FELIPE AUGUSTO MORETTI FERREIRA PINTO

Stenocarpella spp. INOCULUM QUANTIFICATION IN TROPICAL MAIZE STUBBLE

LAVRAS – MG

2016

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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-graduação em Agronomia, área de concentração Fitopatologia, para a obtenção do título de Doutor.

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APROVADA em 28 de Julho de 2016.

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RESUMO GERAL

Espécies do gênero *Stenocarpella* (S.*maydis* e S.*macrospora*) sobrevivem na entresafra na palhada de milho e a utilização de plantio direto e em monocultura de milho está relacionada a surtos de podridão branca da espiga e podridão do colmo, mas pouco se sabe sobre os fatores que contribuem para a sua sobrevivência. Portanto, este trabalho teve como objetivo determinar a contribuição de rotação de culturas de soja e pousio na sobrevivência e na dinâmica da decomposição de resíduos de patógenos do milho e determinar fatores que contribuem para a sobrevivência destes patógenos. No primeiro artigo, áreas de milho com história de elevada podridão branca da espiga foram utilizadas para determinar o tamanho da amostragem dos restos culturais do milho, para analisar a esporulação dos agentes patogênicos e viabilidade, bem como a sua decomposição. A monocultura de milho foi comparada à rotação com soja e pousio. No segundo artigo, campos de milho foram selecionados nas áreas de cultivo de milho no Estado de Minas Gerais para amostragem dos restos culturais. Durante a entresafra, caules de milho, grãos, espigas, folhas de milho caídas, palhas e resíduos de plantas daninhas mortas, foram coletadas e durante a safra de 2015/16 caules e espigas foram coletadas em áreas utilizando plantio convencional, plantio direto, seja com rotação de culturas ou de monocultura de milho. As amostras foram processadas, liofilizadas, moídas, o DNA foi extraído e o inóculo de *Stenocarpella* sp quantificado por qPCR. Os solos das áreas amostradas foram verificados quanto à supressividade aos fungos causadroes de podridão de espiga. Mapas de aquecimento foram construídos com a análise de dados. A sub-amostra de um quilograma foi a menor necessária para estimar a sobrevivência do patógeno. A esporulação do patógeno foi semelhante entre os tratamentos, mas a viabilidade foi reduzida (27,8-33,3%) nas regiões consideradas. A decomposição dos restos culturais foi maior em apenas um local. O tamanho da amostra, os métodos clássicos e moleculares permitiram o levantamento do patógeno em restos culturais de milho e demonstraram o papel de rotação de culturas de soja na redução da viabilidade de *Stenocarpella* sp. Em áreas cultivadas com o sistema de plantio convencional, o patógeno não foi detectado. No entanto, sob áreas manejadas com rotação de culturas em plantio direto, *Stenocarpella* sp foi detectada em menor grau do que em as áreas que adotam sistema de plantio direto com monocultivo de milho. Os resultados corroboram o papel de resíduos de milho na sobrevivência de *Stenocarpella* sp., a rotação de culturas como um método útil para manejar as doenças causadas por *Stenocarpella maydis* e apontam as variáveis que podem estar contribuindo para o aumento do inóculo do patógeno em áreas de cultivo.

Palavras-chave: Podridão branca da espiga, rotação de culturas, restos culturais do milho, *Diplodia maydis*, *Zea mays*.

GENERAL ABSTRACT

Stenocarpella species (*S.maydis* and *S.macrospora*) overwinters in maize stubble and no-till monoculture maize is related to ear and stalk rot outbreaks, but little is known about the factors that contribute to its survival. Therefore this work aimed at determining the contribution of soybean crop rotation and fallow land on pathogen survival and dynamics of maize stubble breakdown, and determining factors that contribute to the pathogens survival. Inn the first article maize fields with history of severe white ear rot were used to determine the stubble sampling size to analyze the pathogen sporulation and viability, as well the maize decomposition. The maize monoculture was compared to one round of soybean rotation and fallow. In the second article, maize fields were selected in the maize growing areas in the state of Minas Gerais for sampling maize stubbles. During the offseason, maize stalks, grains, cobs, fallen maize leaves, straw and dead weed stubble were collected. During the 2015/16 season stalks and cobs were collected in areas under conventional tillage, no-tillage, either with crop rotation or maize monoculture. Samples were processed, freeze-dried, powedered, had their DNA extracted and the *Stenocarpella* sp inoculum quantified through qPCR. Soils from the sampled fields were checked for suppressivity to ear and stalk rot-causing fungi. Heat maps were generated with the obtained data. A sub-sample of one kilogram was the minimum necessary to estimate the pathogen survival. The pathogen sporulation was similar among treatments but viability was reduced (27.8-33.3%) in the considered regions. The stubble decomposition was higher in only one location. The sample size, classical and molecular methods allowed the survey of the pathogen in maize stubble and pointed out the role of soybean crop rotation in the reduction of *Stenocarpella* sp viability. In cultivated areas adopting the conventional tillage system, the pathogen was not detected. However, under no-tillage managed areas, *Stenocarpella* sp was detected to a lesser extent than in areas adopting no-tillage system with maize monoculture. The results endorse the role of maize stubbles in the survival of *Stenocarpella* sp. and crop rotation as a useful method to manage the diseases caused by *Stenocarpella maydis*.

Key-words: White ear rot, crop rotation, maize crop stubbles, *Diplodia maydis*, *Zea mays*.

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1 INTRODUCTION

Maize (Zea mays L.) is the most widely cultivated cereal in the world, used for animal feed, industrial use and human consumption. Brazil appears as the third most important supplier of the grain, following United States and China (UNITED STATES DEPARTMENT OF AGRICULTURE - USDA, 2016). The maize crop has gained major importance in the productive chain, not only because of economic aspects, but as an important crop within a crop rotation system (SWARTZ; MARCHIORO, 2009). However, diseases have become a major concern and yield losses because of pathogen attack have been even more frequent in the principal growing regions (COSTA et al., 2009).

One of the major problems for increasing maize production in Brazil is the disease management. The use of fungicides on maize has increased greatly over the past ten years in the country to control foliar diseases and has been accompanied by an increase in yield (BLANDINO et al., 2012). However, despite the use of fungicides, outbreaks of stalk and ear rots, caused by *Fusarium verticillioides* Sheld (HEFNY et al., 2012), *F. graminearum* Schw, *Stenocarpella maydis* (Berk.) Sacc. and S. *macrospora* Earle are frequently observed (BIGIRWA et al., 2007). Ears rots are one of the most dangerous foods and feed safety challenges to maize production in the world (MESTERHÁZY; LEMMENS; REID, 2012).

Plant pathogens are noticed only when they attack plants and cause diseases, but another phase of their cycle is their survival and buildup as saprophytes in the crop stubble when they infect the plant at a certain stage of

the plant cycle. While the parasitic phase is well known for most plant pathogens, not much has been explained on how they survive when they are not infecting plants, in many cases this important aspect has been neglected. The ways in which parasites survive, between cropping seasons or during overwintering periods when the environment is unfavorable, are important phases to understand in order to develop strategies to reduce the inicial pathogen inoculum and thus manage the disease (BROWN, 1997).

Tillage can bury pathogens deeper in the soil where they are less likely to become a problem (WALTERS, 2009) Tillage can also alter soil texture, aeration, temperature, moisture and density, and also influence nutrient release in the soil with consequent benefits to the crop (BALL et al., 2005). Tillage also leads to clear fluctuations in soil microbial activity and biomass (BRUGGEN et al., 2006)

The intensity of the diseases caused by *Stenocarpella* spp in the no-tillage system is higher in maize monoculture ((FLETT; WEHNER, 1991; MORA; MORENO, 1984). Eleven months after the crop, Flett, Wehner and Smith (1992) observed 39.5 and 24.3% germinated spores, in the crop stubble remaining on the soil surface and buried, respectively.

However, there is no report on the impact of inoculum present in the area. Reis and Mário (2003) determined *Stenocarpella maydis* and *Stenocarpella macrospora* inoculum density from maize stubble existing on the soil surface after planting, randomly sampling ten straw samples, each with an area of 0.25 m². Casa, Reis and Zambolim (2003) proved the saprophytic survival of *Stenocarpella* spp. in the southern region of Brazil and related the quantity of stalks infected with picnidia and its viability but did not establish a methodology for accurately quantify the *Stenocarpella* spp survival.

In four consecutive seasons in South Africa, Flett, McLaren and Wehner (2001) determined that soybean or peanut crops in succession to maize reduced the amount of damaged kernels and the recovery of the pathogen from crop stubble in 75-100%, but in case of planting before the maize, soybean was effective, instead of peanut, to control Stenocarpella. The authors did not inform that the absence of the host during the production cycle reduces the pathogen survival, but with plant succession with legumes, there is also a reduced carbon-nitrogen relation from crop stubble, consequently favoring the growth of soil microorganisms, accelerating decomposition of the contaminated crop stubble, increasing competition with other saprobe microrganisms and thereby reducing pathogen survival (COOK; BAKKER, 1983).

There is considerable knowledge on the epidemiology of other pathogens causing ear rot in maize, such as *Fusarium graminearum* and *Fusarium verticillioides* (DRAGICH; NELSON, 2014; MUNKVOLD, 2003). In contrast, knowledge on the occurrence of the Stenocarpella complex disease epidemiology is scarce, particularly in tropical agriculture. Crop control methods are aimed at protecting crops against pathogens, rather than curing them once they have been infected and it provides the foundation for disease control in crops and yet its importance is often overlooked (WALTERS, 2009).

Crop stubble in the field such as maize stubble are major sources of inocula of necrotrophic plant pathogens (BUDDEMEYER et al., 2004; MAIORANO et al., 2008). According to Reis and Mário (2003) the quantification of the initial Stenocarpella spp inoculum in the maize crop stubble is essential to demonstrate the importance of this source, and to serve as a basis for the development of alternative control strategies, but it has not, as yet, been investigated in a tropical region following an established methodology.

By knowing the biology of plant pathogens, such as where and for how long they survive in the absence of the live host plant, effective disease control strategies can be developed (COLEY-SMITH, 1979).

2 THEORETICAL FRAMEWORK

2.1 Ecology and survival of plant pathogens

Ecosystems change over time due to long-term environmental trends, such as climate change, and short-term events, since fire or species introductions and parasites can influence ecosystem structure, biogeochemical cycles, energy flow, and temporal ecosystem dynamics (PRESTON et al., 2016)

Plant disease cycles are interdependent stages including survival, dormancy, reproduction, dispersal, and pathogenesis. The progression over these stages is decided by a unceasing sequence of interactions among host, pathogen, and environment (WOLF; ISARD, 2007).

Plant pathogens play hidden roles in the structure, dynamics, and evolution of natural plant communities. Pathogens can affect host population dynamics by direct effects on survival and growth. A strong understanding of the life of pathogens as well as plant hosts is necessary to acknowledge the effects that plant disease can have on natural ecosystems (GILBERT, 2002).

Some pathogens can survive by means of specialized resting structures, such as saprophytes, in vital association with living plants, in association with nematodes and fungi, insects or on agricultural materials or surface water. Plant pathogens have two phases one infective and the other saprophytic, in which they remain until infecting the plants again.

Biotrophic fungal pathogens obtain nutrients from living host tissues, often via specialized cells called haustoria that form inside host cells. Necrotrophic pathogens obtain nutrients from dead host tissue, which they kill through the production of toxins or enzymes. Most biotrophic fungi have fairly narrow host ranges—they are specialized on a limited number of plant hosts. Necrotrophic fungi can be either generalists, growing on a wide range of host species, or specialized on a restricted range of hosts. Some plant pathogenic fungi change the way that their hosts grow, either by affecting the level of growth regulators produced by the plant, or by producing growth regulators themselves.

2.2 Strategies for pathogens survival

Once plant pathogens are introduced in a field, they face both a novel environment and novel host species (PARKER; GILBERT, 2004). The first challenge for these microbes is to land in the new environment and secrete the enzymes necessary to obtain their nutrients (BROWN; HOVMØLLER, 2002; GOODELL; PARKER; GILBERT, 2000), but only pathogens suitable to the particular environment can survive there because the abiotic characteristics are compatible with environmental conditions like temperature, moisture, and UV irradiation (PAINI et al., 2016). The survivors need to find and infect susceptible hosts, before reproducing and dispersing (GILBERT, 2002). Each stage presents a selection filter, which makes the rapid adaptation from pathogen to new abiotic conditions and rapid evolution of novel host necessary (PARKER; GILBERT, 2004).

Many plant pathogenic bacteria and fungi survive during intercrop periods as saprophytes on diseased plant stubble or on organic material present in or on soil (BRUGGEN; FINCKH, 2016). For example, the apple scab fungus (*Venturia inaequalis*) infects apple leaves and fruits during spring and summer.

The fungus overwinters as pseudothecial initials in infected leaves and fruit that fall to the ground during autumn.

Some pathogens are obligate biotrophs that require a living host to finish their life cycle (PARKER; GILBERT, 2004). However, many pathogens can persist, grow and sporulate on dead tissue as facultative saprobes (BURDON, 1991; ROY, 2001). Nonetheless, it is expected that invasive microbes are facultative saprobes only via long-distance transportation modes since they survive well without a living host. Long-lived resting stages or saprotrophic abilities in the new habitat should be an advantage for those pathogens (PARKER; GILBERT, 2004).

This phase is characterized by a decrease in the activity of the pathogen, extends beyond the death of the host, the colonization of the plant tissue or the dead pathogen propagules being released to the soil. This phase ends when these seedlings come in contact with a new source of energy that stimulates germination (MICHEREFF et al., 2001).

Several strategies determine the survival period and, consequently, the maintenance of the population of an organism in the soil. There are factors related to the pathogen and factors inherent in propagules. The intrinsic characteristics of the pathogen are critical after the death of the host whose resistance to invasion by other microorganisms will reduce until it stops, not leaving the pathogen with other alternatives but to survive or resist in other ways. Three features are essential to the survival of a pathogen; host range, saprophytic competition ability, and production capacity of resistance structures.

Pathogenic agents have a wide range of alternative hosts, independently of having any other survival mechanisms, they are better prepared to perpetuate themselves, thus lengthening the period in which their soil population levels are high.

The saprophytic competitive ability is the faculty that a pathogen must maintain or even increase in their biomass by saprophytic colonization of dead tissues of its host and / or the use of undifferentiated substrates present in the soil. The decisive attributes of saprophytic competition ability are: rapid seedling germination, high growth rate, enzymatic ability to degrade cellulose and lignin, capacity to produce biostatic substances and tolerance to fungistatic substances produced by other microorganisms (GARRETT, 1970).

The higher the ability to produce resistance structures, the higher the number of propagules in the soil and therefore that of the pathogen population level. Two characteristics determine the longevity of propagating material: ability to withstand adverse conditions and susceptibility to biotic factors (MICHEREFF et al., 2001).

2.3 Crop stubble as source of inoculum

Fungal plant pathogens produce a range of extracellular enzymes to facilitate plant colonization, for the decomposition of crop stubble and degradation of primary cell wall components (LEPLAT et al., 2013). The survival of necrotrophic pathogens is related to the stubble decomposition rate, where stubble on the soil surface provides nutrients and serves as a substrate allowing the pathogen to survive for long periods of time, even up to several years (KHONGA; SUTTON, 1988; PEREYRA; DILL-MACKY; SIMS, 2004).

There is a general relationship between the yield of a given crop and the amount of stubble left on the ground after harvesting. In most cases, the stubble/yield ratio is between 1 and 2 (KUMAR; CAMERON; FLYNN, 2003; SCARLAT; BLUJDEA; DALLEMAND, 2011). The amount of stubble may differ from 2 to 9 tons ha-1(LEPLAT et al., 2013), depending on the type of crop, values are 2.0, 2.5, 3.5, 8.0 and 9.0 tons stubble ha−1in rape, barley, wheat,

alfalfa and maize leaves, respectively (MOREL, 1996; VILAIN, 1989). Part of the stubble is used for animal bedding, animal feed and biofuel production (BERNDES; HOOGWIJK; BROEK, 2003). However, a large part of it is left in and on the soil (Malhi et al. 2011). The stubble is either ploughed under (inversion tillage) or left on the surface when conservation tillage is being practiced, as in zero-tillage or other types of non-inversion tillage.

The effects vary to a great extent with climatic conditions and preceding crop type (BLANDINO et al., 2010; FERNANDEZ et al., 2008). In conservation tillage, more than 30 % of the soil surface is covered by crop stubble (BOCKUS; SHROYER, 1998).

For example, maize production results in a large mass of stubble which increases the inoculum production (CHAMPEIL; DORE; FOURBET, 2004). Comparing four different densities of maize stubble left on the soil surface showed that disease severity and deoxynivalenol occurrence in wheat grains increased with stubble quantity (BLANDINO et al., 2010; MAIORANO et al., 2008).

Crop control methods are aimed at protecting crops against pathogens rather than curing them once they have been infected. It aims to prevent contact with the pathogen, to create environmental conditions unfavorable to the pathogen or at least to avoid favorable conditions, or to reduce the amount of pathogen inoculum available to infect crop plants. Methods used for cultivation control include host eradication, crop rotation, sanitation, irrigation, tillage and improving crop growth conditions. Destroying crop stubble is an important practice, but how it is performed depends upon the type of crop and the type of pathogen (WALTERS, 2009).

2.4 Crop rotation as a control measure

One maize planting strategy is the no-tillage system. This form has many advantages and has been adopted by many maize farmers and constantly constitutes planting maize without crop rotation. However, this system promotes the survival, maintenance and propagation of the inoculum of such fungus (ZAMBOLIM; VALE, 2000).

Stenocarpella maydis e S. *macrospora* are necrotrophic pathogens, thus survive in crop stubble and are transmitted by contaminated seeds. In the off season, the pathogen can multiply and survive adverse conditions in the maize stubble and infect the plants in the next season. Therefore, burying crop stubble after harvest potentially reduces the survival of the infected fungi associated propagation material (FLETT; WEHNER; SMITH, 1992). However, with the benefits of no-till , the adoption of this tillage system is already adopted by about 35 million hectares , ie an estimated 70 % of adoption of this farming system and therefore the burial of crop stubble after cultivation does not seem a plausible strategy. Moreover , this production system is referred to as the biological pump and its benefits are related to the protection of the soil (conservation, reducing compaction and leaching of nutrients) , buffering potential environmental excesses (temperature, dry and wet) , bioremediation pesticides, weed reduction , as well as food for the development of intense biological activity (TIVET, 2010) .

If handled correctly, no-till can be suppressive and not conducivo the necrotrophic pathogens. Is growing planting off-season or winter crop in the south of Minas, but still dominated by producers who plant the maize alone with several years of exclusive planting of the crop , which favors epidemics of the disease (FITT et al., 2011).

The practice of crop rotation can be considered an important control measure for pathogens causing grain rot and knowledge of biological cycles of the

fungal species involved in the production of mycotoxins can contribute to reducing injuried grain incidence and thus reducing the damage to maize grain quantity and quality. (TRENTO; IRGANG; REIS, 2002).

In a recent survey conducted by Embrapa Maize and Sorghum, farmers get productivity of at least 8t / ha are those who rotation with soybeans (91%) (CRUZ et al., 2011). In addition to plant nutrition, the succession with a legume can also contribute to the control of diseases not host the same pathogens that infect maize and with it, the producer has greater success in the management of diseases and hence lower losses arising diseases.

Furthermore, crop rotation has other benefits for crops. Diversified 2- and 4-yr crop rotations increased maize and grain sorghum yields (SINDELAR, 2016). Increasing the cropping sequence diversity promotes greater yields when two species are included in a rotation compared to continuous maize and including soybean and cotton twice increased yields by 6 and 7%, respectively (ASHWORTH et al., 2016).

3 OBJECTIVES

3.1 General objectives

The objective was to determine the contribution of factors that govern the survival and ecology of *Stenocarpella spp.* in maize stubble.

3.2 Specific objectives

A) To verify the presence of *Stenocarpella spp.* on maize growing areas in Minas Gerais State,
B) To identify major inoculum sources of the *Stenocarpella* sp in cultivated areas

C) To verify the contribution of crop rotation or maize monoculture on the pathogen recovery from maize stubble
D) To quantify the survival of *Stenocarpella* spp. in maize stubble according to the following rotation systems: maize monoculture, rotation with soybean or fallow land.
E) To find the size of sample to quantify the density of pycnidia

4 GENERAL PROVISIONS

The study showed how knowledge about fungus survival is important for plant pathogens in annual plants. These results may be used for new management applied in maize growing areas to reduce the inoculum source for the next season. Furthermore, this shows how the plant pathogens have more shelter and can survive over time in different tissues and hosts.

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SECOND PART – ARTICLES

Article 1 The quantification of *Stenocarpella maydis* survival in maize stubble

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The quantification of *Stenocarpella maydis* survival in maize stubble

Abstract

Stenocarpella species (*S.maydis* and *S.macrospora*) overwinters in maize stubble and no-till monoculture maize is related to ear and stalk rot outbreaks. Therefore this work aimed at determining the size of sample for the pathogen survey and validate a PCR-based protocol for the determination of pathogen survival in maize stubblestubble. Maize fields with history of high white ear rot were used to determine the maize stubble sampling size to analyze the pathogens sporulation and viability as well as its decomposition. The maize monoculture was compared to one round soybean rotation and fallow. The sub-sample of one kilogram was the minimum necessary to estimate the pathogen survival. The pathogen sporulation was similar among treatments but viability was reduced (27.8-33.3%) in the considered regions. The sample size, classical and molecular methods allowed the survey of the pathogen on maize stubble and pointed out the role of soybean crop rotation on the reduction of *Stenocarpella* sp viability.

Keywords: White ear rot, crop rotation, maize crop stubbles, *Diplodia maydis*, *Zea mays*.

Highlights

The sampling size plays a role on the *Stenocarpella* sp inoculum quantification

Classical and molecular methods can be used to access *Stenocarpella* sp inoculum.

Soybean crop rotation reduces *Stenocarpella* sp viability on maize stubble.

1. Introduction

Maize *(Zea mays L.)* is the most widely cultivated cereal in the world, used for animal feed, industrial use and human consumption. Brazil stands out as the third most

important supplier of the grain, following United States and China (Usda, 2015) but its average yield and grain quality has to improve to be a more profitable business (Cruz et al., 2009)

One of the major problems for raising maize production in Brazil is the disease management and use of fungicides on maize has increased greatly over the past ten years in the country to control foliar diseases and was accompanied by an increase in yield (Blandino et al. 2012). However, despite the use of fungicides, outbreaks of stalk and ear rots, caused by *Fusarium moniliforme* Sheld (Hefny et al.,2012), *F. graminearum* Schw, *Stenocarpella maydis* (Berk.) Sacc. and *S. macrospora* Earle are frequently observed (Bigirwa et al., 2007). Ear rots are one of the most dangerous foods and feed safety challenges to maize production in the world (Mesterházy et al., 2012).

The improtance of maize diseases is directly related to the production system. Modifications occurred in the production system and resulted in increased crop yield, nevertheless it was accompanied by the increased disease incidence and severity, particularly the one related to the tillage system (Costa et al. 2009). In the last 20 years the use of no-tillage system has increased (Giacomini et al. 2003). This system reduces the soil preparation and provides maintenance of the soil humidity, however through this type of soil management, the conditions favor the overwinter survival and even buildup of most necrotrophic pathogens.

The crop stubbles on the soil surface favor the survival of plant pathogens, turning a neglected disease in one season into an epidemy with successive croppings

(Casa et al. 2006). In areas where no-tillage system was adopted, a higher foliar disease incidence and infected grains by *S. maydis* has been reported (Flett & Wehner, 1991).

In the last years, an increase on intensity of diseases caused by *Stenocarpella maydis* and *Stenocarpella macrospora* has been observed, with 10% estimated losses over a ten year studied timespan considering different hybrids on different Brazilian locations (Dudienas *et al*. 2012).

The species complex *Stenocarpella* in maize encompasses two species: *S.maydis* and *S.macrospora*, which causes stalk rot and white ear rot and *S. macrospora* also causes Diplodia leaf streak. *S.maydis* is recognized as one of the most important and persistent pathogens of ear rots in the world (Rossouw et al., 2009). The higher intensity of Ear rots usually causes higher occurrence of rot grains, undesirable for commercialization of grains to consumption and seeds for planting (Ribeiro et al., 2005).

Besides the losses in the field, these fungi produces mycotoxins, affecting the quality of the grain (Rogers et al., 2014). In some areas of animal production, cases of diplodiosis, a livestock neuromycotoxicosis was reported and associated to *S.maydis*. (Kellerman et al., 1985, Marasas & Van der Westhuizen, 1979, Rao & Achar, 2001, Riet-Correa et al., 1984). Other micotoxins reported as associate to diplodiosis were diplodiatoxin and diplonine. (Snyman et al., 2011, Steyn et al., 1972, Wicklow et al., 2011). *S.macrospora* produces diplosporine e diplodiol, two micotoxins affect small animals (Cutler et al., 1980, Kriek & Marasas, 1979).

The species of *Stenocarpella* are necrotrophic, has two phases: parasite in the living plant and saprophytic in the crop residuals. It is found as a mycelium in seeds (Mcgee, 1988, Casa *et al.*, 1998, Rheeder *et al.*, 1990) and forming picnydia in the seeds (Siqueira et al., 2014) and in maize crop stubbles (Smith & White, 1988).

The incidence of fungi in harvested seed is related to the inoculum of *S. maydis* and *S. macrospora* produced in the crop stubblestubble on the surface and dispersed by the wind. (Mário et al., 1998)

To present only bamboo (*Arundinaria* sp.) (Sutton & Waterston, 1966, Sutton, 1980) has been reported as alternative host of *S. maydis.* Therefore, *Stenocarpella* sp. has likely no other host and it survives also as a saprophyte in the crop stubbles after harvest, colonizing the cellular tissues and forming subepidermic picnidia in stalks presents in the area. The position of the straw in the surface makes the decomposition more slowly and raises the period of survival of the necrotrophic pathogens, during the saprophytic phase. (Casa et al. 2003)

The intensity of the diseases caused by *Stenocarpella spp* in the no-tillage system is higher in maize monoculture (Mora & Moreno, 1984, Flett & Wehner, 1991). Flett *et al.* (1992) observed eleven months after the crop 39.5 and 24.3% of germinated spores, respectively, for the crop stubble remaining on the soil surface and buried.

Reis & Mário (2003) determined *Stenocarpella maydis* and *Stenocarpella macrospora* inoculum density from maize stubble existing on the soil surface after planting, collecting random ten straw samples, each one with an area of 0.25 m². Casa *et*

al. (2003) proved the saprophytic survival of *Stenocarpella* spp. in the southern region of Brazil and related the quantity of stalks infected with picnidia and your viability but did not establish a methodology for accurately quantify the survival of *Stenocarpella* spp.

In four consecutive seasons in South Africa, Flett *et al.* (2001) determined soybeans or peanuts crops in succession to maize reduced the amount of damaged kernels and the recovery of the pathogen from crop stubbles in 75-100 % but the sampling size has not been determined nor molecular based sampling has been attempted and the validation of such methods increase the accuracy of the disease quantification.

According to Reis & Mário (2003) the quantification of initial inoculum from *Stenocarpella* spp in the maize crop stubble is essential to demonstrate the importance of this source, and to serve as a base to the development of effective control.

Therefore, considering the importance of these pathogens in maize and the lack of knowledge about the relationship between this survival in these crop stubble and the quantification of initial inoculum present in the area, the purpose of this study was to quantify the survival of *Stenocarpella* spp. in maize stubble according to the following rotation systems: maize monoculture, rotation with soybean or fallow land.

2. Material and methods

2.1 Selection of areas for conducting the assays

In six maize producing areas where has a history of losses by *Stenocarpella spp*-associated diseases, kernels, stalks and leaves were collected according to confirm the pathogen presence.

The sampled plant tissues were transported and then processed in the laboratory to determinate the presence and viability of *Stenocarpella* sp, according to Casa *et al.* (2004). Briefly, 30 maize stalk pieces, 30 kernels and 30 leaf-pieces laying on the ground were randomly collected for each selected field. These samples were collected right after harvest. They were taken from the field, washed thoroughly in tap water to remove the adhered soil and saprobes, placed in a humid chamber for 24h at room temperature and photoperiod of 12h, optimum conditions for the fungus to sporulate.

The presence of *Stenocarpella* sp. was confirmed by incubating samples for 7 days in moist chamber at 25ºC, examining them in the stereomicroscope and identifying picnidia with cirrus (Casa et al., 2004).

To determine the inoculum density of these pathogens, conidia were harvested from the cirrus and germination was evaluated. Five cirrus from *Stenocarpella* sp. were randomly sampled for each plant material with the aid of a stylet and transferred to microfuge tube of 2mL containing 1mL from saline solution (0,85% m/v de NaCl added one drop of tween 80) submitted to ultrasonic bath for 3 minutes and agitated in vortex for 1 minute.

A 100µL aliquot from each tube was plated on half PDA and incubated in BOD at ca.25ºC for 12h when conidial germination was recorded. The conidia were considered germinated when the germination tube is larger or equal to the larger length of conidia. (Casa et al. 2004).

After the survey, two areas of maize production were chosen, one located in the experimental farm “Center of scientific and technological development in agriculture” of the Federal University of Lavras, at 21°14'S, 44°59'W and another one in Piumhí at 20°15' S e 46°22' W, both in the Minas Gerais state.

2.2 Sampling size for analysis of *Stenocarpella* sp. survival in maize stubble

To quantify the *Stenocarpella* spp survival in maize stubble were collected four replications of stalks naturally infected in the six areas, with 1 to 2 ha of size. The stalks were cleaned in tap water to remove the adhered particles, after they were processed as described above for conidium quantification and germination. The samples were weighed and it was defined four kilogram as maximum weight per collected sample, based on the maximum capacity of litter bags. After this period the picnidia present in the stalks were quantified with the support of the light stereoscopic microscope. To establish which sample size represents the total, was quantified the density of pycnidia (pycnidia/Kg of crop stubble) in sub-samples of one, two and three kilograms, besides the quantification of the total sample, with four kilograms.

2.3 *Stenocarpella* sp survival in maize stubble

In the fields with history of both *Stenocarpella* species and maize no-till cultivation, plots planted with maize DKB-390 pro II, soybeans or fallow land to evaluate the *Stenocarpella* spp survival in maize straw. All treatments were planted in areas where maize had been planted as the previous crop. Samples of six kilograms of maize stubble were packed in “nylon” bags with whole size of 2 ”mesh”, so-called “

litter bags", remained on the ground, allowing the interaction of straw with microbiota from soil without excessive material loss by flood (Karberg et al., 2008). Samples were collected before and after the cultivation in the season of 2013-2014, the first collection was in october 2013 and the second in february 2014.

2.4 Determination of viability of *Stenocarpella* sp in maize stubble

Once the inoculum for each sample was quantified, ten pycnidia with cirrus were randomly chosen for each litter bag. Ten cirrus from *Stenocarpella* sp. were randomly picked on each sample with the aid of a stylet and transferred to microfuge tube of 2mL contain 1mL from saline solution (0,85% m/v de NaCl added one drop of tween 80) submitted to ultrasonic bath for 3 minutes and agitated in vortex for 1 minute.

A 100µL aliquot from each tube was plated on half PDA and incubated in BOD at ca.25ºC for 12h when conidial germination was recorded. The conidia were considered germinated when the germination tube is larger or equal to the larger length of conidia. (Casa et al. 2004).

2.5 Assessing decomposition of crop cultural stubble

The decomposition of stubble was determined by the drying in the kiln at 70°C until constant weight and the weight data obtained for each sample were analyzed collectively by the medium rate of decomposition percentage of remaining stubble through time, was adopted methodology similar to used by Casa *et al.* (2003).

2.6 Nitrogen and carbon total analysis in crop stubble

To understand the differences between each location, the nitrogen and carbon total analysis were performed by Dumas combustion method, which consisted on total sample oxidation in the presence of oxygen at high temperatures, followed by nitrogen oxide reduction and detection of molecular nitrogen produced.

2.7 Sample processing, DNA extraction and Real time PCR

After storage, frozen samples were shredded in squares of 1-2cm. A subsample was weighed and freeze-dried to assess dry matter content until constant weight. Then the dried material was grounded in a laboratory mill with a 1-mm mesh sieve. Powdered subsamples were stored at -18°C until DNA extraction.

DNA was extracted from subsamples of approximately 40 mg using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI), according to the protocol recommended by the manufacturer.

Sybr Green PCR assays were performed in Rotor-Gene 6500 (Corbett Research, Montlake, Austrália). For each reaction, a 2.0µl sample was mixed with 23µl reaction mix containing 12.5µl SYBR Green PCR Kit,(Qiagen), 0.75µM of each forward and reverse primer. The primers P1 (GTTGGGGGTTTAACGGCACG) and P2 (GTTGCCTCGGCACAGGCCGG) described by Xia and Achar (2001), specific for the genus *Stenocarpella*, were used to detect the presence of the fungus in the tissues (Barrocas et al., 2012, Siqueira et al., 2014). The cycle initially consisted of 95 ºC for 3 minutes, denaturation at 94 ºC for 30 seconds, annealing at 60 ºC for 1 minute, and extension of 72 ºC for 1 minute, with final extension of 72 ºC for 10 minutes, for a total

of 40 cycles. A 5-fold serial dilution ranging from 20 ng to 2 pg of DNA of *S.maydis* isolate 698, was included in each run for reference. A melting curve analysis was carried out for each real-time PCR test to confirm specific amplification of the target product, and rule out non-specific amplification and false positive reactions. The Ct was determined as the number of cycles in which the fluorescence generated within a reaction crossed the threshold. The comparative Ct method was used. Samples showing the lowest expression for each gene were used as calibration sam-ples and relative expression was measured using the relative standard curve method. To quantify gene expression using real-time PCR, the values obtained corresponding to sample DNA levels were compared to the control DNA level, using the Relative expression software tool developed by Pfaffl *et al.* (2002) with software Rest 2009 version 2.0.13.

2.8 Statistical analysis

The density of pycnidia data were compared using F test. The values observed in each parcel for survival, viability and decomposition of cultural stubble were submitted to the variance analysis (ANAVA) and for the significant effects, means were compared according to Tukey test (p=0,05), using the Software R. The relative expression were compared by hypothesis test P(H1), with software Rest 2009 version 2.0.13.

3. Results

3.1 Sampling size for analysis of *Stenocarpella* sp. survival in maize stubble

The density of pycnidia on maize stubble from the different fields ranged from 32.12 to 92.9 pycnidia/Kg of crop stubbles (Table 1). The pattern of spatial distribution of the sample in the field was similar (P= 0.1114483) for all samples (Table 1).

Table 1. Maize stubble sampled for quantification of *Stenocarpella* sp. pycnidia according to the sample size.

Field	Sample size (Kg)	Density of pycnidia(pycnidia/kg)[1]
Mean	1	56.70a
	2	58.35a
	3	58.47a
	4	57.88a

There was no significant difference in sample size in accordance with the F test for all sampled size, 1, 2, 3 and 4 Kg maize stubbles.

Since there were no differences between the size of samples, to study the survival of *Stenocarpella* sp., it is proposed the use of one kilogram samples. That choice was based on decrease the time spent in the counting process, facilitating the quantification of picnidia survival, using the density of picnidia, in the next studies, establishing a less complex and more accurate methodology.

3.2 *Stenocarpella* sp survival and viability in maize stubble

There was a significant effect of treatments within each location but no difference between locations within each considered crop treatment.. Soybean was the most efficient in the reduction of *Stenocarpella* spp survival, compared to maize crop and fallow land. The treatments with maize crop and fallow land were similar from each other. (Table 2)

Comparing the treatment with the maize crop, soybean rotation reduced the pathogen viability in 37%. The fallow land decreased the survival in 6.5% compared to maize.

Table 2. *Stenocarpella* spp. reduction in picnidia density after one season of maize, soybean or fallow after maize

Treatment	Lavras	Piumhí
Maize	77.25000 Ab	97.8125 Ab
Soybeans	158.47917 Aa	133.5625 Aa
Fallow land	92.97917 Ab	91.15625 Ab
C.V.	34,35	

*Means followed by the same small letter in the column or capital leter in the line are significantly similar at P<0,05 according to Tukey's test. ** Reduction in picnidia density calculated by difference between picnidia density initial and picnidia density final after crops on plots.

The treatment with soybeans crop reduced the conidia germination more than other treatments, differing statistically in the two performed experiments. In the experiment conducted in Lavras, the treatments with maize and fallow land were similar. However, in the experiment conducted in Piumhí the treatment with maize planting provided a higher *Stenocarpella spp*. viability (table 2).

Table 3. *Stenocarpella* spp. viability in cultural maize stubbles after one season of maize, soybean or fallow after maize

Treatment	Lavras	Piumhí
Maize	86,25 aB	93.75 aB
Soybeans	63,75 bA	62.50 cA
Fallow land	81,25 aA	82.50 bA
C.V.	9,95	

*Means followed by the same small letter in the column or capital leter in the line are significantly similar at P<0,05 according to Tukey's test.

For the conidia germination, there was a significant effect of the interaction between location and crops and the crops were compared for each location (Table 2).

3.3 Decomposition of crop cultural stubbles

There was no effect of the interaction between crops and location and the factors contributed to the decomposition independently. None of the crops contributed to the decomposition but the locations did (P<0.001), where the decomposition in Piumhi was 23,5 % higher(Table 3).

Table 4. Decomposition of maize stubbles in cultural maize stubble after one season of maize, soybean or fallow after maize

Treatment	Lavras	Piumhí
Maize	74,64 aA	64.32 aB
Soybeans	75,68 aA	60.70 aB
Fallow land	74,00 aA	56.52 aB
C.V.	11,8	

*Means followed by the same small letter in the column or capital leter in the line are significantly similar at P<0,05 according to Tukey's test. ** Decomposition of stubble was determined by the drying in the kiln at 70ºC until constant weight.

3.4 Nitrogen and carbon total analysis in crop stubble

The stalks located at Lavras had Carbon (p = 0.9789888) and ratio C/N (p=0.9691483) higher than the stalks from Piumhí. The Nitrogen content (p = 0.3978885) was similar among locations (Table 5).

Table 5. Nitrogen and carbon analysis for stalks collected on locations after one season of maize, soybean or fallow after maize

Location	Nitrogen	Carbon	CN
Lavras	0.5759a	46.80a	82.08a
Piumhí	0.9199a	43.85b	48.71b
C.V.	19.25	2.46	17.25

*Means followed by the same letter in the column are significantly similar at P<0,05 according to Tukey's test.

3.5 Real time PCR

The presence of *Stenocarpella* sp from samples collected at both locations was confirmed through qPCR in 40 out of 48 samples. The amount of pathogen DNA titer was higher on stalks sampled under maize crop, followed by Fallow land and Soybean. Soybean crop and Falllow land were similar from each other. Crop stubble collected on before and after maize had higher relative expression compared to soybean and fallow land at Piumhi. Already in Lavras, crop stubble sampled before soybean had higher relative expression, differing from others, being followed by stalks under maize cultivation and stalks sampled before fallow land. The relative expression was reduced after soybean cultivation and fallow land. (Table 6)

Table 6. Relative Expression analysis of Stenocarpella spp. in cultural maize stubbles one season of maize, soybean or fallow after maize

	Relative Expression	
Treatment	Piumhí	Lavras
Initial Soybean	38,45b*	169,6a
Initial Maize	512,60a	2,78b
Initial Fallow land	1b	1b
Final Soybean	72,17b	0,001c
Final Maize	478,76a	22,56b
Final Fallow land	0,013b	0,002c
Reaction Efficiency	0,94	0,79

*Means followed by the same letter in a column do not differ from each other, according to hypothesis test P(H1), performed with software Rest 2009 version 2.0.13.

4. Discussion

Studying the survival of *Fusarium moniliforme*, *F.subglutinans* and *F.proliferatum* in crop cultural stubbles, Cotten and Munkvold (1998) were able to recover the fungus after 630 days in uncultivated areas, indicating these stubbles could be a source of *Fusarium* inoculum for a long time. *F.moniliforme* survival after 8 months was more found in 30cm buried stubbles. (Nyvall and Kommedahl, 1968), however in the study realized by Cotten and Munkvold (1998), *Fusarium* survival was buried, for the collected samples until 343 days after the experiment started or in the cultural

stubbles presents in the surface in collected samples after 529 days after the experiment installation.

Casa *et al.* (2003) found the average incidence of the occurrence of colonized stalks by the fungus in the three agricultural years was 55% for *S. maydis* and 54,5% for *S. macrospora*, in sample realized in cultural stubbles in the period between December and march, during three years., but they did not quantify the amount of fungi was present in stalks.

The *S.maydis* and *S.macrospora* survival in maize crop stubble is the most important source of initial inoculum for the next crop, being able to maintain the pathogens always present in the area, even without living plants of maize. *S.macrospora* only parasitizes the maize plants (Sutton e Waterston, 1966, White, 1999) and *S.maydis* was found in bamboo and maize (Sutton e Waterston, 1966, Sutton, 1980), since the population of bamboo is restrict, it makes crop stubble and crop rotation even more important in the management of diseases caused by these fungus in comparison with diseases caused by *Fusarium* species, since the genus *Fusarium* has a lot of host plants.

A possible explanation could be the effect of the canopy caused by the soybeans plants, creating a friendly microclimate to the microbiota present in the area and consequently decreasing the *Stenocarpella* spp survival.

In relation to the viability of fungi, the conidia germinationwas higher than 84%, with an average in three years of 89.4% for *S. maydis* and 87.7% for *S. macrospora*. (Casa *et al.* 2003), which is in accordance with our results, under maize at

Piumhi, where the viability was 93.75 and in Lavras 86,66 both higher than 84%. Under fallow land, the viability ranged from 80- 84%.

The results confirmed *Stenocarpella* sp, when present in the infected maize stalks, sustains its conidia viability up to twelve months after grain harvest (Casa *et al.* 2003), under soybean rotation this viability is reduced, but still viable after four months. Therefore, under the tropical weather found in the regions where our trials were carried out, the contribution of one season crop rotation already impact the pathogen viability.

While maize and a wild bamboo are reported as the only host for *Stenocarpella* sp, the fungus is a necrotrophic pathogen and likely survives and multiplies and keeps a viability similar to the one found for maize (ca. 80%) under in vitro tests on substrates based on barley, wheat, oat or sorghum (Kuhnem Junior et al., 2012).

Reis *et al.* (2011) defined the decomposition time of crop cultural maize stubbles in 37 months, Casa *et al.* (2006) estimated the decomposition time of crop stubbles between 32 and 38 months after the harvest in fallow land In the plots with fallow land were observed after four months, same decomposition rate. However those studies analyzed the decomposition rate of maize stubble after maize crop, leaving the area in fallow. Moreover, in the present study the areas were cultivated with maize, soybean and also fallow land.

In accordance with Holtz (1995) the slower decomposition of maize stubble can be attributed to air and soil temperature, since it is less favorable during the winter, but in our data the measurements were taken during the summer season. Another factor can

explain crop decomposition showed in our work is C/N ratio, when it is high, the decomposition can be slow, because on the crop stubble from maize, most of them are stalks and cobs, both are lignified materials. In work developed by Aita and Giacomini (2003) with crop stubble of Black oat, Common vetch and Oil seed radish, with C/N ration 40.3, 14.8 and 30.6, respectively, Common vetch stubble were more decomposed than the other stubble and the results found herein agree with it, since the location with the higher C/N ration, Lavras, had lower decomposition than Piumhi.

Besides the C/N ratio, there are other aspects like size of stubble particles , lignin content, N, polyphenols, lignin/N ratio, lignin +N/polyphenols ratio, presence of toxic elements, physical and chemical conditions from soil and type of microbes present in soil that influence the decomposition of crop stubble (Moreira & Siqueira, 2006).

Two different classes of microrganisms are involved in the decomposition of stubble particles in the surface: one community autoctone limited in the quantity of carbon present and one community limited in the quantity of nitrogen colonizes the cultural stubble (Potthoff et al. 2001). The microrganisms presents in the surface dominates the decomposition of maize straw, although microrganisms colonizing the straw gives one significant contribution to the process. (Potthoff et al., 2008)

Because it is a short period of analysis could be infer a cycle of soybeans culture or fallow land were not enough to modify the dynamic of decomposition in the maize stalks, on soil. It would be necessary to follow the dynamics of decomposition for a longer period to verify if occurs changes in the dynamics in a long time.

Marburger *et al.* (2015) working with maize-soybean-wheat rotation and species of *Fusarium* spp. found a reduce of *F. graminearum* recovered from extracted soil. *F. graminearum* and *S.maydis* are both species causing Maize ear rots and maize-soybean rotation is more commom rotation utilized nowadays on Brazil, so it is convenient if this type of crop rotation reduce the survival of those pathogens, whereas they are increased by adopting no-tillage system.

From the point of view of maize grain quality and safety, reducing the impact of micotoxins remains a major challenge. The present study demonstrated the contribution of crop rotation, used as tool, in diminishing *Stenocarpella maydis* survival under field conditions. The results obtained agree with previous studies, since it showed that crop rotation can influence plant pathogen survival (Flett & Mclaren, 2001).

Different techniques have been addressed in this study to determine the quantification of initial inoculum *Stenocarpella* spp., such as pycnidia density, viability of these and qPCR . The three techniques proved to be useful tools for such analysis. All the techniques have been able to demonstrate that treatment with soybean crop reduced the presence of *Stenocarpella* spp . in the maize stubble. However, pycnidia density, viability and qPCR had 55, 65, and 99% in reduction of inoculum of *Stenocarpella* spp . in the maize stubble in treatment with soybean crop.

5. Conclusions

The minimum tested sample size of one kilogram is enough to accurately determine the pathogen inoculums. Using this sample size, a qPCR protocol has been proposed that accurately estimated the pathogen inoculum compared to picnidia count or

pathogen viability and also the estimate of the contribution of soybean rotation, fallow land or maize monoculture on the percent *Stenocarpella* spp inoculum level.

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Article 2 - Towards understanding the factors favoring the survival of *Stenocarpella* spp. in maize stubble

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Towards understanding the factors favoring the survival of *Stenocarpella spp*. in maize stubble

Abstract

Stenocarpella species (S.*maydis* and S.*macrospora*) survive in maize stubble over the offseason but little is known about the factors that contribute to its survival. Therefore this work aimed at determining factors that contribute to the pathogen survival. Maize fields were selected in the maize growing areas on State of Minas Gerais to sampling maize stubble. During offseason, maize stalks, grains, cobs, fallen maize leaves, straws and stubble of dead weeds were collected in maize field, and during season of 2015/16 stalks and cobs were collected in areas under conventional tillage, no-tillage, either with crop rotation or maize monoculture. Samples were processed, freeze-dried, ground, DNA extracted and the *Stenocarpella* sp population quantified. Soils from the sampled fields were checked for suppressiviness to ear and stalk rot-causing fungi. Heat maps were generated based on the obtained data. In areas cultivated adopting conventional tillage system, the pathogen was not detected. However, under no-tillage managed areas, *Stenocarpella* sp was detected at a smaller extent than areas adopting no-tillage system and monocultivated with maize. The results endorse the role of maize stubbles on the survival of *Stenocarpella* sp. and crop rotation as a useful method to manage the diseases caused by *Stenocarpella maydis*.

Introduction

Ear rot diseases of maize result in significant economic losses after harvest (Duarte et al., 2009) and reduce quality and quantity of grains (Matiello et al., 2015). Multiple fungi are related as cause of Ear rot as *Aspergillus flavus, Colletotrichum graminicola* (Ces) G.W. Wils., *Stenocarpella maydis* (Berk.) Sutton [Sin. *Diplodia maydis* (Berk.) Sacc., S. *macrospora* (Earle) Sutton [Sin. D. *macrospora* Earle in Bull.], *Fusarium graminearum* Schwabe (Gibberella zeae Schw.) and *Fusarium verticillioides* [Sin.= *Fusarium moniliforme* J. Sheld (*Gibberella fujikuroi* Sawada)] (Reis et al., 2004; Ahsan, S. et al., 2010). There is a growing market demand for grains with a restricted percentage of maximum values of rot grains: 2% for exportation and 6% to Brazilian intern market (Mendes, 2009). In this situation it is urgent both to understand the factors that contribute to the disease development and in turn, this will impact the development of efficient disease management strategies.

The evolution of maize diseases is directly related to cropping systems. Modifications occurred in the production system, resulted in increased crop productivity, nevertheless also was accompanied by increased disease incidence, particularly the one related to tillage system

(Costa et al. 2009). In the last 20 years the use of no-tillage system has increased worldwide (Giacomini et al. 2003). This system reduces the soil preparation time and provides maintenance of the soil humidity, reduced erosion however this favors the overwintering and inoculums buildup of most necrotrophic pathogen.(Casa et al. 2006)

Crop stubble on soil surface benefit many plant pathogens survival, turning a neglected monocyclic diseases in one season into an epidemy with successive planting of crops within the same plant host range (Casa et al. 2006). In areas that no-tillage system has been adopted, a higher foliar *macrospora* leaf spot incidence, stalk and ear rot caused by S. *maydis* has been reported (Flett & Wehner, 1991).

There is considerable knowledge on the epidemiology of other pathogens causing ear rot on maize as *Fusarium graminearum* and *Fusarium verticillioides* (Munkvold, 2003; Dragich and Nelson, 2014). In contrast, knowledge on the occurrence of the *Stenocarpella* complex diseases epidemiology is scarce, particularly in tropical agriculture.

Considering, the lack of knowledge on the epidemiology of *Stenocarpella* complex disease, the objectives of our study were to

identify major inoculum sources of the *Stenocarpella* sp., to verify the presence of *Stenocarpella* spp on maize growing areas of Minas Gerais State, on cultivated areas and to verify the contribuition of long-term crop rotation or maize monoculture on the pathogen recovery from maize stubble. This knowledge on the disease survival is essential for the development of preventive measures to reduce the risk of grain infections during the growing season.

Materials and Methods

Assay to define the major source of *Stenocarpella* on field

To determine the major source of the pathogen inoculum, samples were taken in one field at Lavras. The following the target substrates were separately considered: stalks, grains, cobs, fallen maize leaves, straws, or a pool of dead weeds.

Sampling in maize fields

Maize fields were selected in the maize growing areas on State of Minas Gerais. It were collected 3 samples in each area. All maize fields were managed according to technical conventional recommendations, i.e.

fungicide and herbicide sprays as needed for plant protection. They were cultivated with different systems of production: maize succession(33%), crop rotation(61%), no-tillage and tillage(6%). It is estimated that , today, the No-tillage system covers about 50% of the area with annual crops in Brazil (Cruz et al., 2006). All of them were cultivated following the management pattern.

Samples of maize plant stubble were collected in the 31 fields during the season of 2015/16 (Figure 2) to investigate their role as potential inoculum source for the *Stenocarpella* spp. Samples were taken in January 2016(Table 1).

For each sampled field, following crop stubbles encompassed : stalks, grains, cobs, fallen maize leaves, straws, and volunteer plants of maize. Actually the composite sampling encompassed the kind of sample available in the field and not always all the mentioned samples were found. For each type of substrate, three sub-samples taken were pooled so that samples for each field, substrate type and sampling date were available. Sub-samples were stored at -18°C until processing.

Detection of *Stenocarpella* spp.

To verify the presence of *Stenocarpella* within samples, each sub-sample containing stalks was classified in: entire, disintegrated and partially disintegrated (Table 1). All of sub-samples classified in entire or partially disintegrated were putted on humid chamber for 15 days and incidence of *Stenocarpella* was checked after this period, using a stereoscopic microscope.

A total of 140 samples were collected, encompassing 91 samples of stalks, 48 samples of cobs and one sample of volunteer plants of maize in 31 areas. Cobs were found in 24 areas from the total of 31 areas used during the season and in one area there was volunteer maize plants. From the total of 91 stalks, 36 were classified as entire, 21 partially disintegrated and 34 disintegrated (Table 1).

Sample processing and DNA extraction and Real time PCR

After storage, frozen samples were shredded in squares of 1-2cm. A subsample was weighed and freeze-dried using freeze dryer (model L-101, Liotop, São Carlos-SP, Brazil) to assess dry matter content until constant weight. Then the dried material was grounded in a laboratory

mill with a 1-mm mesh sieve. Powdered subsamples were stored at -18°C until DNA extraction.

DNA was extracted from subsamples of approximately 40 mg using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI), according to the protocol recommended by the manufacturer.

All sub-samples collected on each area were poolled into a mixed sample. Sybr Green PCR assays were performed in Rotor-Gene 6500 (Corbett Research, Montlake, Austrália). For each reaction, a 2.0µl sample was mixed with 23µl reaction mix containing 12.5µl SYBR Green PCR Kit,(Qiagen), 0.75µM of each forward and reverse primer. The primers P1 (GTTGGGGGTTTAACGGCACG) and P2 (GTTGCCTCGGCACAGGCCGG) described by Xia and Achar (2001), specific for the genus *Stenocarpella*, were used to detect the presence of the fungus in the tissues (Barrocas et al., 2012; Siqueira et al., 2014). The cycle initially consisted of 95 ºC for 3 minutes, denaturation at 94 ºC for 30 seconds, annealing at 60 ºC for 1 minute, and extension of 72 ºC for 1 minute, with final extension of 72 ºC for 10 minutes, for a total of 40 cycles. A 5-fold serial dilution ranging from 20 ng to 2 pg of DNA of S.*maydis* isolate 698, was included in each run for reference.

The Ct was determined as the number of cycles in which the fluorescence generated within a reaction crossed the threshold. The comparative Ct method was used. Samples showing the lowest expression for each gene were used as calibration sam-ples and relative expression was measured using the relative standard curve method. To quantify gene expression using real-time PCR, the values obtained corresponding to sample DNA levels were compared to the control DNA level. To calculate expression levels, the following were considered: Ct (exponential increase in PCR product) of the target gene and endogenous control, ΔCt = Ct (sample) - Ct (endogenous control) and $\Delta\Delta Ct = \Delta Ct$ (sample) - ΔCt (calibrator). Expression levels were then calculated using the formula: $RQ = 2^{-\Delta\Delta Ct}$ (LIVAK, 2001).

Suppressiveness soil assay

To identify possible cause of low *Stenocarpella* sp recovery and check if the possible supressiveness was general or specific, soil samples (0-5cm depth) were obtained from each site (Table 1) and checked for suppressiveness against *Stenocarpella maydis, Fusarium graminearum* and *Fusarium verticillioides,* following the methodology proposed by Pereira(2015) with the following modifications. The soil samples were

individually loaded into 9mm-petri dishes, watered to 60% field capacity, this value was enough to high production on maize (Bergamaschi et al., 2006).and infested with 5 mL suspension of each $1x10^5$ of each pathogen per sample and a spore. One week after soil infestation, seven autoclaved maize stalks were deposited on each plate over the soil and baits were checked daily for 7 days for the presence of the pathogen by visually examined through stereo-and light microscopes.

It was realized the Shapiro Wilk test, since the data were not normalized they were transformed. The data were transformed to arc sen(p/100), were p is the percentage of colonized baits. After transformation was realized the Shapiro Wilk test, the data in each replicate was submitted to the variance analysis (ANAVA) and for the significant effects, means were compared according to Skott Knott's test (p=0.05) using the Software R.

Data processing and statistical analysis

The data of stalks size, classified into entire, disintegrated and partially disintegrated for each sample, processed for qPCR and the Ct

values were submitted to Anova one-way and Tukey multiple comparisons of means at 95% family-wise confidence level.

The data of relative quantification and the location were submitted an agglomerative hierarchical cluster analysis based on Ward's grouping method and correlation matrix (Webster and Oliver, 1990) was conducted to group of 17 cities, the cluster was based on dissimilarity matrix of Euclidean distances between individuals.

The data of incidence in each replicate from suppressiveness assay was submitted to the variance analysis (ANAVA) and for the significant effects, means were compared according to Skott Knott's test (p=0,05), using the Software R.

The data of relative quantification were submitted an agglomerative hierarchical cluster analysis based on Ward's grouping method and correlation matrix (Webster and Oliver, 1990) was conducted to 31 areas sampled, the cluster was based on dissimilarity matrix of Euclidean distances between individuals and the result was used to define categories for heat map of *Stenocarpella* survival.

Heat maps were generated using interpolation method, Inverse Distance Weighting for the classes of relative quantification of *Stenocarpella* spp., suppressiveness assay, annual average temperature, annual average precipitation, altitude,maize yield, maize production, using the Software Arcgis.

Results

In areas cultivated adopting conventional tillage system, the pathogen was not detected, until 200 picograms regardless of the succession crop in previous season, even in maize over maize systems.

However, under no-tillage managed areas, *Stenocarpella* sp was detected at a smaller extent than areas adopting no-tillage system and being monocultivated with maize. In one area being cultivated with maize there was no presence of pathogen, already on areas using crop rotation were four without *Stenocarpella* (Table 1).

According the analysis of Anova one-way and Tukey's multiple c omparisons of means, the size of Stalk present on the area contributed to the pathogen survival, measured by *Stenocarpella* spp DNA quantificatio n. Relative quantification of Stalks classified as entire was different from

disintegrated and same as partially disintegrated. Areas with disintegrate d stalks were different of areas with partially disintegrated stalks (Table 1).

Relative quantification data and location showed presence of neighbouring cities within the same group, i.e. Piedade do Rio Grande, São Vicente de Minas, Minduri, Cruzília, Conceição do Rio Verde and Machado in one group and Nepomuceno, Boa Esperança, Campos Gerais and Alfenas in the second group, which in turn grouped close to Campo Belo, Arcos, Iguatama and Madre de Deus de Minas. On the first group are include Bambuí and São Gotardo (Figure 1).

Among the different maize stubble found in the field, i.e. stalks, grains, cobs, fallen maize leaves, straws and dead weeds, stalks were the substrate encountered at all areas. However, *Stenocarpella* spp DNA was found in higher amounts on grains and cobs followed by stalks, fallen maize leaves and straw on the samples collected during offseason and there was no DNA of these pathogens found on dead weeds (Figure 4).

From 31 total fields, samples 19, 16 and, 23 (Table 1) were conducive, respectively, to *Stenocarpella maydis*, *F. verticillioides* and

F.graminearum. Three soils were classified as suppressive against all pathogens, six, eight and, three soils presented as suppressive, respectively, to *Stenocarpella maydis*, *F. verticillioides* and *F.graminearum.* Others soils were classified as intermediate (Table 1).

The percentage of colonized baits ranged from 0 to 100% in all pathogens tested. The average of colonized baits ranged from 4,76 to 100% on *Stenocarpella maydis* and *F. verticillioides* assays. Already for *F.graminearum* average ranged from 14,28 and 100% colonized baits.

The soils were classified in three groups: suppressive, conducive and intermediate for each pathogen. The soils 3, 4, and 12 were classified as suppressive to all tested pathogens. Besides these, the soils 8, 15, and 17 were suppressive to *Stenocarpella maydis* and 1, 8, and 15, against *Fusarium verticillioides*. The soils 2, 6, 13, 16, 18, 19, 22, 23, 24, 25, 26, 28, 29, and 30 were classified as conducive for the three pathogens tested. The Soils 7, 10, 11, 17, 20,21, were conducive to *Fusarium graminearum*, 17 and 21 to *Fusarium verticillioides* and 1 and 11 to *Stenocarpella maydis* (Table 1).

Discussion

Both crop rotation and tillage systems have proven to impact pathogen survival (Flett et al., 2001; Casa et. al. 2006) but for the first time, it the contribution of those cropping systems have been addressed for *Stenocarpella* sp survival using DNA-based quantification in crop stubbles on maize fields. Crop stubbles can serve as shelter as well as nutritional source to phytopathogens that show saprophytic phase in their life cicle (Scott *et al.*, 1994; Reis & Casa, 1998) and it is known that *Stenocarpella* sp's survival on the maize stubble during offseason (Smith & White, 1988; Shurtllef, 1992), and focus on the previous studies were on grains and stalks (Reis & Mario, 2003) or seeds (Siqueira et al. 2014) but with our results we demonstrated that other sources such as cobs, can be even more important pathogen reservoirs.

It is important to identify all inoculum sources (Casa et al., 2006), because each one can be perpetuated in the area for a different time. This persistence of maize stubble will depend of C/N ratio, size of particles from stubbles, lignin content, N, polyphenols, lignin/N ratio, lignin +N/polyphenols ratio, presence of toxic elements, physical and chemical conditions from soil and type of microbes present on soil influence the decomposition of crop stubbles (Moreira & Siqueira, 2006).

In all areas were the pathogen's DNA was found it was also possible to check the presence in sub-samples that were placed in moinst chamber, this result show that both ways are enough to verify the presence of *Stenocarpella* in different areas.

Marburger et al. 2015 working with maize-soybean-wheat rotation and species of *Fusarium* spp. found a reduced F. *graminearum* recovery from extracted soil DNA. F. *graminearum* and fungi of genus *Stenocarpella* are causing Maize ear rots and maize-soybean rotation is the most commom rotation adopted by Brazilian growers, whereas they are increased by adopting no-tillage system. Among the sampled areas there were three with wheat on the crop rotation, but it was not possible found relation between the use of wheat and *Stenocarpella* spp. survival and is not common the use of wheat as component of crop rotation by Brazilian farmers,. Moreover maize-soybean-wheat rotation may contribute very little to the reduction of inoculum of F. *graminearum*, because the pathogen has the capacity to infect all three hosts. (Broders et al., 2007)

The presence of maize crop stubbles can also indicate the *Stenocarpella* sp. inoculum on cultivated area (Reis & Mario, 2003).

Working with different fungicides and three different plant growth stages, Romero Luna and Wise (2015) did not consistently reduced white ear rot or improved yield under low and high disease pressure. This absence of control by different fungicides support the importance to use crop rotation, when farmer is adopting no-tillage system or tillage as management method to reduce severity of this disease and the sources of inoculum for next season and with the results showed on our work is expected that survival of *Stenocarpella maydis* will reduce. Moreover, strategies to White Ear rot control can be based on elimination of maize crop stubbles by crop rotation (Scott et al., 1994).

There is a requirement to rise the cultivated area using crop rotation with vegetables species considered no host of *Stenocarpella maydis* as soybean, on summer season (Reis & Mario, 2003). In four consecutive seasons in South Africa, Flett et al. (2001) determined that soybeans or peanuts crops in crop rotation with maize reduced the amount of damaged kernels and the recovery of *Stenocarpella maydis* from crop stubbles in 75-100 %, but in case of planting before the maize, that authors showed soybean was effective, instead of peanut to control *Stenocarpella*. Both findings corroborated our findings, since most of

sampled areas were from crop rotation with soybean and they were reducing the survival of the pathogen.

The results about relation size of stalks and relative quantification are expected, because they are disintegrated and a hypothesis is it could have been caused by the type of crop succession adopted or other factors such as type of soil, altitude, weather conditions. In tillage system, occurs partial incorporation of maize stubble and they are mechanically defragmented with more intensity and provides more contact between soil and maize stubble (Summerel & Burgess, 1989) and this increase of contact surface can explain the difference found on present work on the size of stalks present on maize stubble and their influence on relative quantification of *Stenocarpella spp*. Moreover, more contact between soil and stubble reduces temperature and wheather flutation, allowing more benefitial microbiome activity (Casa et al., 2003).

Cluster analysis for relative quantification by city showed next cities grouped, what is expected because they are theoretically exposed at the same conditions. Working with survey of mycoflora in maize grains, Ramos et al. (2010) found *Stenocarpella spp*. on the weather zones

SA(700 meters of altitude), TA(above 700 meters) and TB(below 500 meters).

Soil supressiveness may explain reduced survival of *Stenocarpella maydis* in maize monocultivated found in specific cases. The supressiveness found on soils three, four and 12 against all of tested pathogens suggests to treat general supressiveness. However, is important highlight most suppressive soils are from areas adopting crop rotation and no-tillage system. Another point may explain those soils with maize monocultivated showing reduced survival of *Stenocarpella maydis* is over time of monocultive, for some pathosystems, monoculture for long time has led to suppressiveness as the case of specific area controlling Take-all root rot caused by *Gaeumannomyces graminis* var. *tritici*, (Hornby, 1983). Besides, in these areas sampled the maize is harvested for silage, it has fewer stalks and nothing cobs, reducing the survival of the pathogen in the area.

Pankhurst et al. (2002) in work carried out in Australia observed suppression towards *Gaeumannomyces graminis* var. *tritici* and *Rhizoctonia solani* was greater in soils under no-tillage than in soils under conventional tillage. Garbeva et al. (2006) reported that soils with higher

plant diversity increased microbial diversity and showed higher levels of suppressiveness against *R. solani* AG3. Working with *F.graminearum*, Lisboa et al. (2015) found higher fungistasis for soil under no-tillage management in comparison with conventional tillage, similar to found in present work, since all supressive soils to *F.graminearum* were from no-tillage system. Already for S. *maydis* and F. *verticillioides*, one supressive soil was from Conventional tillage as well as, Sipilä et al. (2012) identified a positive correlation between no-tillage system and soil suppressiveness to *Fusarium culmorum*, concluding that management have strong influences on soil microbiological processes. Furthermore, no-tillage and the crop system with highest input of plant stubbles and species diversity altered the bacterial community structure, increased microbial biomass and activity and, as a consequence, increased the suppressive potential of the soil (Campos et al., 2016).

Crop systems with high plant diversity can also positively affect microbial community by altering the microbial diversity and increasing the abundance of microbial groups associated with soil suppressiveness, such as Bacillus and Pseudomonas (Garbeva et al., 2006). Development of suppressive soils may be a more cost effective and efficient alternative

to microbe application. (Lapsansky et al., 2016). Hence, can be a good next step to study microbiome of the supressive soils used on present work. The microbial diversity can come from the diversity of weeds, which in turn, have found not to be hosts of *Stenocarpella* sp. (Figure 4).

Analyzing the heat maps, it is not possible see clearly determine the relationship between survival of *Stenocarpella* and maize yield, maize production, temperature. However it seems that there is a trend between survival of *Stenocarpella* and suppressiveness, altitude and precipitation. Where there is more *Stenocarpella* sp DNA, suppressiveness is low or absent, since soil suppressiveness can reduce the survival of *Stenocarpella*, this result corroborates this hypothesis.

Areas with high altitude had higher *Stenocarpella* sp than in low altitudes (Figure 3). Instead it Malusha (2016) working with maize, found low altitude had influence on aflatoxin occurrence and aflatoxin positivity in contaminated maize was higher in low altitude area than in high altitude.

Comparing the heatmaps Classes of *Stenocarpella* survival and precipitation, it seems high precipitation favored the survival of

Stenocarpella. More humid enviroments are favourable to pathogen (Casa et al., 2007), affording their survival.

The approach adopted on work was macro, since samples were taken at several different areas. However, same idea can be used when soil samples are collected to fertilizer analisys at specific areas, inferring where the pathogens will survive more or where there are more suppressiveness on that specific area and it can be a good tool in precision agriculture. Next step is look at areas located on high altitude and check which factors are more important to affect the *Stenocarpella* survival.

The results found indicates there is a high relation between crop rotation and reduced *Stenocarpella* survival or by increasing the suppressiveness or any other aspect that encourages greater enzyme activity and consequently the decomposition of crop stubbles, the main source of inoculum.

Results found herein show for the first time how crop rotation infludes the *Stenocarpella* spp. survival in maize crop stubbles and how is possible to reduce the initial inoculum using other crop like soybean. Some soils have general and specific supressiveness to pathogens causing

maize rot grains. There are several factors that alters the *Stenocarpella* spp. survival dynamics in maize crop stubbles.

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Table 1. Stubble sampled in different fields within Minas Gerais state and evaluated for t Stenocarpella spp. Inoculums and suppressiveness to ear and stalk rot causing pathogens (Fusarium graminearum, Fusarium verticillioides, Stenocarpella maydis)

								Colonized baits		
Field	First season	Second season	System	Place	Stalk	Location	RQ[2]	*Fusarium gramiearum*	*Fusarium verticillioides*	*Stenocarpella maydis*
1	Maize	Soybean	No-tillage Crop rotation	Madre de Deus de Minas	D[1]	21º24'15"S 44º13'08"W	1	85 B[3]	33 C	95 A
2	Maize	Soybean	No-tillage Crop rotation	Madre de Deus de Minas	P	21º24'15"S 44º13'08"W	8,E-11	100 A	100 A	100 A
3	Maize	Bean-Soybean	No-tillage Crop rotation	Madre de Deus de Minas	D	21º26'5"S 44º14'11"W	6,E-11	14 C	10 C	10 C
4	Maize	Soybean	No-tillage Crop rotation	Madre de Deus de Minas	E	21º24'58"S 44º12'34"W	7,E-10	24 C	5 C	5 C
5	Maize	Bean	No-tillage Crop rotation	Madre de Deus de Minas	P	21º24'58"S 44º12'34"W	1E-10	62 B	38 C	71 B
6	Maize	Soybean	No-tillage Crop	Piedade do Rio	E	21º23'14"S 44º10'31"W	1E-10	95 A	100 A	95 A

			rotation	Grande						
7	Maize	Soybean	No-tillage Crop rotation	São Vicente de Minas	P	21º39’32”S 44º32’58”W	3E-09	100 A	90 B	71 B
8	Maize	Soybean	No-tillage Crop rotation	São Vicente de Minas	E	21º39’32”S 44º32’58”W	8E-10	67 B	29 C	33 C
9	Maize	Wheat-Soybean	No-tillage Crop rotation	Minduri	P	21º40’01”S 44º34’35”W	4E-11	62 B	71 B	71 B
10	Maize	Maize	No-tillage Crop rotation	Minduri	P	21º42’46”S 44º39’59”W	6E-11	95 A	86 B	76 B
11	Maize	Maize	No-tillage Crop rotation	Cruzília	E	21º42’46”S 44º47’52”W	1E-10	100 A	86 B	100 A
12	Maize	Wheat-Maize	No-tillage Crop rotation	Conceição do Rio Verde	E	21º56’32”S 45º02’59”W	3E-10	29 C	43 C	29 C
13	Soybean	Wheat-Maize	No-tillage Crop rotation	Conceição do Rio Verde	P	21º55’45”S 45º07’08”W	9E-07	95 A	100 A	95 A
14	Maize	Maize	No-tillage Crop rotation	Nepomuceno	E	21º14’22”S 45º19’08”W	7E-11	71 B	76 B	86 B

15	Maize	Bean	Conventional Tillage	Nepomuceno	D	21º15’22”S 45º20’24”W	1	76 B	33 C	38 C
16	Maize	Soybean	No-tillage Crop rotation	Nepomuceno	P	21º14’47”S 45º21’16”W	1	100 A	100 A	100 A
17	Maize	Maize	No-tillage Crop rotation	Boa Esperança	E	21º09’50”S 45º33’56”W	1E-11	100 A	100 A	33 C
18	Maize	Soybean	No-tillage Crop rotation	Boa Esperança	P	21º09’50”S 45º33’56”W	1	100 A	95 A	95 A
19	Maize	Maize	No-tillage Monoculti ve	Campos Gerais	D	21º17’37”S 45º49’28”W	2E-12	100 A	100 A	100 A
20	Maize	Soybean	No-tillage Crop rotation	Campos Gerais	D	21º17’53”S 45º50’09”W	1	100 A	81 B	76 B
21	Maize	Maize	No-tillage Monoculti ve	Alfenas	D	21º38’26”S 45º54’03”W	1	90 A	95 A	86 B
22	Maize	Soybean	No-tillage Crop rotation	Machado	E	21º40’52”S 45º51’27”W	5E-12	95 A	100 A	100 A
23	Maize	Maize	No-tillage	Carmo da	E	21º32’56”S	1E-11	100 A	95 A	100 A

			Monoculti ve	Cachoeira		45º14’05”W				
24	Maize	Maize	Conventio nal Tillage	Campo Belo	D	20º52'50"S 45º15'38"W	1	100 A	100 A	95 A
25	Maize	Soybean	No-tillage Crop rotation	Arcos	D	20º23’21”S 45º31’16”W	1	100 A	100 A	100 A
26	Maize	Maize	No-tillage Monoculti ve	Arcos	P	20º23’06”S 45º30’33”W	7E-12	100 A	100 A	100 A
27	Maize	Soybean	No-tillage Crop rotation	Bambuí	D	20º03’04”S 45º53’57”W	8E-11	71 B	71 B	81 B
28	Maize	Soybean	No-tillage Crop rotation	Bambuí	E	19º53’55”S 45º58’34”W	4E-08	100 A	100 A	95 A
29	Maize	Maize	No-tillage Monoculti ve	São Gotardo	D	19º20’44”S 46º05’24”W	5E-05	100 A	100 A	100 A
30	Maize	Soybean	No-tillage Crop rotation	São Gotardo	E	19º21’00”S 46º06’11”W	1E-10	100 A	100 A	100 A

31	Maize	Soybean	No-tillage Crop rotation	Iguatama	D	20º08’29”S 45º44’13”W	1	86 B	86 B	81 B

[1]Stalks size: D-desintegrated, P-parcially disintegrated and E-entire.

[2]RQ: Relative quantification of *Stenocarpella spp.*

[3]Colonized baits followed by same letter do not differ.

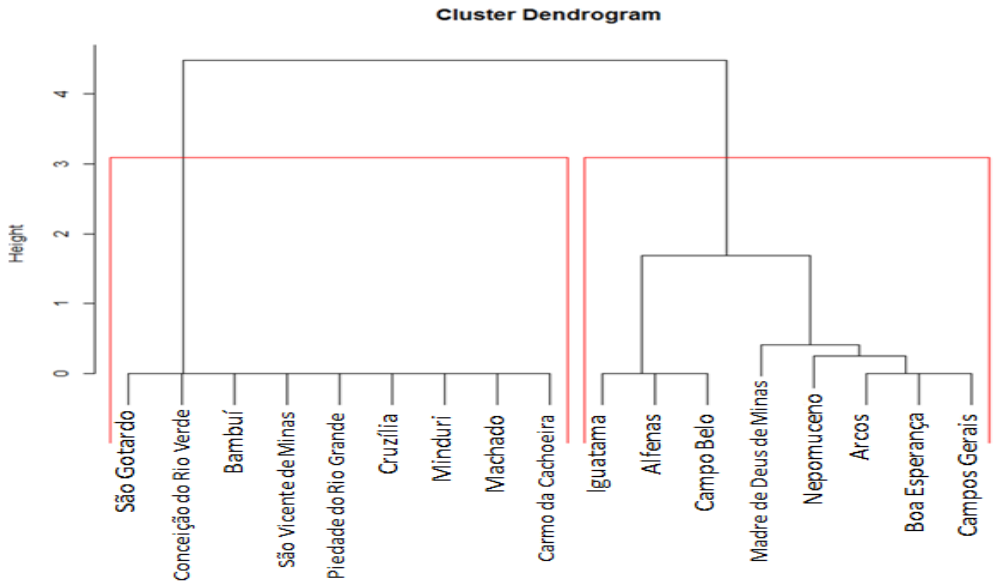


Figure 1. Cluster dendrogram for DNA rtPCR relative quantification of Stenocarpella spp. by sampled location. Dendrogram generated by data submitted an agglomerative hierarchical cluster analysis basedon Ward's grouping method and correlation matrix (Webster and Oliver, 1990)

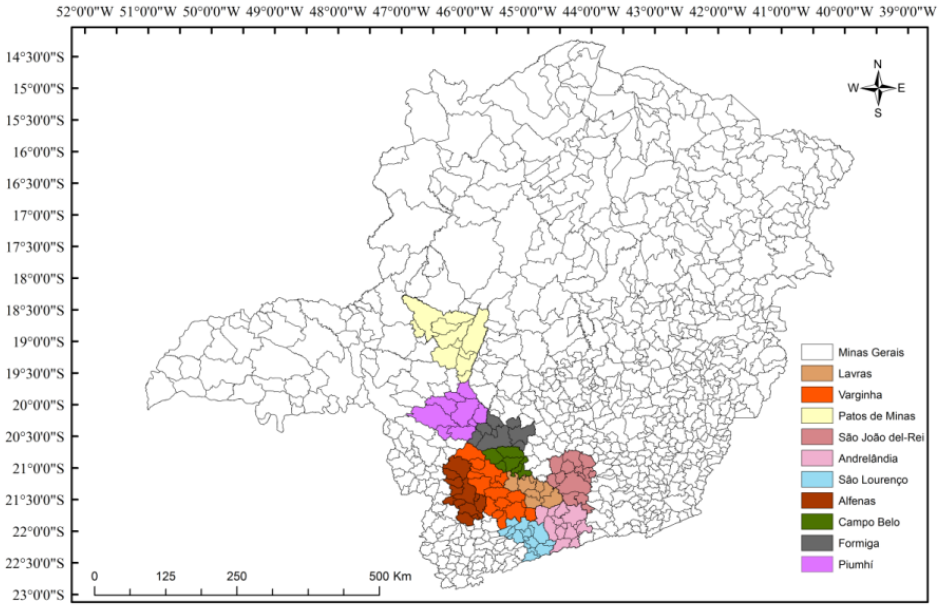


Figure 2. Location map of sampled areas in Minas Gerais, Brazil

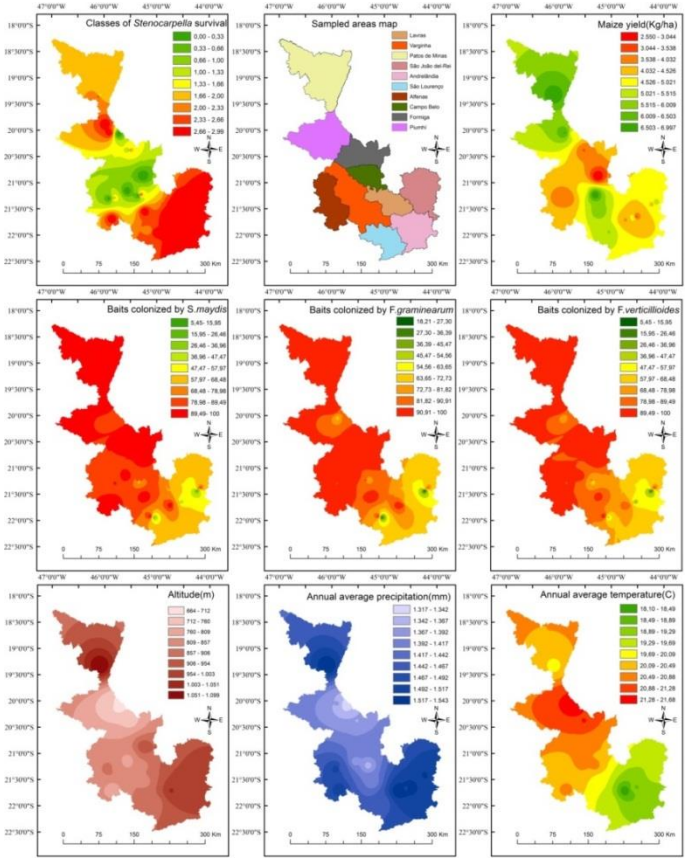
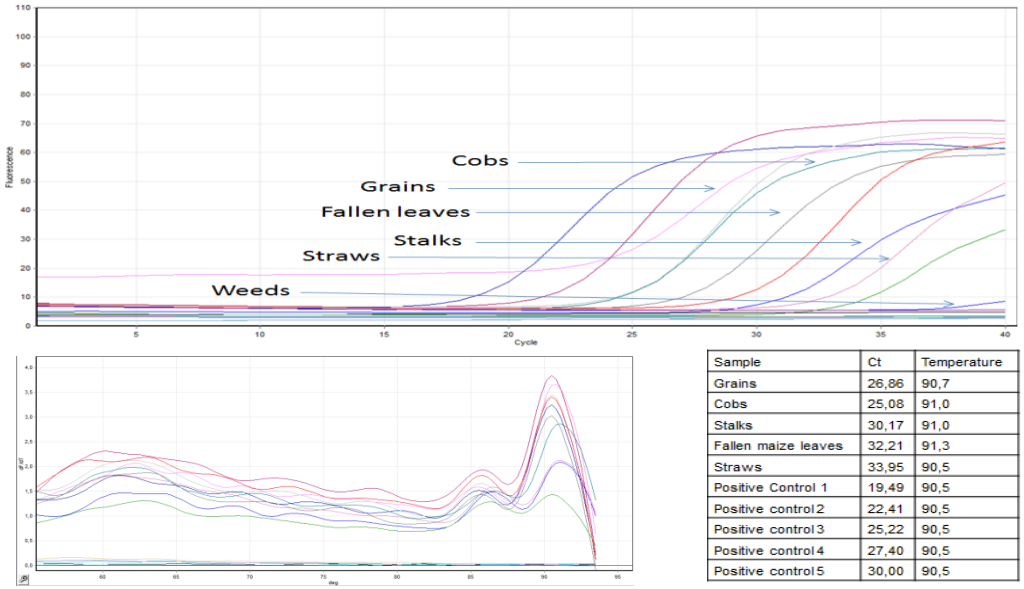


Figure 3. Heatmaps of Classes of *Stenocarpella* survival generated using arcgis

(classes were grouped by cluster analisys of *Stenocarpella* DNA relative quantification) , Maize yield(Kg/ha), Maize production(Kg), Soil suppressiveness to *Stenocarpella maydis*, *Fusarium graminearum*, *Fusarium verticillioides* (% of colonized baits), Altitude(m), average temperature(ºC), rainfall (mm).



Sample	Ct	Temperature
Grains	26,86	90,7
Cobs	25,08	91,0
Stalks	30,17	91,0
Fallen maize leaves	32,21	91,3
Straws	33,95	90,5
Positive Control 1	19,49	90,5
Positive control 2	22,41	90,5
Positive control 3	25,22	90,5
Positive control 4	27,40	90,5
Positive control 5	30,00	90,5

Figure 4. Melting curve analysis for Stenocarpella spp. using real-time polymerase reaction(PCR) assay . A. amplification curve of different substrates and controls obtained with primers P1/P2 (Xia and Achar,2001). B. Melting

curve analysis of the same samples shows the presence of the specific PCR product. C. Comparison chart with cycle threshold(Ct) values and melting temperatures.