3.2x in roots, -2.3x in tassel, -2.2x in leaves, -1.9x in cob and -1.8 in cob sheath (Table 3.3.). The levels of Cry1Ab in and between tissues over the growing season were still significantly different (Table 3.4. and 3.5.). The F1 plants had observed decreased fitness, were generally stunted and had reduced ear production compared to the commercial MON810 hybrid. These data support the suggestion of Krupte *et al*. (2009) that IR plants with reduced fitness were less effective at controlling insect pests due to lower levels of Cry1Ab. In addition, almost all the F1 plants had visible *B. fusca* damage either in the stems or cob sheath and cob (Table 3.7.). The effect of segregation on Cry1Ab expression is typical for maize production practice in subsistence farming, where farmers save and share seed. Thus, while IR technology can benefit subsistence farmers, it is expected that subsistence farming practice will result in the exposure of the target insect to a sub-lethal dose of Cry1Ab that in turn will promote resistance development.

Table 3.1.: Mean levels of Cry1Ab (µg g^{-1} dry weight) in different tissues over the 2009/2010 growing season for F1 plants. *The Cry1Ab data is presented as the mean with standard deviation, range and number of samples.*

Plant tissue		Cry1Ab (µg/g dry weight)			
		V20 (Pre-flowering)	R1 (Flowering)	R4 (Green cob)	R6 (Seed maturity)
Roots	Mean ±SD	7.5 ± 3.0	7.8 ± 2.4	3.7 ± 2.0	6.6 ± 3.2
	Range	1.1 - 13.6	3.1 - 12.3	1.1 - 8.6	0.5 - 12.2
	n	28	30	22	23
Stems	Mean ±SD	1.7 ± 1.9	4.1 ± 1.3	4.6 ± 1.8	6.6 ± 2.0
	Range	0.5 - 10.8	1.7 - 7.7	0.3 - 8.4	2.7 - 10.3
	n	28	30	22	23
Leaves	Mean ±SD	30.2 ± 13.3	19.1 ± 10.4	25.8 ± 8.1	5.1 ± 2.4
	Range	11.3 - 67.2	5.9 - 42.3	13.8 - 44.0	1.7 - 10.0
	n	28	30	22	23
Silk	Mean ±SD	NA	6.9 ± 6.5	NA	NA
	Range		0.7 - 27.8		
	n		30		
Tassel	Mean ±SD	NA	12.8 ± 2.7	NA	NA
	Range		6.7 - 17.2		
	n		30		
Cob sheath	Mean ±SD	NA	NA	3.8 ± 1.8	1.4 ± 0.9
	Range			1.7 - 9.1	0.5 - 3.2
	n			22	8
Cob	Mean ±SD	NA	NA	1.7 ± 0.7	0.4 ± 0.2
	Range			0.6 - 3.1	0.2 - 0.7
	n			22	8

NA – Not applicable

Table 3.2.: Mean levels of Cry1Ab (µg g^{-1} dry weight) in different tissues over the 2009/2010 growing season for F1 plants, with a 95% CI. *The Cry1Ab data is presented as the mean with standard deviation, range and number of samples.*

Plant tissue		Cry1Ab (µg/g dry weight)			
		V20 (Pre-flowering)	R1 (Flowering)	R4 (Green cob)	R6 (Seed maturity)
Roots	Mean ±SD	7.8 ± 0.6	8.0 ± 0.6	3.7 ± 0.5	5.9 ± 0.4
	Range	6.8 - 8.7	7.3 - 8.7	3.1 - 4.2	5.4 - 6.8
	n	8	8	5	9
Stems	Mean ±SD	1.5 ± 0.4	4.0 ± 0.3	6.7 ± 0.5	6.5 ± 0.5
	Range	1.0 - 2.4	3.8 - 4.4	4.0 - 5.4	6.1 - 7.3
	n	15	5	9	6
Leaves	Mean ±SD	29.6 ± 2.5	18.9 ± 2.2	26.2 ± 0.7	4.8 ± 0.6
	Range	26.1 - 31.7	16.2 - 22.3	25.3 - 26.9	4.3 - 5.9
	n	7	7	4	6
Silk	Mean ±SD	NA	6.5 ± 1.8	NA	NA
	Range		4.5 - 9.1		
	n		9		
Tassel	Mean ±SD	NA	12.6 ± 0.4	NA	NA
	Range		11.9 - 13.4		
	n		10		
Cob sheath	Mean ±SD	NA	NA	3.8 ± 0.4	1.3 ± 0.5
	Range			3.1 - 4.1	0.8 - 1.8
	n			7	5
Cob	Mean ±SD	NA	NA	1.6 ± 0.2	0.4 ± 0.1
	Range			1.4 - 1.9	0.3 - 0.5
	n			5	4

NA – Not applicable

Table 3.3.: The skewness of data for levels of Cry1Ab in tissue type over the 2009/2010 growing season for F1 plants. *The data from most sampling points was moderately to highly skew.*

Tissue type	Growth stage V20 to R6	Comment[1]
Roots	-0.07 to 0.7	Normal to moderately skewed
Stems	-0.5 to 4.2	Normal to moderate to highly skewed
Leaves	0.6 to 1.0	Moderate to highly skewed
Silk	1.7	Highly skewed
Tassel	-0.1	Normal
Cob sheath	1.3 to 1.4	Highly skewed
Cob	0.5 to 0.6	Moderately skewed

[1] Skewness between -1 and 1 is considered moderately skewed. Skewness <-1 and >1 is considered is highly skewed.

Table 3.4.: Statistical differences (p-value) using Bonferroni-Holm test in levels of Cry1Ab, within and between different tissue at different growth stages for the 2009/2010 growing season for F1 plants (p<0.01).

Plant tissue		Roots				Stems				Leaves				Silk	Tassel	Cob sheath		Cob	
		V20	R1	R4	R6	V20	R1	R4	R6	V20	R1	R4	R6	R1	R1	R4	R6	R4	R6
Roots	V20		0.7	3.7E-6*	0.3	7.6E-12*	2.9E-7*	0.0002*	0.2	4.9E-12*	4.9E-7*	8.5E-15*	0.002*	0.6	2.6E-9*	5.0E-6*	1.8E-6*	4.9E-12*	1.1E-7*
	R1	0.7		4.1E-8*	0.1	8.6E-15*	7.5E-10*	5.2E-6*	0.06	1.3E-12*	2.7E-7*	1.0E-15*	0.0002*	0.5	3.3E-10*	4.8E-8*	1.5E-8*	1.3E-15*	4.4E-10*
	R4	3.7E-6*	4.1E-8*		0.0007*	0.0009*	0.4	0.1	1.9E-5*	2.9E-12*	9.7E-9*	1.1E-15*	0.04	0.02	3.8E-18*	0.8	0.003*	3.6E-5*	7.3E-5*
	R6	0.3	0.1	0.0007*		1.5E-8*	0.0002*	0.02	0.9	6.1E-11*	9.2E-7*	1.5E-13*	0.07	0.8	4.2E-10*	0.0009*	8.1E-5*	6.7E-9*	6.9E-6*
Stems	V20	7.6E-12*	8.6E-15*	0.0009*	1.5E-8*		1.3E-6*	1.7E-6*	1.5E-11*	9.4E-16*	5.0E-12*	5.0E-20*	1.0E-6*	0.0001*	8.8E-25*	0.002*	0.6	0.9	0.07
	R1	2.9E-7*	7.5E-10*	0.4	0.0002*	1.3E-6*		0.2	1.6E-6*	3.3E-15*	9.5E-11*	1.5E-19*	0.05	0.02	8.4E-23*	0.6	3.9E-6*	2.7E-10*	3.9E-9*
	R4	0.0002*	5.2E-6*	0.1	0.02	1.7E-6*	0.2		0.002*	8.4E-12*	4.2E-8*	4.0E-15*	0.5	0.1	1.1E-16*	0.2	4.1E-5*	6.2E-9*	4.9E-7*
	R6	0.2	0.06	1.9E-5*	0.9	1.5E-11*	1.6E-6*	0.002		4.0E-11*	5.8E-7*	3.7E-14*	0.03	0.8	1.7E-12*	0.06	1.1E-7*	6.9E-14*	2.3E-9*
Leaves	V20	4.9E-12*	1.3E-12*	2.9E-12*	6.1E-11*	9.4E-16*	3.3E-15*	8.4E-12*	4.0E-11*		0.0008*	0.2	7.2E-12*	8.8E-12*	3.2E-9*	3.3E-12*	6.8E-7*	2.1E-13*	3.7E-7*
	R1	4.9E-7*	2.7E-7*	9.7E-9*	9.2E-7*	5.0E-12*	9.5E-11*	4.2E-8*	5.8E-7*	0.0008*		0.01	5.9E-8*	1.0E-6*	0.002*	1.2E-8*	3.6E-9*	2.7E-10*	1.3E-5*
	R4	8.5E-15*	1.0E-15*	1.1E-15*	1.5E-13*	5.0E-20*	1.5E-19*	4.0E-15*	3.7E-14*	0.2	0.01		5.0E-15*	1.5E-12*	7.9E-11*	1.2E-15*	3.6E-9*	2.3E-17*	1.6E-9*
	R6	0.002*	0.0002*	0.04	0.07	1.0E-6*	0.05	0.5	0.03	7.2E-12*	5.9E-8*	5.0E-15*		0.2	7.9E-15*	0.06	0.0002*	6.2E-8*	6.7E-6*
Silk	R1	0.6	0.5	0.02	0.8	0.0001*	0.02	0.1	0.8	8.8E-12*	1.0E-6*	1.5E-12*	0.2		2.3E-5*	0.04	0.02	0.000*4	0.008*
Tassel	R1	2.6E-9*	3.3E-10*	3.8E-18*	4.2E-10*	8.8E-25*	8.4E-23*	1.1E-16*	1.7E-12*	3.2E-9*	0.002*	7.9E-11*	7.9E-15*	2.3E-5*		2.5E-18*	8.4E-14*	2.2E-24*	5.8E-15*
Cob sheath	R4	5.0E-6*	4.8E-8*	0.8	0.0009*	0.002*	0.6	0.2	0.06	3.3E-12*	1.2E-8*	1.2E-15*	0.06	0.04	2.5E-18*		0.0007*	2.1E-6*	8.3E-6*
	R6	1.8E-6*	1.5E-8*	0.003*	8.1E-5*	0.6	3.9E-6*	4.1E-5*	1.1E-7*	6.8E-7*	2.9E-5*	3.6E-9*	0.0002*	0.02	8.4E-14*	0.0007*		0.3	0.01
Cob	R4	4.9E-12*	1.3E-15*	3.6E-5*	6.7E-9*	0.9	2.7E-10*	6.2E-9*	6.9E-14*	2.1E-13*	2.7E-10*	2.3E-17*	6.2E-8*	0.0004*	2.2E-24*	2.1E-6*	0.3*		2.0E-5*
	R6	1.1E-7*	4.4E-10*	7.3E-5*	6.9E-6*	0.07	3.9E-9*	4.9E-7*	2.3E-9*	3.7E-7*	1.3E-5*	1.6E-9*	6.7E-6*	0.008*	5.8E-15*	8.3E-6*	0.01	2.0E-5*	

* Statistically significantly different at p<0.01

Table 3.5.: Statistical differences (p-value) in levels of Cry1Ab, between F1 plants for 2009/2010 growing season and a commercial MON810 maize hybrid for the 2008/2009 and 2009/2010 growing season. *Statistically significantly different at p<0.01.*

Plant tissue	V20 (Pre-flowering)	R1 (Flowering)	R4 (Green cob)	R6 (Seed maturity)
Roots	3.4E-17*	2.2E-13*	7.7E-16*	0.004*
Stems	5.8E-12*	7.2E-34*	4.8E-25*	9.7E-7*
Leaves	2.4E-14*	6.9E-35*	2.5E-6*	5.6E-7*
Silk	NA	1.4E-18*	NA	NA
Tassel	NA	2.9E-19*	NA	NA
Cob sheath	NA	NA	4.0E-13*	0.7
Cob	NA	NA	0.004*	0.0009*

NA – Not applicable

* Statistically significantly different at p<0.01

Table 3.6.: The reduction in mean levels of Cry1Ab between F1 plants and a commercial MON810 maize hybrid.
A negative value indicates that the mean level of Cry1Ab in the F1 plants was lower than the commercial MON810 maize hybrid.

Plant tissue	V20 (Pre-flowering)	R1 (Flowering)	R4 (Green cob)	R6 (Seed maturity)
Roots	-4.1	-4.4	-5.1	-1.9
Stems	-7.2	-4.4	-3.5	-1.8
Leaves	-1.9	-3.4	-1.4	-2.2
Silk	NA	-4.3	NA	NA
Tassel	NA	-2.3	NA	NA
Cob sheath	NA	NA	-2.5	-1.1
Cob	NA	NA	-1.5	-2.3

NA – Not applicable

Table 3.7.: ***The number of F1 plants that had observable B. fusca damage in relation to total number of plants sampled.***

Description	**V20**	**R1**	**R4**	**R6**
Plants with observable *B. fusca* damage	3	3	12	20
Total number of plants	28	30	22	23

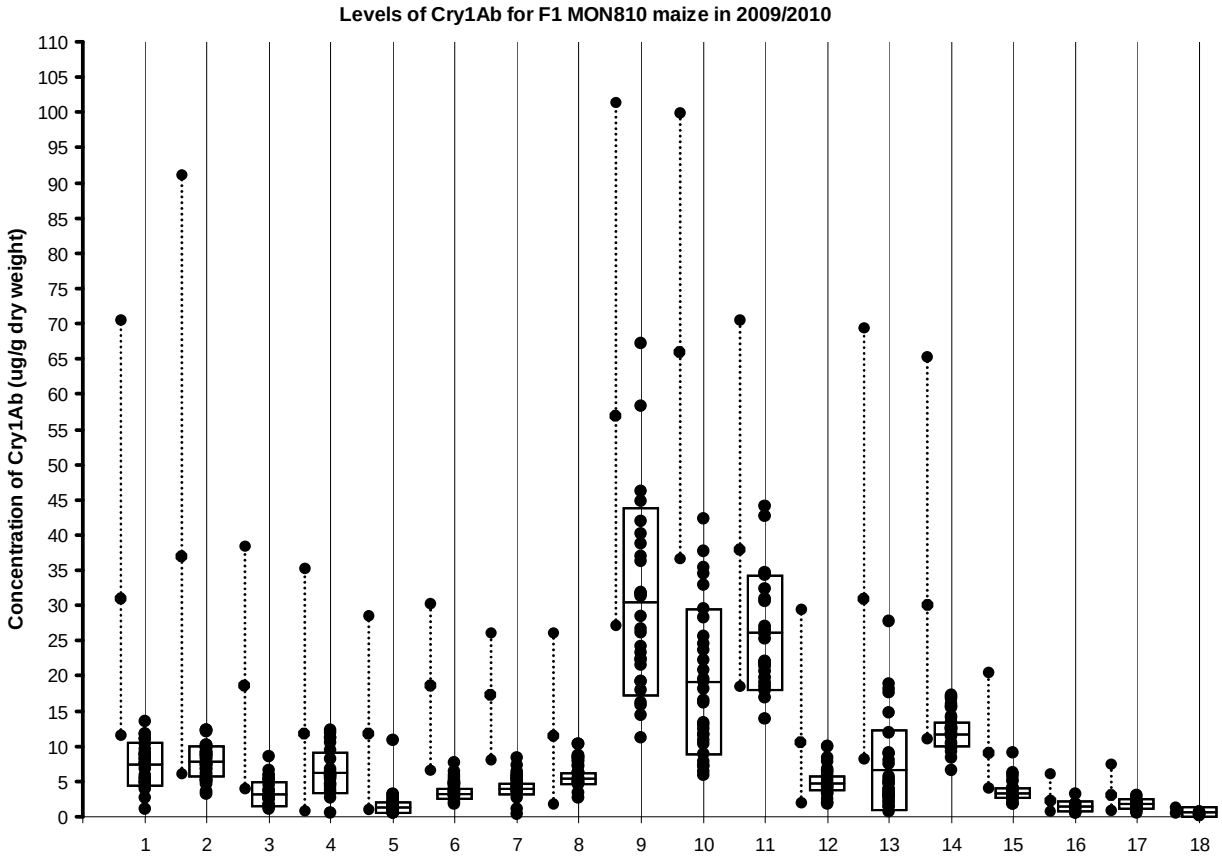

Figure 3.2.: ***Scatter plot of the levels of Cry1Ab (µg g^{-1} dry weight) in different tissues at V20, R1, R4 and R6 growth stages over the 2009/2010 growing season in F1 plants.*** *The standard deviation and mean is indicated by the box and horizontal line. The dotted line indicates the combined range and mean in levels of Cry1Ab in the commercial MON810 maize hybrid reported in Chapter 2. 1 – roots at V20, 2 – roots at R1, 3 – roots at R4, 4 – roots at R6, 5 – stems at V20, 6 – stems at R1, 7 – stems at R4, 8 – stems at R6, 9 – leaves at V20, 10 – leaves at R1, 11 – leaves at R4, 12 – leaves at R6, 13 – silk at R1, 14 – tassel at R1, 15 – cob sheath at R4, 16 – cob sheath at R6, 17 – cob at R4 and 18 – cob at R6.*

3.4. Conclusion

It has been suggested that IR maize can contribute towards improving agriculture and sustainable food production in Africa (Raybould and Quemada, 2010). In no way do the results from the current study disprove this, nor do they intend to. However, from these data it is evident that the agricultural practice of saving and exchanging seed by communal and subsistence farmers may have a detrimental effect on the levels of Cry1Ab in saved seed. Due to the practice of saving seed, the levels of Cry1Ab in IR maize will become diluted and the requirement for the 'high dose' will not be met. Thus, the effects of gene flow may counter the positive benefits of IR technology in an African subsistence and communal farming environment. Therefore, without considerable farmer education and extensive support to discourage traditional seed practices, resistance to Cry1Ab is likely to evolve more quickly compared to non-compliance to refugia. Finally, the incorrect management of IR technology by subsistence and communal farmers may jeopardise the use of pyramid *cry* genes for commercial farmers in South Africa in the medium to long term.

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Summary

In 2007, the first case of field resistance to Cry1Ab was reported in South Africa, which is a concern as it negates the benefit of this technology. It has been suggested that a major contributing factor to the development of resistance in the target insect was the lack of compliance by commercial farmers to plant refugia. However, another possible mechanism of resistance development is the production of sub-lethal doses of Cry1Ab that could have resulted in a high selective pressure for resistance alleles.

Although there are studies that have determined levels of Cry1Ab in different tissue in MON810 maize, the available data is not complete, especially for important feeding tissue of *B. fusca* larvae, such as silk and cob sheath. In this study, a comprehensive analysis of levels of Cry1Ab within and between different tissue over the growing season was conducted, taking the effect of gene flow also into account. Field trials were performed over the 2008/2009 and 2009/2010 growing seasons under conventional farming practice. Gene flow was allowed to occur between IR and non-IR maize in the 2008/2009 growing season, and the F1 seed was planted in the 2009/2010 growing season. The levels of Cry1Ab were monitored over both growing seasons, including the F1 plants in the second season. Notably, this study was the first to determine levels of Cry1Ab in cob sheath, which is considered one of the primary food sources for *B. fusca* larvae.

It was found that there was considerable variation in levels of Cry1Ab within and between different tissue over the growing season. The data for the majority of the sampling points was moderately to highly skewed, indicating the non-parametric range in variation of Cry1Ab levels. There was a significant difference in Cry1Ab production between the two growing seasons, which was attributed to the lower than average rainfall in the 2008/2009 growing season and a higher than average rainfall in the 2009/2010 growing season. The overall trend in Cry1Ab production was congruent with the pattern of target insect larval survival after feeding on different tissue as reported by Van Rensburg (2009). Based on these data we suggest that important insect feeding tissue, namely silk, cob sheath and cob, could be producing sub-lethal doses of Cry1Ab that may result in ineffective control of insect pests. It appears that the decline in Cry1Ab production at late growth stages, in conjunction with variable levels of Cry1Ab between different tissue, may compromise the high dose/refugia strategy, resulting in selective pressure for the evolution of resistance.

The gene flow study determined that outcrossing between IR and non-IR maize adversely affects the level of Cry1Ab in F1 plants. The levels of Cry1Ab were significantly lower in F1 maize when compared to a commercial MON810 maize hybrid, possibly as a result of reduced fitness. These data support the observation of increased insect larvae damage to F1 plants, suggesting that F1 maize may produce sub-lethal doses of endotoxin, and consequently will not effectively control insect pests. The considerably lower expression of Cry1Ab in F1 plants is a consideration in respect to subsistence

farming practice in Africa, where seed is saved or exchanged among farmers. We postulate that the introduction of IR maize in subsistence farming could promote the development of insect resistance if not managed correctly.

In conclusion, the current study has determined that there is a wide range of level of Cry1Ab within and between different tissue over the growing season. Gene flow adversely affects Cry1Ab production, potentially due to reduced fitness of the F1 plants. These data support the observation of differential rates of larvae survival when feeding on different IR maize tissue. Finally, the study provides an important basis for understanding the potential role that variable levels of Cry1Ab may have had on the development of resistance in *B. fusca* in South Africa.

Keywords

Bt maize

Cry1Ab expression

Gene flow

GM

Insect resistance development

Opsomming

Die eerste geval van veld weerstand in insekte teen Bt was in 2007 gerapporteer. Hierdie verskynsel was van uiters belang omdat dit die gebruik van Bt tegnologie tot niet kan maak. Studies het aangetoon dat die bydraende faktor teenoor die ontwikkeling van weerstand was die gebrek aan die korrekte bestuur in terme van die plant van nie-Bt deur kommersiele boere. Ten spyte hiervan, is dit ook moontlik dat die produksie van sub-letale vlakke van Bt ook bygedra het tot die ontwikkeling van weerstand.

Alhoewel verskeie studies die vlakke van Bt in verskillende weefsels aangetoon het, is die beskikbare data nie volledig nie, ten opsigte van belangrike insek voedings weefsel soos die baard en stonkskede. Hierdie studie het ‘n volledige analise van die vlakke van Bt in verskillende weefsel oor die groei seisoen en tussen groei seisoene behels. Die effek van geenvloei is ook in ag geneem. Veld proewe is geloods tydens die 2008/2009 en 2009/2010 groei seisoene onder normale landboupraktyk. Geen vloei is toegelaat tussen Bt en nie-Bt mielies tydens 2008/2009 en die F1 saad is geplant tydens 2009/2010. Die vlakke van Bt is bepaal oor twee groei seisoene asook vir F1 plante. Hierdie is die eerste studie wat Bt vlakke in baard en stronkskede van mielies bepaal, beide belangrike bronne van voeding vir *B. Fusca* larvas.

Dit is gevind dat daar aansienlike variasie in vlakke van Bt tussen weefsel was oor die groei seisoen. Die data vir die meeste monsters was oneweredig

versprei. Daar was ook betekenisvolle verskille tussen die twee groei seisoene waarskynlik as gevolg van die verskil in reënval. Die algemene tendens in Cry1Ab produksie was ooreenstemmend met die patroon van teiken insek oorlewing na voeding op verskillende weefsel. Gebaseer op die data, wil dit voorkom of die baard, stronk en stronkskede van mielies sub-letale vlakke van Bt bevat wat onvoldoende beheer van insekte veroorsaak. Dit wil ook voorkom dat Bt vlakke aan die einde van die groei seisoen afneem en dat dit die oorlewing van insek lavas bevoordeel en weerstand te weeg bring.

Die geen vloei studie het bepaal dat uit-kruising tussen Bt en nie-Bt mielies ‘n newe effek het op die vlakke van Bt in die F1 plante. Die vlakke van Bt was betekenisvol laer in F1 plante in vergelyking met ‘n kommersiele Bt mielie hibried, moontlik as gevolg van lewensvatbaarheid. Hierdie data ondersteun die opmerking van toenemende insek skade in F1 plante as gevolg van sub-letale vlakke van endotoksien wat veroorsaak dat daar nie genoegsame beskerming gebied word nie. Die aansienlike laer vlakke van Bt in F1 plante is ook ‘n oorweging in kleinskaalse boerdery in Afrika, waar saad terug gehou en tussen bure uitgeruil word. Ons stel voor dat die implementering van Bt mielies in sulke kleinskaal boerderye in Afrika die ontwikkeling van weerstand as gevolg van saad terug hou en uitruiling moontlik kan bevoordeel.

Ten slotte, die huidige studie het bepaal dat daar ‘n wye reeks van vlakke van Bt in weefsel bestaan oor die groei seisoen. Geenvloei het ‘n newe effek op die Bt vlakke in F1 plante. Hierdie data verduidelik ook die graad van insek

oorlewing ten opsigte van verskillende Bt vlakke in verskillende weefsel. In die finale instansie, hierdie studie lê ‘n belangrike grondslag om die rol van Bt vlakke ten opsigte van die ontwikkeling van insek weerstand te verduidelik in *B. fusca* in Suid Afrika.