

## Indirect effects of transgenes on resistance to northern leaf blight of maize under chemical and manual management practices of weeds

Efeitos indiretos de transgenes na resistência a helmintosporiose do milho sob práticas de manejo químico e manual de plantas daninhas

Efectos indirectos de los transgenes sobre la resistencia al tizón de la hoja del norte del maíz bajo prácticas de manejo químico y manual de plantas arvenses

Received: 03/02/2022 | Reviewed: 03/09/2022 | Accept: 03/17/2022 | Published: 03/25/2022

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### Abstract

The effects of transgenes on plant resistance to non-target diseases caused by foliar fungi are still poorly studied. This study aimed to evaluate the indirect effects of the TC1507 event, isolated and in combination with NK603, on the maize genetic resistance to northern leaf blight, in the presence and absence of chemical control of weeds. Three isogenic hybrids were used as treatments in this study: the non-genetically modified conventional hybrid BG7060 (NGM); the genetically modified hybrid BG7060H (Hx), carrying transgenes *cry1F* and *pat* (TC1507 event); and the genetically modified hybrid BG7060HR (Hxrr), carrying transgene *cp4epsps* (NK603 event) combined with the TC1507 event. To evaluate the resistance of the three isogenic hybrids to that pathogen, three experiments were carried out in Florianópolis, southern Brazil, in complete randomized blocks with three replications in experiment 1 (2012/2013 harvest) and four replications in experiments 2 and 3 (2016/2017 harvest). The control of weeds was done by manual weeding, in experiments 1 and 2, and with the application of the herbicide Finale in a single dose, in experiment 3. The severity of northern leaf blight was evaluated under natural conditions of disease occurrence, in experiment 1, and from inoculation of the pathogen, in experiments 2 and 3. The isogenic hybrid Hxrr showed higher incidence and shorter incubation and latency periods than the Hx and conventional isogenic hybrids in the two management environments of weeds. Pathogen resistance, measured from these three variables, tended to be lower in the genetically modified hybrid Hxrr, which contains two events (TC1507 and NK603), followed by the hybrids Hx and NGM, in increasing order of resistance. The addition of the herbicide for the control of weeds increased the susceptibility of the genetically modified hybrids Hx and Hxrr, as well as the conventional hybrid not submitted to this control, but cultivated close to the hybrids Hx and Hxrr managed with the use of the herbicide.

**Keywords:** Genetically modified maize; *Zea mays* L.; Northern leaf blight; Ammonium glufosinate.

### Resumo

Os efeitos dos transgenes sobre a resistência das plantas a doenças não-alvo, causadas por fungos foliares, ainda são pouco estudados. O objetivo deste estudo foi avaliar os efeitos indiretos do evento TC1507 isolado e em combinação com NK603 sobre a resistência genética do milho à helmintosporiose, na presença e ausência de controle químico de plantas daninhas. Três híbridos isogênicos foram utilizados como tratamentos nesse estudo: BG7060 (NGM) não geneticamente modificado (convencional); BG7060H (Hx) geneticamente modificado, carregando os transgenes *cry1F* e *pat* (evento TC1507); BG7060HR (Hxrr) geneticamente modificado, carregando o transgene *cp4epsps* (evento NK603), combinado com o evento TC1507. Para avaliar a resistência dos três híbridos isogênicos ao referido patógeno, foram conduzidos três experimentos, em Florianópolis, sul do Brasil, em blocos completos casualizados com três

repetições no experimento 1 (safra 2012/2013) e quatro repetições nos experimentos 2 e 3 (safra 2016/2017). O controle das plantas daninhas foi feito por capina manual, nos experimentos 1 e 2, e com a aplicação do herbicida Finale, em dose única, no experimento 3. A severidade a helmintosporiose foi avaliada em condições naturais de ocorrência doença, no experimento 1, e a partir da inoculação do patógeno, nos experimentos 2 e 3. O híbrido isogênico Hxrr apresentou maior incidência e menores períodos de incubação e latência do que os híbridos isogênicos Hx e convencional. A resistência ao patógeno, medida a partir destas três variáveis, tendeu a ser menor no híbrido geneticamente modificado Hxrr, que contém os dois eventos (TC1507 and NK603), seguido pelos híbridos Hx e NGM, em ordem crescente de resistência. A adição do herbicida para o controle de plantas daninhas aumentou a suscetibilidade dos híbridos geneticamente modificados Hx e Hxrr, assim como do híbrido convencional não submetido a este controle, mas cultivado próximo aos híbridos Hx e Hxrr manejados com uso de herbicida.

**Palavras-chave:** Milho geneticamente modificado; *Zea mays* L.; Helmintosporiose; Glufosinato de amônio.

## Resumen

Los efectos de los transgenes sobre la resistencia de las plantas a las enfermedades no diana causadas por los hongos foliares no son aún objeto de estudio. El objetivo de este estudio fue evaluar los efectos indirectos del evento TC1507 aislado y en combinación con NK603 sobre la resistencia genética del maíz al tizón foliar del norte, en presencia y ausencia de control químico de plantas arvenses. Se utilizaron tres híbridos como tratamientos en este estudio: BG7060 (NGM) no modificado genéticamente (convencional); BG7060H (Hx) genéticamente modificado, que lleva transgenes cry1F y pat (evento TC1507); BG7060HR (Hxrr) genéticamente modificado, que transporta el transgén cp4epsps (evento NK603) combinado con el evento TC1507. Para evaluar la resistencia de los tres híbridos isogénicos a ese patógeno, se realizaron tres experimentos, en Florianópolis, sur de Brasil en bloques completos al azar con tres repeticiones en el experimento 1 (cosecha 2012/2013) y cuatro repeticiones en los experimentos 2 y 3 (cosecha 2016/2017). El control de plantas arvenses se realizó mediante deshierbe manual, en los experimentos 1 y 2, y con la aplicación del herbicida Finale, en una sola dosis, en el experimento 3. La severidad de tizón foliar del norte se evaluó en condiciones naturales de ocurrencia de la enfermedad, en el experimento 1, y a partir de la inoculación del patógeno, en los experimentos 2 y 3. El híbrido isogénico Hxrr mostró mayor incidencia y períodos de incubación y latencia más cortos que los híbridos isogénicos Hx y convencional. La resistencia al patógeno, medida a partir de estas tres variables, tendía a ser menor en el híbrido genéticamente modificado Hxrr, que contiene los dos eventos (TC1507 y NK603), seguido de los híbridos Hx y NGM, en orden creciente de resistencia. La adición del herbicida para el control de plantas arvenses incrementó la susceptibilidad de los híbridos genéticamente modificados Hx y Hxrr, así como del híbrido convencional no sometido a este control, pero cultivado cerca de los híbridos Hx y Hxrr manejados con el uso de herbicidas.

**Palabras clave:** Maíz modificado genéticamente; *Zea mays* L.; Tizón foliar del norte; Glufosinato de amonio.

## 1. Introduction

The Brazil became the second largest producer of transgenic crops in the world, reaching a planted area with soybean, maize, and cotton of 52.8 million hectares, behind only the USA, with 71.5 million hectares (ISAAA, 2020). The maize reached in the country the mark of 18.5 million hectares in 2020, and it is estimated that more than 93% of this area was occupied by transgenic maize (Pereira Filho & Borghi, 2020). The importance of biotechnology for maize crop in the country can also be evidenced by the high number of transgenic cultivars (131) available commercially in the 2019/2020 harvest in relation to total cultivars (196) (Pereira Filho & Borghi, 2020).

By March 2020, 94 events were released for plants by the National Technical Commission on Biosafety (Comissão Técnica Nacional de Biossegurança - CTNBio), of which 50 were for corn (CTNBio, 2020). The events TC1507 and NK603 are the most frequent in transgenic maize hybrids in Brazil, especially the ones grown in Santa Catarina state, in southern Brazil. TC1507 confers resistance to insects of the Lepidoptera order and tolerance to herbicide based on glufosinate of ammonium - HBGA (active ingredient: L-fosfonitricina), while the NK603 event confers tolerance to herbicide based on glyphosate - HBG (active ingredient: Sal de Isopropilamina).

The resistance of maize to lepidopteran pests - *Spodoptera frugiperda*, *Diatraea saccharalis* and *Helicoverpa zea* - carrying event TC1507 is conferred by a truncated form of the insecticidal protein Cry1F (endotoxin), encoded by the *cry1F* gene and isolated from PS81I strain (NRRL B-18484) of the bacterium *Bacillus thuringiensis* var. *aizawai*. The tolerance of maize carrying TC1507 event to HBGA is conferred by the PAT enzyme (phosphinothricin-N-acetyltransferase), encoded by the *pat* gene and isolated from Tu494 strain obtained from the soil microorganism *Streptomyces viridochromogenes* (AGBIOS,

2008). In turn, the NK603 event contains the *cp4epsps* gene isolated from bacterium *Agrobacterium sp.*, CP4 strain, which gives tolerance to the HBG. This transgene is responsible for the expression of the CP4-EPSPS enzyme (CP4-5 enolpyruvylshikimate-3-phosphate synthase), functionally identical to the endogenous EPSPS protein of plants (Padgett et al., 1993).

The genetic basis of a transgenic maize hybrid is derived from a conventional hybrid (non-transgenic or not genetically modified), developed by classical breeding methods. The insertion of one or more events into the conventional hybrid, isolated or combined in the same basic genetic material, constitutes a series of isogenic hybrids that differ basically by the absence (in conventional hybrid) or the presence of one or more events (in genetically modified hybrids). Thus, theoretically, the isogenic hybrids of maize should not present differences in their genetic composition, in addition to that provided by the DNA technology used to create them. However, recent studies have shown that these differences between isogenic hybrids exist and are presented at the metabolome, proteome, and high-throughput RNA sequencing level (Agapito-Tenfen, Vilperte, Traavik & Nodari, 2018; Ali, Draxler, Poelzl, Agapito-Tenfen, Hochegger, et al., 2020; Benevenuto, Agapito-Tenfen, Vilperte, Wikmark, Van Rensburg, et al., 2017; Liu, Zhang, Liu & Jin, 2020; Wang, Zhang, Yang & Wang, 2018;). In addition, other changes were detected: damage caused by pests, changes in morphological, structural and chemical composition of parts of the plant (Balieiro Neto, Cividanes, Branco, Bueno, Possenti, et al., 2013), harmful effects on non-target organisms (Cheeke, Darby, Rosenstiel, Bever & Cruzan, 2014), reduction of agronomic performance of plants (Prado, Estevão, Maeda, Carlesso, Gonçalves, et al., 2016) and effects on *in vitro* cultures (Holderbaum, Traavik, Nodari & Guerra, 2019).

Studies evaluating the negative effects of the transgenes on the physiological reaction of the plants are available in the literature for over a decade. In some studies with glyphosate in maize cultivars carrying the technology *Roundup Ready 2 (RR2)* under the application of HBG were observed negative effects on nutritional balance, water use efficiency, photosynthesis, rhizosphere, biomass accumulation, amino acid synthesis and secondary compounds, seed quality and grain yield in maize, as well as phytotoxic effects, significant linear decline in the chlorophyll index and nutrient contents in the leaves (Albrecht, Braccini, Scapim, Ávila, Albrecht, et al., 2011; Krenchinski, Albrecht, Albrecht, Cesco, Rodrigues, et al., 2017; Thomas, Plinesnic, Thomas, Edmisten, Wells, et al., 2004; Zobiolo, Bonini, Oliveira Jr., Kremer & Ferrarese-Filho, 2010b; Zobiolo, Oliveira, Kremer, Constantin, Yamada, et al., 2010a; Zobiolo, Oliveira Jr., Kremer, Constantin, Bonato, et al., 2010c).

However, works are scarce as to the indirect effects of transgenes on plant resistance to non-target phytopathogenic microorganisms. Few investigations have studied the effects on maize resistance to pathogenic fungi of the genus *Fusarium* (Clements, Campbell, Maragos, Pilcher, Headrick, et al., 2003; Gatch & Munkvold, 2002; Munkvold & Desjardins, 1997; Munkvold & Hellmich, 1999; Munkvold, Hellmich & Showers, 1997). Gatch and Munkvold (2002), for example, observed a higher level of *Fusarium graminearum* infection in the height of transgenic maize compared to the respective non-transgenic version. Munkvold and Hellmich (1999), Munkvold and Desjardins (1997), Munkvold et al. (1997) and Clements et al. (2003), evaluating corn ears containing grains infected with fungi of the genus *Fusarium*, from areas with attack of the *Ostrinia nubilalis*, also verified a lower occurrence of infection of ears of the non-transgenic hybrid, compared to the genetically modified isogenic hybrid with the *cryIAb* transgene. In contrast, a study realized by Kmoch, Šafránková, Holková, Polišenská, Krédl et al. (2012) showed the absence of effects of the *cryIAb* transgene on maize grain infection caused by *Fusarium*.

Despite these studies conducted with the *Zea mays*-*Fusarium* pathosystem, there are no records of works in order to observe the effects of transgenes on non-target leaf fungal diseases. With regard to the effects caused by the TC1507 and NK603 events on the leaf severity of the maize to northern leaf blight (NLB), the works are nonexistent. This finding reinforces the need for research involving the established interactions between microorganisms, plants, and transgenes.

Northern leaf blight or helminthosporiosis, caused by *Exserohilum turcicum*, is a severe foliar disease, which can lead to up to 90% reduction in crop yield (Mueller, Wise, Sisson, Allen, Bergstrom, et al., 2016; Ramathani, Biruma, Martin, Dixelius & Okori, 2014; Wang, Xiao, Wang, Xiao, Zhao, et al., 2012), as a consequence of extensive defoliation during the period of

grain filling, especially if the infection occurs before flowering (Nwanosike, Mabagala & Kusolwa, 2013). Temperatures between 20° C to 25° C, relative humidity of 90% to 100% and low luminosity are favorable environmental conditions for the reproduction of this phytopathogen (Mallowa, Esker, Paul, Bradley, Chapara, et al., 2015; Wise & Mueller, 2011). In Brazil, epidemics occur more frequently in the south region and central-west plateaus, where production losses are more severe (Ogliari, Guimarães & Camargo, 2007; Ogliari, Guimarães, Geraldi & Camargo, 2005). Therefore, the pathosystem in question is promising for studies involving the effects of transgenes on the resistance reaction of the plant to leaf pathogens.

Considering the importance of the maize crop for the Brazilian agricultural economy, the extensive cultivated area with transgenic cultivars in the country, the scarcity of studies focused on the effects of transgenes on the reaction of maize to non-target foliar pathogens, and the relevance of the losses in yield caused by NLB pathogen, then this work aimed to evaluate the indirect effects of the TC1507 event, isolated and in combination with NK603, on the maize genetic resistance to NLB, in the presence and absence of chemical control of weeds.

## 2. Methodology

### 2.1 Plant Material

Three isogenic hybrids of Dupont do Brasil S/A – Pioneer, belonging to the Biogene BG7060® series (Biogene, 2016), were used as treatments of the present study, namely: the non-genetically modified conventional hybrid BG7060 (NGM); the genetically modified hybrid BG7060H (Hx), carrier of *cry1F* and *pat* transgenes (TC1507 event, *Herculex I* technology); and the genetically modified hybrid BG7060HR (Hxrr), carrier of *cp4epsps* transgene (NK603 event, *Roundup Ready 2* technology), combined with TC1507 event (AGBIOS, 2008). There is no isogenic hybrid belonging to the Biogene BG7060® series carrying the isolated *cp4epsps* transgene.

### 2.2 Field Experiments

Three experiments were carried out under field conditions, one in the 2012/2013 harvest (experiment 1) and two in the 2016/2017 harvest (experiments 2 and 3). The experiments were conducted at the Experimental Farm of Santa Catarina University (UFSC), in Florianópolis, Santa Catarina state, southern Brazil (27°41'06.28"S, 48°32'38.81"W). The region presents climate mesothermic wet (Cfa Köppen) and soil typical Hydromorphic Quartzeneic Neosol (EMBRAPA, 2006).

During the period of conduction of the experiment of 2012/2013 harvest, the average temperature was of 20.3 °C, with a minimum of 8.4 °C and maximum of 31.8 °C. The total rainfall in the period was of 522.5 mm and the average relative humidity was of 81%. In the period of conduction of the two experiments of 2016/2017 harvest, the average temperature was of 24 °C, with a minimum of 13.3 °C and maximum of 36.7 °C. The total rainfall in the period was of 583.8 mm and the average relative humidity was of 81% (ICEA, 2017).

The experiment 1 (2012/2013 harvest) was conducted in completely randomized blocks design, with three replications to evaluate the three isogenic hybrids (NGM, Hx and Hxrr). Each experimental unit consisted of four rows, 5.0 m long with 1.0 m between rows, under a plant density of 60,000 plants ha<sup>-1</sup> after thinning. The useful plot of 8.0 m<sup>2</sup> comprised 48 plants of the central rows excluding the two ends of each row. The experiments 2 and 3 (2016/2017 harvest) were conducted in the same experimental area in completely randomized blocks design, with four replications to evaluate the same isogenic hybrids. Each experimental unit consisted of four linear rows (4.0 m in length), at 0.8 m spacing between rows, under a plant density of 75.000 plants ha<sup>-1</sup> after thinning. A useful plot of 6.4 m<sup>2</sup> consisted of 48 plants of the central rows excluding the two ends of each row. The plant densities used in the experiments were based in the recommendation for maize cultivation in the south of Brazil (Argenta, Silva & Sangoi, 2001).

The chemical fertilization was performed based on soil analysis results and following fertilization and liming recommendations for the states of Rio Grande do Sul and Santa Catarina (ROLAS, 2004).

Different strategies of weeds control were adopted in order to verify the effect of herbicide use on attributes related to disease resistance in maize. The control of weeds was done by means of manual weeding, in experiments 1 (2012/2013 harvest) and 2 (2016/2017 harvest), and with applying the herbicide Finale in a single dose, in experiment 3 (2016/2017 harvest). The herbicide Finale ( $2.0 \text{ L ha}^{-1}$ ) –  $200 \text{ g L}^{-1}$  of ammonium glufosinate + 0.2% v/v of Joint Oil AgroSciences mineral oil – was applied when the weeds were with one pair of leaves and the maize plants were in the V4 (four leaf developed) to V5 (five leaf developed) vegetative stage.

### **2.3 Collection, isolation, monosporic culture, and pathogen inoculation**

The experiment 1 (2012/2013 harvest) was conducted on the basis of the natural occurrence of NLB and allowed a first analysis of the effects of transgenes on the resistance of the plant to this phytopathogen through the leaf severity evaluation. The results obtained from the experiment 1 led to the planning of the two additional experiments (2 and 3) conducted in the 2016/2017 harvest, but through controlled inoculation of the pathogen. For such, maize leaves containing typical lesions of NLB caused by *Exserohilum turcicum* were collected in the Experimental Farm of Santa Catarina University, in Florianópolis.

The pathogen was isolated from parts of the leaf containing lesions, kept in a humid chamber inside Petri dishes, at room temperature for 24 hours. After sporulation, the reproductive structures were placed, scraped and then diluted in sterile distilled water. After the filtering of suspension containing the conidia,  $10 \mu\text{L}$  of the liquid-based was pipetted and transferred on a hollow blade to obtain of the monosporic cultures. The monosporic cultures were located, collected, and transferred to Petri dish, containing potato-dextrose-agar culture medium (PDA), and kept in a growth room under a temperature of  $25 \text{ }^\circ\text{C}$ , in the dark, for seven days. To obtain the inoculum, fragments of PDA culture medium were added, containing structures from the monosporic culture of *Exserohilum turcicum* packed in Erlenmeyer containing grains of sorghum. The fungus was incubated at  $25 \text{ }^\circ\text{C}$ , in the dark, for 15 days, being that each Erlenmeyer flask was shaken periodically to allow more homogeneous colonization of all the grains. About 20 sorghum grains colonized by the fungus were added in sterile Petri dishes, containing 10 ml of PDA. The fungal material was incubated at  $25 \text{ }^\circ\text{C}$ , in the dark, for seven days. The colonies generated were "scraped" and added to 100 mL of distilled water, until they reached the concentration of  $10^4$  spores/mL, with the aid of a Neubauer chamber according to the methodology described by De Rossi, Reis and Brustolin (2015).

The inoculation of the conidia in the plants in the field was performed 36 days after seeding, in plants at the V6 (six leaf developed) to V7 (seven leaf developed) vegetative stage. For the conditions of the present study, 2 mL of suspension/plant were inoculated, with the aid of pre-compression sprayer Guarany of six liters with adapted atomizing nozzle (0.15 mm conical type). The inoculation was carried at the end of the afternoon, under low light intensity and in the absence of rainfall.

### **2.4 Evaluation of the severity caused by northern leaf blight**

The disease severity was evaluated in experiment 1 (2012/2013 harvest) at 105, 111, 123, 137 and 146 days after seeding (DAS), whose dates corresponded to the reproductive phenological stages between R3 (doughy grain) and R5 (hard grains). Eight random plants of the useful plot area (identified with string) were evaluated. The leaf severity was estimated by using a diagrammatic scale elaborated by Bleicher (1988) from injury percentages of 0, 1, 3, 6, 10, 25, 50 and  $>50\%$ . The average disease severity was estimated for the whole plant from the analysis of three leaves per plant containing lesions.

The variables incidence (INCI), incubation period (INCU), latent period (LATE) and disease severity were evaluated in experiments 2 and 3 (2016/2017 harvest). The incidence was measured in 10 plants of the useful plot, 14 days after inoculation of the fungi and, from this, the percentage or relative frequency of the plants affected with the disease was estimated. The

incubation period was computed from the number of days from inoculation to the appearance of the first symptoms. The latent period was evaluated using a magnifying glass (10x magnification) and measured based on the number of days from inoculation to sporulation (Ferreira, Marino & Furtado, 2017). The severity evaluations were performed at 56, 63, 70, 77, 84, 91, 98 and 105 DAP, between the V6 vegetative stage (six complete leaves) and R5 reproductive stage (hard grains), in twenty random plants of the useful plot (identified with string). Each plant was divided into three thirds (lower, middle and higher), and from each third, three leaves were evaluated for the inference of disease progression. The average severity of each plant was estimated from the average of the nine leaves. The leaf severity of the experiments 2 and 3 was estimated by means of a diagrammatic scale elaborated by AGROCERES (1996) from injury percentages of 0, 1, 3, 6, 10, 25, 50 and >50%.

The evolution of the disease, for the three trials, was estimated through the area below of the disease progression curve (AUDPC), based on severity data obtained at each evaluation and applied to formula of Campbell and Madden (1990):  $AUDPC = \sum_{i=1}^{n-1} \frac{(Y_{i+1} + Y_i)}{2} \cdot (t_{i+1} - t_i)$ , where:  $Y_i$  = severity of disease at time of evaluation  $i$  ( $i=1,2,\dots,n$ );  $Y_{i+1}$  = severity of disease at time of evaluation  $i+1$ ;  $t_i$  = time of evaluation  $i$ ;  $t_{i+1}$  = time of evaluation  $i+1$ ;  $n$  = total number of observations.

## 2.5 Statistical analyses

The individual variance analyses of the experiments followed the mathematical statistical model given by  $Y_{ij} = \mu + t_i + b_j + e_{ij}$ , where  $Y_{ij}$  corresponds to the observation of the treatment  $i$ -th ( $i = 1, 2, 3$ ) of the block  $j$ -th ( $j = 1, 2, 3$ , in experiment 1, 2012/2013 harvest; and  $j = 1, 2, \dots, 4$ , in experiments 2 and 3, 2016/2017 harvest);  $\mu$  is the overall mean of the experiment (constant);  $t_i$  is the effect of treatments (fixed);  $b_j$  is the effect of blocks (random);  $e_{ij}$  is the random effect associated with the experimental error.

The joint variance analyses of the experiments were performed based on the statistical-mathematical model given by  $Y_{ijk} = \mu + t_i + m_j + tm_{ij} + bk(j) + e_{ijk}$ , where  $Y_{ijk}$  is the observation of the treatment  $i$ -th ( $i = 1, 2, 3$ ) of handling  $j$ -th ( $j = 1, 2$ ) and block  $k$ th ( $k = 1, 2, 3, 4$ );  $\mu$  is the overall mean (constant);  $t_i$  is the effect of treatments (fixed);  $m_j$  is the weed handling effect (fixed);  $tm_{ij}$  is the effect of interaction between treatment and handling (fixed);  $bk(j)$  is the effect of blocks within handlings, assumed random and;  $e_{ijk}$  is the random effect associated with the experimental error.

In order to calculate AUDPC as a function of time, the linear regression model was used: (2)  $Y_i = \beta_0 + \beta_1 x_i + \varepsilon_i$ , for  $i = 1, \dots, n$ , where:  $Y_i$ : is a random variable and represents the value of the response variable (dependent variable) in the  $i$ -th observation;  $x_i$ : represents the value of the explanatory variable (independent variable, regressive variable) in the  $i$ -th observation;  $\varepsilon_i$ : is a random variable that represents the experimental error;  $\beta_0$  and  $\beta_1$  are the parameters of the model, which will be estimated, and which define the regression line;  $n$ : is the sample size.

The individual and joint analyses were carried out with the STATISTICA 13.3 program (Tibco, 2017) and the regression analyzes with the R program (R Core Team, 2017). Variables showing significant differences between the treatments or handlings by the F test at 5 % of probability ( $p \leq 0.05$ ) were subjected to the Tukey test, at the same significance level.

## 3. Results

### 3.1 Indirect effects of TC1507 event isolated and combined with NK603 on resistance to NLB

The coefficients of variation (CV %) were lower than 20 % for most the traits in the individual and joint analyses of variance (Table 1). This outcome indicated a good experimental accuracy. Isogenic hybrids differed significantly between them ( $p \leq 0.05$ ) for the INCI, INCU and LATE variables, in the individual and joint analyses (2016/2017 harvest). However, for these same variables, the differences were not significant for the effects associated with management systems (environment) and the interaction of hybrids (genotypes) with management environments (interaction genotype x environment) (Table 1).

In the joint variance analyses including the two experiments conducted in the 2016/2017 harvest, the NGM isogenic hybrid differed significantly from the others, with the better result for INCI and INCU variables associated to the NLB (Table 1). The hybrid NGM presented mean values of the INCI lower than the hybrids Hx (5.4 times lower) and Hxrr (14.2 times less), as well higher mean values for INCU, compared to the values of the Hx (1.1 times greater) and Hxrr (1.4 times greater) hybrids, and for LATE, compared to the average of the Hxrr hybrid (1.5 times greater). This tendency among the means of the three hybrids was also observed in the individual analyses of variance for the two management systems of weeds (hand weeding and ammonium glufosinate herbicide application). The Hxrr isogenic hybrid showed higher incidence and shorter incubation and latency periods than the Hx and conventional isogenic hybrids in the two management environments.

For the area under disease progress curve (AUDPC), the isogenic hybrids differed significantly between them ( $p \leq 0.05$ ) in the experiment conducted in the 2012/2013 harvest, where the severity was evaluated between 105 and 146 DAS on the basis of the natural occurrence of NLB (Table 2; Fig.1). In this experiment, the conventional hybrid (NGM) was more resistant than the two transgenic isogenic hybrids (Hx and Hxrr), which is evidenced by an estimate of AUDPC about 16% lower than the general mean. In the experiments conducted in the 2016/2017 harvest, where the severity was evaluated between 55 and 105 DAS through controlled inoculation of the pathogen in plants at the vegetative stage, the isogenic hybrids did not differ significantly from variable AUDPC (Table 2; Fig.1).

In the joint analysis of variance of the experiments conducted in the 2016/2017 harvest, significant differences occurred only between management environments for AUDPC (Table 2), reinforcing on the influence of the management system used for the control of weeds on the resistance reaction of the plant to NLB. In the 2016/2017 harvest, the three hybrids (NGM, Hx and Hxrr) subjected to management with ammonium glufosinate herbicide showed mean estimates for AUDPC  $\approx 18\%$  higher in relation to the average of hybrids cultivated with the manual weeding. Data analysis comparing the different managements of weeds, but in the different harvests, showed this same tendency. The AUDPC mean estimated for the hybrids evaluated in the experiment with herbicide (2016/2017 harvest) was  $\approx 25\%$  higher than the average of hybrids cultivated with the manual weeding (2012/2013 harvest) (Table 2).

**Table 1.** Means and coefficients of experimental variation estimated from individual and joint analyses of variance applied to incidence (INCI), incubation period (INCU) and latency (LATE) variables of three maize isogenic hybrids belonging to the Biogene BG7060® series – NGM (non-genetically modified), Hx (TC1507), Hxrr (TC1507 + NK603) –, in two management systems of weeds (hand weeding – H/W and ammonium glufosinate herbicide application – W/H). Florianópolis, 2016/2017 harvest.

Hybrids	INCI (%)			INCU (days)			LATE (days)		
	H/W	W/H	Mean <sup>1</sup>	H/W	W/H	Mean <sup>1</sup>	H/W	W/H	Mean <sup>1</sup>
NGM	4.75 b	3.50 b	4.13 c	22.50 a	22.75 a	22.63 a	28.25 a	28.00 a	28.13 a
Hx	23.75 b	21.25 b	22.50 b	19.75 a	22.00 a	19.88 b	24.75 ab	26.25 a	25.50 ab
Hxrr	60.00 a	57.50 a	58.75 a	16.00 b	16.75 b	16.13c	19.50 b	18.50 b	19.00 b
Mean <sup>2</sup>	29.50	27.42		19.42	20.50		24.17	24.25	
CV (%) <sup>3</sup>	40.81	33.73		8.62	6.17		15.99	10.67	
Genotype (p) <sup>4</sup>	0.00	0.00		0.00	0.001		0.00	0.002	
CV (%) <sup>5</sup>	37.30			6.96			12.26		
Genotype (p) <sup>6</sup>	0.000			0.000			0.000		
Environment (p) <sup>7</sup>	0.64 <sup>ns</sup>			0.66 <sup>ns</sup>			0.95 <sup>ns</sup>		
Genotype x Environment (p) <sup>8</sup>	0.99 <sup>ns</sup>			1.00 <sup>ns</sup>			0.69 <sup>ns</sup>		

<sup>1</sup>Mean of treatments independent of environments from of joint variance analysis. <sup>2</sup>Mean of management environments independent of treatments from joint variance analysis. <sup>3</sup>Coefficient of variation of the individual variance analysis. <sup>4</sup>Probability value for difference between treatments from individual variance analysis (*p* value). <sup>5</sup>Coefficient of variation of the joint analysis of the experiments. <sup>6</sup>Probability value for difference between treatments from joint variance analysis (*p* value). <sup>7</sup>Probability value for difference between the management environments from joint variance analysis (*p* value). <sup>8</sup>Probability value for genotype x environment interaction (*p* value); <sup>ns</sup>Not significant. The averages followed by the same lowercase letter in the column do not differ statistically from each other by the Tukey test at the 5% probability level. Source: Authors.



**Table 2.** Means and coefficients of experimental variation estimated by individual and joint analyses of variance applied to area under disease progress curve variable (AUDPC) of three isogenic hybrids belonging to the Biogene BG7060® series – NGM (non-genetically modified), Hx (TC1507), Hxrr (TC1507 + NK603) –, in two management systems of weeds (hand weeding – H/W and ammonium glufosinate herbicide application – W/H). Florianópolis, 2012/2013 and 2016/2017.

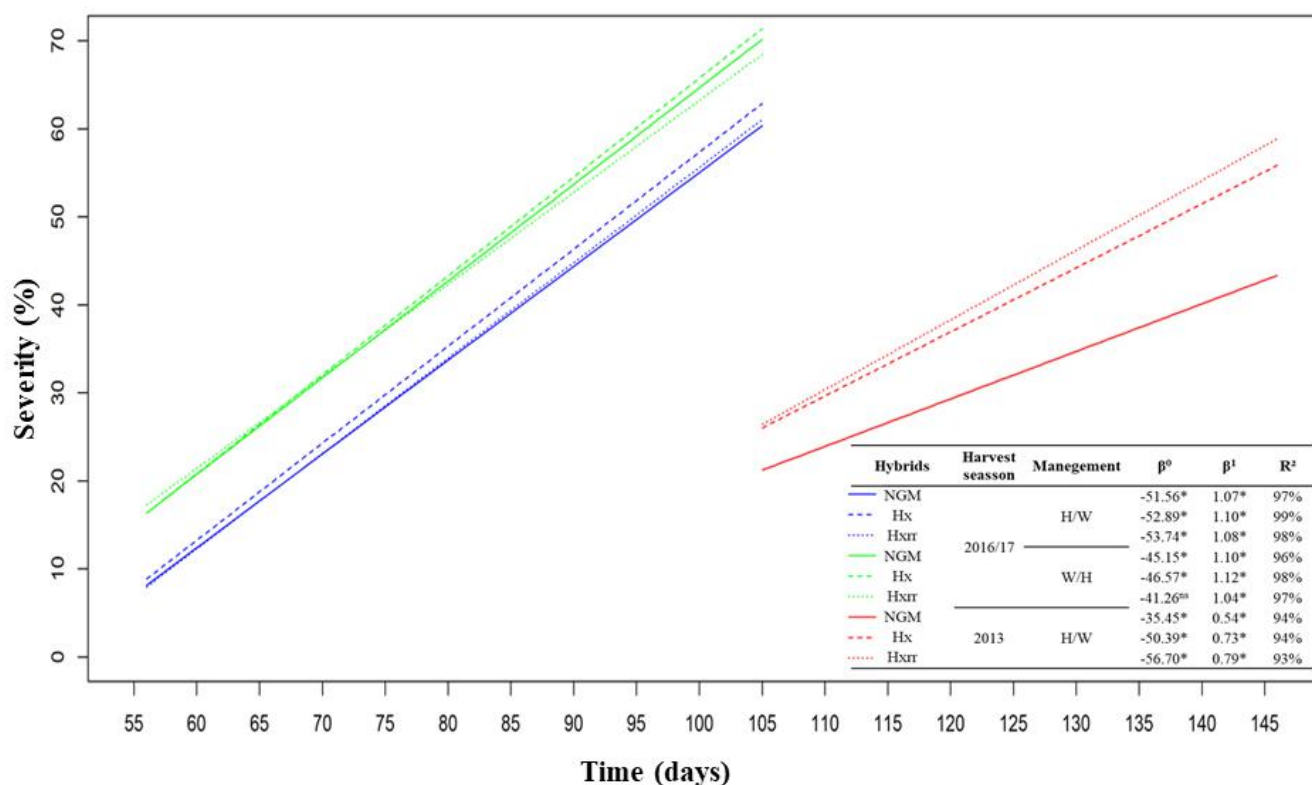
Hybrid	AUDPC								
	Harvest			Harvest			Harvest		
	2016/2017		Mean <sup>1</sup>	2012/2013		2016/2017	Mean <sup>1</sup>	2012/2013	
H/W	W/H		H/W	H/W			H/W	W/H	Mean <sup>1</sup>
NGM	1688.33	1918.98	1803.66	1300.02 b	1688.33	1494.18	1300.02 b	1918.98	1803.66
Hx	1633.66	1951.57	1792.62	1633.68 a	1633.66	1633.67	1633.68 a	1951.57	1792.62
Hxrr	1602.35	1948.56	1775.46	1722.59 a	1602.35	1662.47	1722.59 a	1948.56	1775.46
Mean <sup>2</sup>	1641.45 A	1939.70 B		1552.10	1641.45		1552.10 A	1939.70 B	
CV (%) <sup>3</sup>	17.33	8.36		9.25	17.33		9.25	8.36	
Genotype (p) <sup>4</sup>	1.00 <sup>ns</sup>	1.00 <sup>ns</sup>		0.01	1.00 <sup>ns</sup>		0.01	1.00 <sup>ns</sup>	
CV (%) <sup>5</sup>		14.42			13.60			9.43	
Genotype (p) <sup>6</sup>		0.89 <sup>ns</sup>			0.15 <sup>ns</sup>			0.06 <sup>ns</sup>	
Environment (p) <sup>7</sup>		0.00			0.12 <sup>ns</sup>			0.05	
Genotype x Environment (p) <sup>8</sup>		0.96 <sup>ns</sup>			0.21 <sup>ns</sup>			0.22 <sup>ns</sup>	

<sup>1</sup>Mean of treatments independent of environments from of joint variance analysis. <sup>2</sup>Mean of management environments independent of treatments from joint variance analysis. <sup>3</sup>Coefficient of variation of the individual variance analysis. <sup>4</sup>Probability value for difference between treatments from individual variance analysis (*p* value). <sup>5</sup>Coefficient of variation of the joint variance analysis of the experiments. <sup>6</sup>Probability value for difference between treatments from joint variance analysis (*p* value). <sup>7</sup>Probability value for difference between the management environments from joint variance analysis (*p* value). <sup>8</sup>Probability value for genotype x environment interaction (*p* value); <sup>ns</sup>Not significant. The averages followed by the same lowercase letter in the column and upper case in the row do not differ statistically from each other by the Tukey test at the 5% probability level. Source: Authors.

The isogenic hybrids formed three distinct groups for the AUDPC established from the regression analysis of leaf severity over time, considering two systems of management (hand weeding and with herbicide application), and two harvests (2012/2013 and 2016/2017) (Table 2; Fig.1).

The estimated values for the regression coefficients ( $r^2$ ) were equal to or greater than 93%. This indicates a good fit of the linear regression equations to the severity data evaluated for all genotypes, harvest seasons and weed management systems. The highest AUDPC values were estimated for the hybrids grown in the managed environment with herbicide, in the experiment conducted in the 2016/2017 harvest. The second group with the highest AUDPC values were formed by the isogenic hybrids grown in the same harvest, but whose weeds were controlled by manual weeding. The third group was formed by the hybrids cultivated in the 2012/2013 harvest, where weeds were controlled by manual weeding. The second e third group, both with manual weeding of weeds, not significantly differed.

**Figure 1.** Linear regressions of northern leaf blight severity over the time for the three isogenic hybrids belonging to the Biogene BG7060® series – NGM (non-genetically modified), Hx (TC1507), Hxrr (TC1507 + NK603) –, in two management systems of weeds (without herbicide application, by hand weeding - H/W and with herbicide application - W/H), in two harvest seasons: 2012/2013 (105, 111, 123, 137 and 146 DAS) and 2016/2017 (77, 84, 91, 98 and 105 DAS).



Source: Authors.

## 4. Discussion

### 4.1 Effects on non-target phytopathogenic fungi and their biological associations

Studies evaluating plant resistance to fungal pathogens as non-target organisms of transgenic maize hybrids are scarce, and the existing ones generally do not show the effect of the presence of transgenes on the plant reaction, and consequently,

development of foliar diseases. In relation to other non-phytopathogenic leaf microorganisms, the studies of the interactions between endophytic microorganisms and transgenic plants are also scarce. Important to highlight that these microorganisms establish an intimate symbiotic interaction with the host plants and changes in the diversity of endophytic communities may entail significant changes in plant growth and adaptation capacity (Vandenkoornhuysse, Quaiser, Duhamel, Le Van & Dufresne, 2015), such as the plant resistance reaction to phytopathogens. A study conducted by Silva, Armas, Soares and Ogliari (2016a) is illustrative, particularly since the authors evaluated the structure of bacterial and fungal foliar endophytes using the same isogenic hybrids of this study, i.e., transgenic (Hx and Hxrr) and conventional (NGM) hybrids belonging to the Biogene BG7060® series. The results showed that the maize foliar endophytic fungi suffer changes in the structure of their communities, depending on the plant genotype, presence and absence of transgenes and number of transgenes/events inserted into the genetically modified plant. Still, the results suggests that pyramiding of transgenes/events in Hxrr (TC1507 + NK603) provided a further change in the structural diversity of endophytic fungal communities of maize leaves in relation to the others (NGM and Hx).

Based in the results of individual and joint analyses of the experiments conducted in the 2016/2017 harvest of the present study, it can be stated that the events/transgenes reduced the ability of the plant to resist pathogen in the initial stages of establishment of the disease. The lower incidence ( $p \leq 0.05$ ) presented by the conventional isogenic hybrid and the shorter incubation and latent periods of the Hxrr hybrid in relation to the others suggest that the transgenic condition favors the initial establishment of NLB in maize. The effect was stronger in the Hxrr hybrid that accumulates the combination of two events (TC1507 and NK603) and three transgenes (*cry1F*, *pat* and *cp4epsps*) (Table 1).

These differentiated effects between genotypes may be associated with the changes observed by Silva et al. (2016a) in the structure of fungal endophytic communities, since studies prove that the fungal endophytes present benefits for plant defense mechanisms against biotic stress factors (Hardoim, van Overbeek, Berg, Pirttilä & Compant, 2015; Ibrahim, Sorensen, Jenkins, Ejim, Capretta, et al., 2017; O’Keeffe, Carbone, Jones & Mitchell, 2017).

Considering that the genetic difference between the three hybrids arises only from the absence of transgenes or from the insertion of one or two combined events, the variations in the degree of resistance between the three hybrids, in the early stage of establishment of NLB, can be attributed to the transgenic condition and especially to the pyramiding of events/transgenes.

The development of a transgene pyramid has been a strategy used to control biotic factors under the claim of the presence of synergistic effects resulting from the combination of the proteins produced by the plants carrying the combined transgenes in the same genotype (Gressel, Gassmann & Owen, 2017; Owen, Beckie, Leeson, Norsworthy & Steckel, 2015). However, these studies disregard the negative effects of these exogenous gene combinations on the expression of other plant characters, especially with respect to the reaction on non-target organisms of transgenes (Bowers, Hellmich & Munkvold, 2013; Lazebnik, Arpaia, Baldacchino, Banzato, Moliterni, et al., 2017; Smith, Lepping, Rule, Farhan, & Schaafsma, 2017). These finding reinforce the need for research involving interactions between microorganisms, plants and transgenes isolated and in combination (pyramiding of transgenes) before the release of new genetically modified maize cultivars for commercial cultivation.

#### **4.2 Effects on non-target phytopathogenic fungi triggered by chemical control of weeds**

In the 2016/2017 harvest trials, the inoculation of the conidia was performed 36 DAS (days after seeding), at a vegetative stage of plants (V6 to V7). The early exposure of plants to the pathogen for a long period of time may have contributed to reduce the differences between genotypes as to the resistance degree. In contrast, in the experiment of 2012/2013 harvest, where the disease manifested itself naturally after the male and female flowering and the severity was evaluated between 105 and 146 DAS, the isogenic hybrids differed significantly between them for AUDPC (Table 2; Fig.1). These results show that it is possible to

discriminate the resistance to NLB of isogenic hybrids among themselves based on the AUDPC variable when the disease symptoms begin at more advanced stages of plant development.

The absence of differences between isogenic hybrids for AUDPC (Table 2) in experiments of the 2016/2017 harvest is a result that deserves warning, since the resistance reaction of plants, expressed by this variable, was not associated with transformation events and pyramidal events in isogenic hybrids. Instead, the differences occurred among hybrids managed in different control systems of weeds (manual weeding and HBGA) regardless of genotype, so that the average of the three isogenic hybrids were significantly ( $p \leq 0.05$ ) more susceptible in the environment handled by the herbicide (Table 2; Fig.1).

The AUDPC value for the conventional hybrid (NGM), cultivated alongside the genetically engineered hybrids (Hx and Hxrr) and chemically managed for control of weeds, was higher than the estimated value for it in the experiment with hand weeding, although this treatment (NGM) had not been handled with herbicide in both experiments. Severns, Estep, Sackett and Mundt et al. (2014) and Estep, Sackett and Mundt et al. (2014)'s works were enlightening in this regard, based on the studies of different resistant and susceptible strains of wheat to the wheat stripe rust (*Puccinia striiformis*). In both works, the authors concluded that regardless of the difference in resistance between the genotypes in the initial outbreak of the disease, the epidemic produced by the most susceptible genotypes exerts a dominant influence on the subsequent epidemic, considering the spatial proximity between the genotypes with different degrees of resistance.

Although the NGM hybrid was more resistant at the initial establishment of the disease (INCI and INCU), independently of the weed management system (Table 1), this difference was not maintained with the progress of disease development. Instead, the three hybrids showed a remarkably similar NLB development rate within each of the management environments, which resulted in the impossibility of distinguishing them from the AUDPC. This difference in inoculum pressure on the conventional hybrid between chemical and manual management systems also explains the lower resistance of this hybrid when cultivated alongside transgenic hybrids handled with HBGA. The higher susceptibility of the transgenic hybrids (particularly the isogenic hybrid Hxrr) cultivated in the chemically managed environment with herbicide generated a higher inoculum pressure on the conventional hybrid cultivated in this experiment, in relation to the environment managed completely by manual weeding. This differentiated inoculum pressure also had repercussions on the degree of resistance presented by this treatment (NGM) in the two control environments of weeds, so much that the conventional hybrid of the experiment handled by manual weeding was more resistant.

The present study detected an increase in susceptibility of 18 to 25% in the mean of the most and least resistant genotypes, due to the management of weeds and harvests, and under the small dimensions of experimental plots. The inoculum pressure generated from genotypes less resistant to NLB may be even higher under real growing conditions (Kaiser, Kluth & Märlander, 2010), since a higher inoculum pressure is provided on smaller areas of cultivation from larger areas with susceptible genotypes (Marcuzzo, Duarte, Fernandes, Hilleshein & Scheidt, 2015).

In Brazil, it is estimated that the area planted with transgenic maize accounts for approximately 94% of the total area under maize in the country (ISAAA, 2019) and most confers insect resistance and tolerance to one or more herbicides (CTNBio, 2020). Particularly in the municipalities of Anchieta e Guaraciaba, in far west of the state of Santa Catarina, in the south of the country, where family production units predominate (86.3 and 88.5%, in this order) (IBGE, 2017), it is estimated that the area cultivated with transgenic maize represents at least 40% of the total area cultivated with this crop (Ogliari, 2018). Depending on the farm dimension class, the average area cultivated with transgenic maize in this region is 2.7 to 31.7 times higher than the area grown with conventional maize hybrids, open pollinated and local varieties (unpublished results). Under these conditions, genotype differences in resistance and inoculum pressure may contribute to the increase in the demand for fungicides, aiming at the control of helminthosporiosis, mainly in corn growing areas with diversity regarding the control management systems of weeds. Based on these results, further studies should focus on the impacts of these findings, particularly in areas of the country,

such as far west of the state of Santa Catarina, where a rich diversity of local varieties and their wild relatives is conserved on farm (Silva, Vidal, Costa, Vaio & Ogliari, 2015; Costa, Silva & Ogliari, 2016; Silva, Vidal & Ogliari, 2016b). The impact of chemical control of weeds on resistance to NLB is a relevant concern for those regions with rich genetic diversity, where two contrasting technological systems of maize production coexist, i.e., local varieties and commercial cultivars.

As previously reported, a greater AUDPC was observed both in the Hx and Hxrr hybrids managed with HBGA, and in the NGM hybrid with manual weeding, but close to the treatments treated with chemical control (Table 2). The results provide evidence that the application of HBGA compromises not only the resistance to NLB of transgenic hybrids, but also the conventional hybrid (NGM), which shares the same experimental environment managed chemically by spatial proximity. They also suggest that maize genotypes more resistant to NLB, in environments with lower inoculum pressure, may be adversely affected when cultivated near genotypes most affected by the disease, due to the use of chemical technology to control weeds.

With respect to transgenic hybrids of this study (Hx and Hxrr), it is still possible to infer that the herbicide application for the control of weeds in plots cultivated with transgenic hybrids left them more susceptible to NLB (Tables 1 and 2; Fig.1).

Based on these results, the impacts of fungal diseases on non-genetically modified maize may be relevant even for those production areas that follow the recommendations of the National Technical Commission on Biosafety (Comissão Técnica Nacional de Biossegurança – CTNBio), aiming the insect resistance management through refuge areas. The direct adverse effects could be evaluated in the non-genetically modified hybrid grown in refuge areas close to the production fields of genetically modified isogenic hybrids carrying transgenes of resistance to lepidopteran pests combined with transgenes of tolerance to herbicides. With regard to the NLB, our results highlight the relevance of studying how the weed management with HBGA and the inoculum pressure generated from the hybrid Hxxx carrying *cry1F*, *pat* and *cp4epsps* transgenes impact the resistance to NLB of the conventional hybrid grown in relatively smaller refuge areas.

When analyzing the work done by Carbonari, Latorre, Gomes, Velini, Owens et al. (2016) with transgenic cotton resistant to HBGA, the Liberty Link IMACD 6001 cultivar (LL), resistant to herbicide, presented intoxication in the range of 15% for the highest dose of 3.0 L ha<sup>-1</sup> at 36 days after application (DAA). The WideStrike FM 975WS cultivar (WSFM), with some resistance to HBGA, there was greater intoxication, in the range of 35% of the death of the plants to the higher applied dose. One of the most interesting results is related to the electron transport rate (ETR); it was observed in plants of LL cultivar the maximum reduction of the electron transport rate of 18% at 22 DAA, and WSFM cultivar showed the reduction maximum of 24% in ETR at two DAA. Reduction in ETR causes significant effects on photosystem I and II interfering on the reduction of photosynthesis.

Although it is not possible to separate the action of HBGA and NLB on the reduction of the photosynthesis in the present study, this may have occurred with HBGA resistant maize cultivated in the herbicide application system. The effects generated by the action of HBGA on the reduction of the photosynthesis, may be the initial trigger for the increase in the NLB severity on the Hx and Hxrr isogenic hybrids managed with HBGA. Other part of this presumed reduction of photosynthetic rate may be resultant from the effects of NLB itself on their host plants.

In fact, in plants susceptible to NLB, about 90% reduction in the photosynthesis rate has been reported when the severity exceeds 50% (Reddy, Reddy & Reddy, 2014). This is because biotic stresses, such as pathogen attack, which reduce photosynthetic activity, causing massive loss of organelle and chlorophyll integrity (Del Río, Pastori, Palma, Sandalio, Sevilla, et al., 1998), reducing the production of carbohydrates, fats, and proteins (Reddy, et al., 2014), and consequently, causing senescence of the leaves due to the accumulation of reactive species of oxygen (Prochazkova, Sairam, Srivastava & Singh, 2001).

The reduction in the photosynthetic potential of the plant occurs because many proteins involved in energy metabolism, destination and storage of proteins, and defense of the disease, present altered levels in response to NLB. In inoculation with *E. turcicum*, some defense-related proteins, such as  $\beta$ -glucosidase, superoxide desmutase, polyamines oxidase and cis/trans

peptidylprolilisomerase, are up-regulated, whereas photosynthesis and metabolism-related proteins are down-regulated. These results indicate that resistant maize plants to NLB can directly release defense proteins, modulating the primary metabolism, affecting photosynthesis and carbohydrate metabolism (Zhang, Si, Fan, Li & Wang, 2014).

Based on the above, further works should evaluate the influence of the herbicide in the behavior of transgenic isogenic hybrids belonging to the BG7060 series and other similar hybrids as to the agronomic performance and plant development, as well as the effects of the gene flow between transgenic and non-transgenic maize taking into account regions containing diversity rich of local varieties as in far western Santa Catarina.

## 5. Conclusion

The genetically modified isogenic hybrids carrying event T1507, isolated or in combination with NK603, presented higher susceptibility to northern leaf blight in relation to conventional isogenic hybrid, when the disease started naturally at advanced stages of plant development. This advantage of the conventional hybrid was not observed when the disease occurred well before the reproductive stage through controlled inoculation of the pathogen and under high inoculum pressure.

The genetically modified isogenic hybrid carrying the two events (T1507 and NK603) presented lower resistance to the northern leaf blight in the disease initial establishment steps, with higher incidence and shorter incubation and latency periods, regardless of the management system of weeds. This finding reinforces the need for research on transgenes combined in the same genotype (pyramiding of transgenes) and their relations with the maize genetic resistance to non-target fungal pathogens before the release of new genetically modified cultivars for commercial cultivation.

The non-genetically modified hybrid becomes more susceptible to northern leaf blight when it is under higher inoculum pressure from areas adjacent to the genetically modified isogenic hybrids carrying event T1507, isolated or in combination with NK603, and cultivated with the addition of the ammonium glufosinate herbicide. This finding is an indicative of the need for studies involving interactions between genetically modified maize that has insect resistance and herbicide tolerance traits, management systems of weeds, and plant genetic resistance to non-target pathogens.

The addition of the ammonium glufosinate herbicide on areas cultivated with hybrids carrier of the TC1507 event, isolated and combined with NK603, favors for the greater susceptibility of the maize to northern leaf blight, expressed by higher severities, compared to the same transgenic hybrids on areas cultivated without the addition of herbicide.

## Acknowledgments

To the team of Experimental Farm of Ressacada at UFSC and to the Núcleo de Estudos em Agrobiodiversidade – NEABio/UFSC, for structural supporting given to this research; to the scholarship was granted by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES to the first author.

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